uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Julie Deschênes-Furry
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Neuroscience)
GRADE / DEGREE

Department of Cellular and Molecular Medicine
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Acetylcholinesterase Expression in Excitable Cells: Post-transcriptional Regulation and Role of the Hu Family of RNA-binding Proteins

TITRE DE LA THÈSE / TITLE OF THESIS

Bernard Jasmin
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

S. Brimijoin

L. Megeney

M. Holcik

O. Laneuville

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Role of the Hu Family of RNA-binding Proteins in Regulating Acetylcholinesterase Expression in Excitatory Cells

Julie Deschênes-Furry

This thesis is submitted as a partial fulfillment of the Ph.D. program in Neuroscience

August 31, 2006

Ottawa, Ontario, Canada

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-25865-1
Our file Notre référence
ISBN: 978-0-494-25865-1

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

© Julie Deschênes-Furry, Ottawa, Canada, 2006

To my husband

Authorization

Authorization to reproduce in their entirety the journal articles cited below is granted by the American Society for Biochemistry and Moleclular Biology in accordance with their copyright permission policy.

1) Deschênes-Furry J, Bélanger G, Perrone-Bizzozero N, and Jasmin BJ (2003) Post-transcriptional Regulation of Acetylcholiesterase mRNAs in Nerve Growth Factor-treated PC12 Cells by the RNA-binding Protein HuD. J Biol Chem 278: 5710-5717.

2) Deschênes-Furry J, Bélanger G, Mwanjewe J, Lunde JA, Parks RJ, Perrone-Bizzozero N, and Jasmin BJ (2005) The RNA-binding Protein HuR Binds to Acetylcholinesterase Transcripts and Regulates Their Expression in Differentiating Skeletal Muscle Cells. J Biol Chem 280: 25361-25368.

Authorization to reproduce sections of text from the review article cited below is granted by John Wiley and Sons, Inc.

1) Deschênes-Furry J, Perrone-Bizzozero N and Jasmin BJ (2006) The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity. BioEssays 28: 822-833.

Abstract

Post-transcriptional mechanisms regulating acetylcholinesterase (AChE) expression in muscle and neurons are relatively ill-defined, even though several studies have implicated mRNA stability as a means of controlling expression. We have, therefore, undertaken studies examining the role of post-transcriptional mechanisms in regulating AChE mRNA levels during neuronal differentiation, myogenic differentiation and *in vivo* following axotomy of the rat superior cervical ganglion (SCG). Specifically, using a combination of molecular techniques we have investigated the role of AChE's 3'-untranslated region (UTR) and have identified both cis- and trans-acting factors that modulate AChE mRNA levels.

During neuronal differentiation of PC12 cells we determined that post-transcriptional mechanisms were predominantly responsible for the sustained increase in AChE mRNA since a modest increase in gene transcription occurred early and transiently. We examined the role of the AU-rich element (ARE) found in the 3'-UTR and of the RNA-binding protein (RBP) HuD in modulating AChE mRNA levels. Using a combination of complementary binding assays and transfection assays we demonstrated direct binding of HuD to the ARE and the stabilizing effect of this interaction. Given these results, we examined whether the ubiquitous Hu family protein, HuR, is implicated in post-transcriptional regulation of AChE during C2C12 myogenesis. Using similar approaches, we observed that the ARE and interactions with RBP complexes increased with differentiation and are important elements to AChE expression. We showed that HuR associates with the ARE and is involved in increased AChE transcript stability measured during myogenesis. Finally, we examined the relationship between HuD and AChE

mRNA in neurons *in vivo* following SCG axotomy. Initially, we demonstrated that human HuD expressed in the hippocampus of HuD transgenic mice could bind AChE transcripts and increase AChE mRNA levels. Following SCG axotomy, we observed dramatic decreases in AChE mRNA levels and interactions with RBP complexes, including HuD. HuD protein and mRNA levels were also decreased as a result of axotomy. Exogenous expression of HuD in the SCG prior to axotomy prevented the decrease in AChE mRNA. Accordingly, during the course of these studies we have identified an ARE in the AChE 3'-UTR and its binding partners HuD and HuR, as essential mediators of post-transcriptional mechanisms regulating AChE expression in neurons and muscle, respectively.

Table of contents

List of tables

CHAPTER 1:

List of figures

CHAPTER 4:

CHAPTER 5:

List of abbreviations

3'-UTR	3'-untranslated region
5'-UTR	5'-untranslated region
A4, A8, A12	Asymmetric AChE molecular forms
Aβ	Amyloid-β
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AD	Alzheimer's disease
APP	Amyloid precursor protein
ARE	AU-rich element
BChE	Butyrylcholinesterase
BDNF	Brain derived neurotrophic factor
BRF	Butyrate response factor
α-CaMKII	Ca^{2+}/Calmodulin kinase II α-subunit
CARM1	Coactivator-associated arginine methyltransferase 1
CAT	Chloramphenicol acetyltransferase
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyl transferase
CMS	Congenital myasthenic syndromes
CNS	Central nervous system
CPEB	Cytoplasmic polyadenylation element binding
CRE	cAMP-response element

CREB	cAMP-response element binding
CSF	Cerebral spinal fluid
DRB	5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole
DRG	Dorsal root ganglion
dsRBD	Double-stranded RNA binding domain
DTT	Dithiothreitol
ELAV	Embryonic lethal abnormal vision
EST	Expressed sequence tags
FL	Full-length AChE 3'-UTR
FMN	Facial motor nerve
FMRP	Fragile-X mental retardation protein
G1	Monomeric AChE molecular form
G2	Dimeric AChE molecular form
G4	Tetrameric AChE molecular form
GABP α and β	GA-binding protein alpha and beta
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycophosphatidylinositol
GPI-G2	Glycophosphatidylinositol-associated AChE dimer
GRAP	Giant rat acetylcholinesterase promoter
GRE	Glucocorticoid response element
H	Hydrophobic AChE transcript
hnRNP	Heteronuclear ribonucleoprotein
HSPG	Heparan sulfate proteoglycans

HSV	Herpes simplex virus
IP	Immunoprecipitation
ISH	*In situ* hybridization
KH	K-homology
KSRP	K-homology-type splicing regulatory protein
MEL	Murine erythroleukemia
MOPS	4-morpholinepropanesulfonic acid
MuSK	Muscle specific kinase
nAChR	Nicotinic acetylcholine receptor
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NMD	Non-sense mediated decay
NMJ	Neuromuscular junction
NT 4/5	Neurotrophin 4/5
ORF	Open reading frame
PABP	Poly(A)-binding protein
PAN	Poly(A) nuclease
PARN	Poly(A) ribonuclease
PAS	Peripheral anionic site
PBS	Phosphate-buffered saline
PKC	Protein kinase C
PNS	Peripheral nervous system
Poly(A)	3'-polyadenylate

PRAD	Proline-rich attachment domain
PRiMA	Proline-rich membrane anchor
PTSD	Post-traumatic stress disorder
R	Readthrough AChE transcript
RBP	RNA-binding protein
REMSA	RNA-based electrophoretic mobility shift assay
RGG	Arginine-glycine
RNP	Ribonucleoprotein
RPA	RNase protection assay
RT	Reverse transcription
S12	S12 ribosomal protein
SCG	Superior cervical ganglion
siRNA	Small interfering RNA
T	Tailed AChE transcript
trkA and B	Tyrosine kinase A and B
TTP	Tristetraprolin
TTX	Tetrodotoxin
UTR	Untranslated region
UV-XL	Ultraviolet-crosslinking
WAT	Tryptophan amphiphilic tetramerization
ZBP1	Zipcode binding protein 1

Acknowledgements

This work was conducted under the supervision of Dr. Bernard Jasmin. Dr. Jasmin has been an exceptional mentor and I would like to thank him for guiding my scientific development. Through the years, Dr. Jasmin has been a supportive and encouraging supervisor. Although very busy, he has always been available for guidance and advice and has always had my best interests in mind. It has been a privilege and a pleasure to learn from such a driven and devoted researcher.

This work was supported by funding from Canadian Institutes of Health Research and Ontario Neurotrauma foundation (to Dr. Jasmin). I gratefully acknowledge the support through postgraduate scholarships from the Ontario Neurotrauma Foundation and the Ottawa-Brennan Bookstore through an Ontario Graduate Scholarship in Science and Technology.

This research has been performed in collaboration with Dr. Nora Perrone-Bizzozero at the University of New Mexico. I would like to thank Dr. Perrone-Bizzozero for her invaluable input into these research projects and fruitful discussions. I would also like to thank the members of my Advisory Committee, Dr. Paul Albert, Dr. Stephen Gee, and Dr. Odette Laneuville, for their input and valuable advice throughout my training.

I wish to thank my colleagues and lab-mates, past and present, for their individual support and encouragement over the years, and for many hours of, sometimes animated but always productive, discussion and problem-solving.

To my family and friends, who have wondered why anyone would choose to pursue such an endeavor, but never doubted my ability to accomplish it successfully, I offer my most heartfelt thanks. Specifically, I wish to thank my Father who has always

encouraged me to go beyond anyone's expectations, and my Mother for teaching and reminding me about "balance". This work would not have been possible without their support and confidence.

Finally, I wish to thank my husband, Jeff, who has believed in me and offered constant support, in more ways than one, throughout the years. This work could not have been accomplished without his understanding and encouragement; for this reason I owe him an eternal debt of gratitude.

Chapter 1: General introduction

1.1 Acetylcholinesterase

Acetylcholinesterase (AChE), a serine esterase of the cholinesterase family of proteins (see table 1), is the enzyme responsible for rapid hydrolysis of acetylcholine (ACh) at cholinergic synapses of the central and peripheral nervous systems (CNS and PNS, respectively) (see for review Massoulie et al., 1993;Taylor and Radic, 1994;Rotundo, 2003;Legay, 2000;Soreq and Seidman, 2001). In fact, this enzyme is one of the fastest acting enzymes with the ability to hydrolyze acetylcholine within one millisecond after its release from the pre-synaptic terminal (Rosenberry, 1979;Bazelyansky et al., 1986 and see for review Quinn, 1987;Taylor and Radic, 1994). ACh can also be hydrolyzed, although less efficiently, by the closely related enzyme butyrylcholinesterase (BChE), which is also a member of the cholinesterase family. BChE's role in the CNS and different tissues is different from AChE and is still emerging (see for review Darvesh et al., 2003;Mack and Robitzki, 2000;Massoulie et al., 1993). Existence of AChE was initially suggested in 1914 by Henry Dale (Dale, 1914) and was later identified and localized in the electric organ of *Torpedo californica* electric ray (*Torpedo*) and *Electrophorus electricus* electric eel (*Electrophorus*), and to the neuromuscular junction (NMJ) (Marnay, 1937;Marnay and Nachmansohn, 1937;Marnay and Nachmansohn, 1938).

In vertebrates, AChE is expressed broadly throughout the body, but is found predominantly within the CNS, ganglia of the PNS, muscle (skeletal, cardiac and smooth, see for review Rotundo, 2003), and hematopoietic cells such as erythrocytes (see for review Chan and Jasmin, 1999). AChE catalytic activity is constant, regardless of the

tissue; however, the molecular structure of the protein and its expression pattern are unique to each tissue. Given the diverse range of tissues expressing AChE, its complexity and its essential activity at synapses, this enzyme has been the focus of numerous studies aimed at characterizing its function and expression in the body.

Table 1: Cholinesterase related proteins *

Family	Protein and origin	Catalytic triad residues
Cholinesterases	AChE	S E H
	BChE	S E H
	Cholinesterase-like (insect)	S E H
Esterases	Carboxylesterase	S E H
	Cholesterol esterase	S D H
	Esterase (insect)	S E H and S D H
	Juvenile hormone esterase (insect)	S E H
	Carboxylesterase-lipase (fungi)	S E H
Non-catalytic	Thyroglobulin	– – H
	Neuroligin	– E H
	Neurotactin (*Drosophila*)	– E H
	Glutactin (*Drosophila*)	– – –
	Gliotactin (*Drosophila*)	– – –

* These related families of proteins belong to the α/β-fold superfamily of proteins based on their protein folding patterns (Hotelier et al., 2004). Unless otherwise stated the protein origin is mammalian. Catalytic triad residues are serine (S), glutamate (E) or aspartate (D), and histidine (H).

1.1.1 Catalytic activity

The catalytic site, responsible for cleavage of ACh into choline and acetate subfragments, is found at the base of a long and narrow gorge (~20 Å) (Sussman et al., 1991). This site consists of the catalytic triad of amino acids serine, histidine, and glutamate; in which serine is the active residue that acts on the ester bond in ACh (see Figure 1). Mutation of this residue to another less reactive residue completely abolishes enzymatic activity, as demonstrated in mutant Zebrafish (Behra et al., 2002). At the opening of the catalytic gorge is a peripheral binding site, termed the peripheral anionic site (PAS), which is not directly involved in catalysis, but can modulate enzymatic activity as demonstrated by AChE inhibitors that target this site. The PAS is also involved in other non-catalytic functions (see *1.4.1 Alzheimer's disease and 1.6.1 Neurite outgrowth*). Given its critical role in cholinergic neurotransmission, AChE is the target of naturally occurring and synthetic inhibitors, including specifically, green mamba snake venom (fasciculin) (Bourne et al., 1995;Radic et al., 1994), nerve gases (soman and sarin), and organophosphate insecticides.

1.1.2 Molecular forms and non-cholinergic subunits

AChE is a heteromeric glycoprotein consisting of monomers or oligomers of the catalytic subunit and non-catalytic anchoring subunits (see Figure 2) (Bon et al., 1976 and see for review Brimijoin, 1983). The quaternary structure of AChE molecular forms, termed globular or asymmetric, was initially characterized based on sedimentation values of proteins extracted from *Torpedo* or *Electrophorus* electric organs (Bon et al., 1976;Cartaud et al., 1975;Lwebuga-Mukasa et al., 1976;Rieger et al., 1973;Bon et al., 1979). The basic and smallest molecular form isolated from tissues is the monomer

globular form termed G1. Monomers exist in a dynamic equilibrium of catalytically active and inactive molecules (see *1.2.4 Activity-dependent modulation of expression* for further details) (Lazar et al., 1984;Rotundo, 1988;Chatel et al., 1993;Eichler and Silman, 1995 and see for review Fernandez et al., 1996).

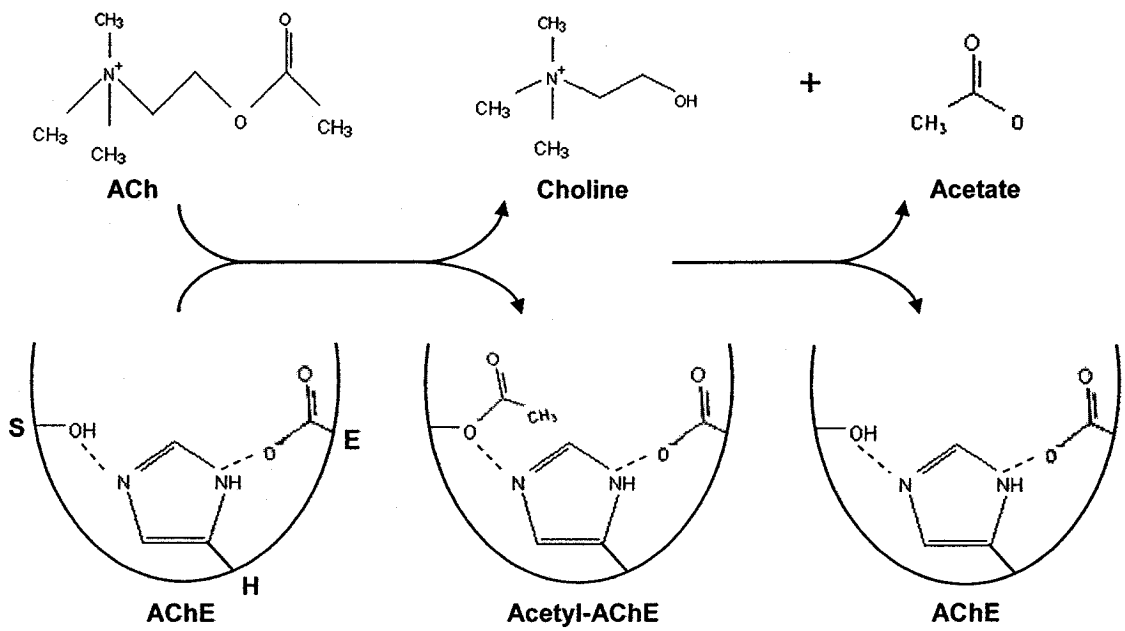

Figure 1: AChE catalytic activity. Diagram of acetylcholine (ACh) hydrolysis by the AChE catalytic catalytic triad of serine (S), histidine (H) and glutamate (E) (adapted from Soreq and Seidman, 2001).

Within the endoplasmic reticulum, monomers can form dimers (G2), via the interaction of two alpha-helices of each catalytic subunit (Sussman et al., 1991;Morel et al., 2001;Bourne et al., 1995), or form tetramers (G4), and associate with anchoring subunits (Rotundo, 1984;Brockman et al., 1986). Expression and formation of specific molecular forms is regulated in a tissue-specific manner. G2 subunits, for example,

associate with glycophosphatidylinositol (GPI) anchors via C-terminal residues of the catalytic subunit (see for review Silman and Futerman, 1987). The GPI-G2 molecular

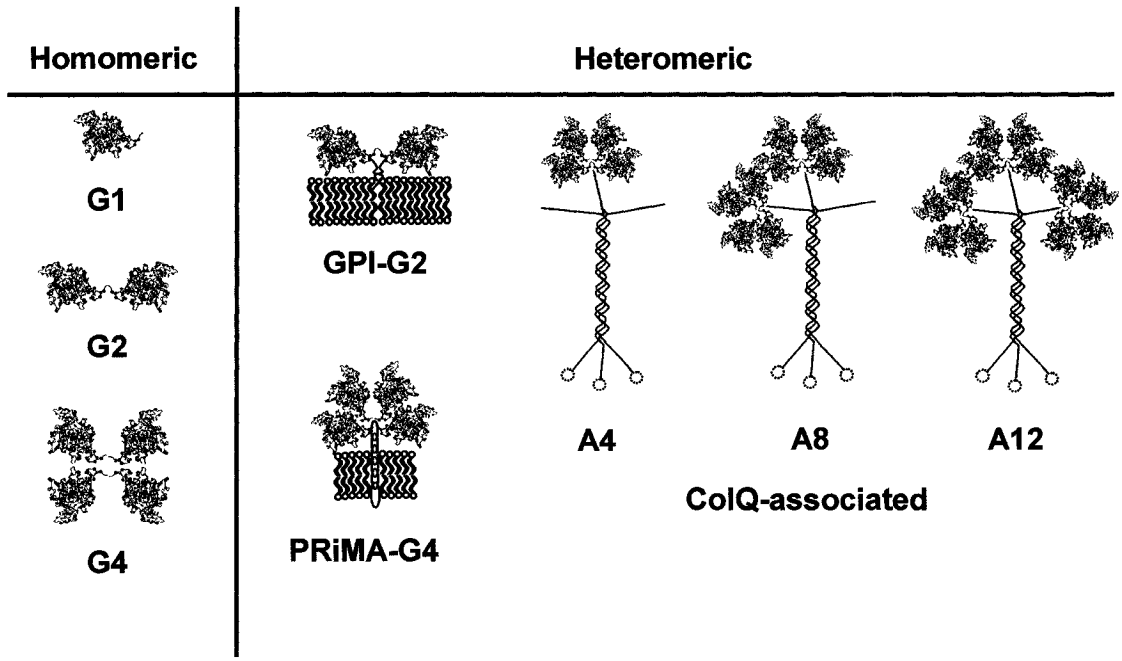

Figure 2: AChE molecular forms. AChE catalytic subunits are expressed as globular homomeric monomers (G1), dimers (G2) and tetramers (G4), or as globular heteromeric dimers (GPI-G2) and tetramers (PRiMA-G4) associated with non-cholinergic subunits, GPI and PRiMA. Asymmetric forms of AChE consist of one (A4), two (A8), or three (A12) tetramers associated with ColQ.

form is widely expressed amongst different species with the exception of birds and is localized to the NMJ in *Torpedo* and insects, but is predominantly associated with hematopoietic cells, such as erythrocytes, in most mammals (see for review Rotundo, 2003). In comparison, G4 molecular forms associate with the proline-rich membrane anchor (PRiMA) via disulfide bonds between AChE dimers and the PRiMA proline-rich attachment domain (PRAD) (Fuentes et al., 1988;Fuentes and Inestrosa, 1988;Gennari et al., 1987;Inestrosa et al., 1987;Perrier et al., 2002 and see for review Fernandez et al., 1996). PRiMA is a type 1 transmembrane protein that is essential for targeting AChE to

the plasma membrane (Perrier et al., 2002). This molecular form is predominantly expressed at the neuronal cell membrane in the CNS.

Asymmetric forms, A4, A8, and A12, are composed of one, two or three tetramers of the catalytic subunit, respectively, and the collagen-like tail formed by the triple helical association of three collagenic subunits termed ColQ (Q for Queue, which is French for tail). Asymmetric forms are assembled in the golgi apparatus via interactions between the C-terminal domain of AChE, termed the tryptophan amphiphilic tetramerization (WAT) domain and the PRAD of the ColQ subunit (Rosenberry and Richardson, 1977;Bon et al., 1997;Camp et al., 1995;Krejci et al., 1991;Bon et al., 1997;Bon and Massoulie, 1997). The collagen-like tail functions to target and anchor the catalytic subunits in the extracellular matrix of the post-synaptic domain. Specifically, asymmetric forms interact with glycosaminoglycans and heparan sulfate proteoglycans (HSPG), such as perlecan, in the basal lamina, or with membrane associated proteins of the NMJ, like MuSK (Arikawa-Hirasawa et al., 2002;Vigny et al., 1983;Cartaud et al., 2004;Kimbell et al., 2004;Peng et al., 1999). ColQ is essential to accumulation of asymmetric AChE at the neuromuscular junction (Feng et al., 1999), as was demonstrated by development of myasthenic symptoms and endplate deficiencies in AChE when the heparan sulfate binding domain of ColQ is mutated (Kimbell et al., 2004). Importantly, AChE is structurally homologous amongst all vertebrates and, regardless of the molecular form, the catalytic activity of the enzyme subunit is the same (Bon et al., 1979).

1.1.3 Gene

The *AChE* gene was initially cloned from the invertebrate *Drosophila melanogaster* (Hall and Spierer, 1986). Subsequently, *AChE* has been cloned in most vertebrates including human, mouse, rat, bovine, *Torpedo* and Zebrafish (Rotundo et al., 1988;Doctor et al., 1990;Soreq et al., 1990;Bertrand et al., 2001;Rachinsky et al., 1990;Legay et al., 1993b;Schumacher et al., 1986;Sikorav et al., 1987;Gibney et al., 1988). In all species, with the exception of *Caenorhabditis elegans* (*C. elegans*), which has four *AChE* genes (ACE-1 to –4), a single *AChE* gene has been identified (Hall and Spierer, 1986;Combes et al., 2000;Combes et al., 2003;Johnson et al., 1988). Comparison of the available gene sequences revealed that the G+C rich gene is highly conserved and shares a great deal of similarity between different species (Chatonnet and Lockridge, 1989;Legay et al., 1993b). For instance, mouse *AChE* shares 88%, 61%, and 37% identity with bovine, *Torpedo*, and *Drosophila AChE* genes, respectively (Legay et al., 1993b;Rachinsky et al., 1990). The syntenic region where the gene is located is conserved amongst human, mouse, and Zebrafish, even though it is located on a different chromosome for each species, chromosome 7 (7q22) in humans (Getman et al., 1992;Ehrlich et al., 1992), chromosome 5 in mouse (Rachinsky et al., 1992), and linkage group 7 in Zebrafish (Bertrand et al., 2001).

The gene generally consists of six exons, of which the first is non-coding and the last two (exon 5 and exon 6) are alternatively spliced (see Figure 3 and *1.1.4 Transcripts and alternative splicing*) resulting in different transcripts and molecular forms of AChE. The basic intron/exon structure of the gene is conserved between vertebrates and invertebrates and across species; although, the relative size of the intronic regions varies. Thus the

open reading frame (ORF) of the mouse *AChE* gene is encoded within 4.5-4.7 kb of DNA whereas *Torpedo AChE* stretches over 25 kb of DNA (Li et al., 1991;Maulet et al., 1990). In the mammalian gene, exons 2, 3, and 4 encode the catalytic or common domain and exons 5 and 6 encode the variable C-terminal domains and thus, the different molecular forms (Legay et al., 1993b;Rachinsky et al., 1990;Schumacher et al., 1986).

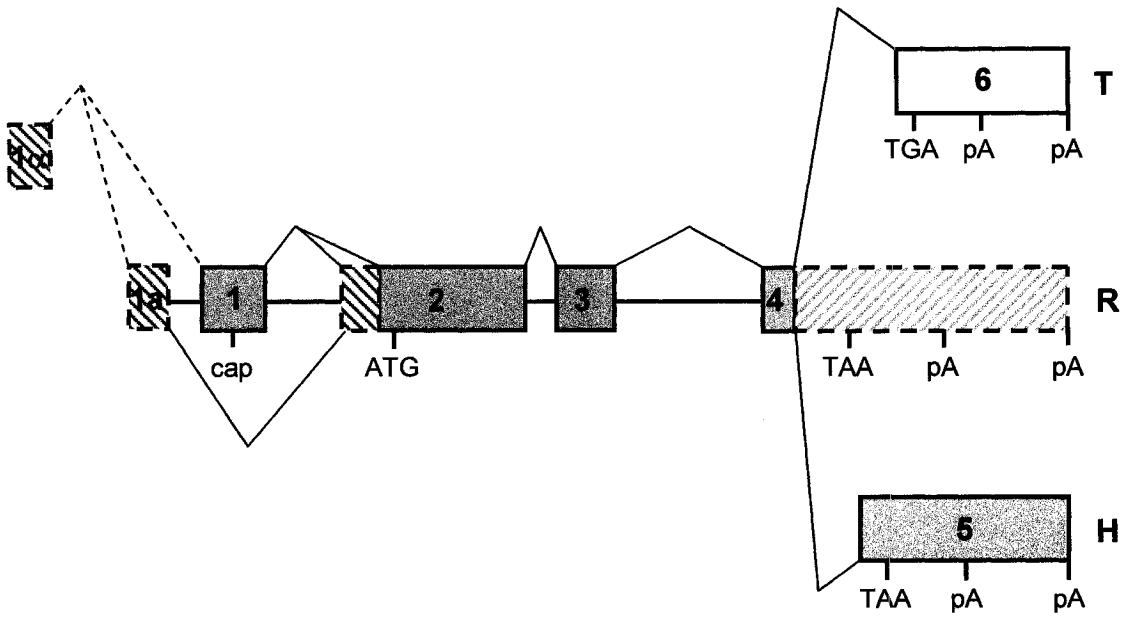

Figure 3: AChE gene organization and alternative splicing. The *AChE* intron/exon organization and three principal transcripts (T, R and H) are depicted. The 5'-cap site (cap) and translation initiation (ATG) sites are indicated in exons 1 and 2, respectively. Translation termination (TGA and TAA) and polyadenylation signals (pA) in the 3'-exons are indicated. Splicing of alternate exon 1 variants (1α and 1a) is hypothetical and therefore, indicated with a dashed line. Figure adapted from Legay (2000) and Taylor and Radic (1994).

Although the function and translation of the ORF is well defined, less is known about the roles and sequences of the more variable 5' and 3' non-coding regions. Initial cloning and sequencing results of the *AChE* gene in *Torpedo* and mouse indicated that there are alternative 5'non-coding exons (Ekstrom et al., 1993). RNase protection assays (RPA)

performed with probes designed to the 5' non-coding region confirmed that at least two other upstream non-coding exon 1 variants exist for mouse *AChE* (Li et al., 1993), one of which is expressed specifically in the brain. Subsequently, Atanasova *et al.* (1999), using 5' rapid amplification of cDNA ends, identified an upstream non-coding exon, termed exon 1α, in the mouse *AChE* gene that encoded an mRNA highly expressed in brain (Atanasova et al., 1999). More recently, a search of the mouse and human expressed sequence tags (EST) databases using the 5' region of mouse and human *AChE* genes, respectively, revealed the presence of multiple putative upstream exon 1 splice variants (Meshorer et al., 2004). In particular, a putative coding exon 1 variant, termed exon 1e in mouse and exon 1d in human, was identified that shares 79% similarity between mouse and human. This exon 1 variant could add ~ 60 amino acid residues to the exon 2 coding region and thereby, modify the N-terminal domain of the protein. Whether this additional domain has any significance to protein function remains to be determined.

In addition to the 5'-end, the 3'-end of the gene is also variable. Northern blots performed with different tissues demonstrated the presence of two or more different sized transcripts. This banding pattern was suggested to result primarily from choice of polyadenylation signals in the 3'-untranslated region (3'-UTR) of the last coding exon (exon 6) (Li et al., 1991). Although the number of polyadenylation signals and the length of the 3'-UTR is variable between species, the sequence identity between the short 3'-UTRs of mouse, rat and human is 93% and 83%, respectively (Maulet et al., 1990); therefore, suggesting that this domain is functionally important to AChE expression (see *2.5 Results*).

1.1.4 Transcripts and alternative splicing

As mentioned above, the *AChE* gene encodes for alternative transcripts, which in turn encode for different molecular forms. Alternative splicing was initially suggested by alignment of the sequences obtained following AChE peptide digestion, which differed predominantly and significantly in the C-terminal domain (Gibney et al., 1988). Subsequently, RNase protection assays, performed with different probes to the C-terminal half of AChE, confirmed the existence of three different protected transcripts resulting from splicing within the 3'-end of the gene (Schumacher et al., 1988;Maulet et al., 1990;Rachinsky et al., 1990). Closer analysis of the mouse *AChE* gene demonstrated the presence and alternative usage of exons found in the 3'-end that were originally termed 3R, 3H and 3A (Li et al., 1991) and following sequencing of the rat gene were termed exon 4-readthrough, exon 5, and exon 6, respectively (Legay et al., 1993a;Legay et al., 1993b see for review Legay, 2000). Results of this later study demonstrated that exon 6 (3A), encoding the tail (T) transcript, is predominantly used in most tissues. This exon encodes a terminal cysteine allowing for formation of dimers and tetramers via disulfide bonds and formation of disulfide bonds with ColQ and PRiMA. Exon 5 (3H), termed the hydrophobic (H) transcript, encodes for one or two terminal cysteines that form disulfide bonds and form a cleavage signal that enables addition of a GPI-anchor to the protein. This transcript, therefore, generates GPI-anchored dimers of AChE. The exon4-readthrough (3R) containing transcript consists of a transcribed domain that extends beyond exon 4 and continues to exon 5 and is, therefore, described as a readthrough (R) transcript. This transcript encodes for a secreted form of the protein. Note, however, that there are very few studies that have reported measurable amounts of

the molecular form encoded by this splice variant (see Perrier et al., 2005 and compare with Kaufer et al., 1998;Nijholt et al., 2004;Sternfeld et al., 2000). Further 3' in the gene are two poly-adenylation signals, which can be used independently of the choice of upstream exon (Legay, 2000). Accordingly, two transcripts of different lengths, 2.4 and 3.2 kb, have been observed on Northern blots.

Several studies have shown that the alternatively spliced transcripts are expressed in a tissue-specific manner. For example, many tissues, such as muscle, spleen, and brain, express mRNAs corresponding to the T transcript but not the H transcript (Legay et al., 1993b;Legay et al., 1999). Furthermore, injection of a cDNA corresponding to the T transcript into Xenopus embryos resulted in expression of the transgene product in muscle and neurons only, whereas injection of a cDNA of the R transcript resulted in expression of the transgene product in epidermal tissues (Seidman et al., 1995;Legay et al., 1999). Since both cDNA constructs used in this later study had identical unrelated promoter regions, the observed pattern of expression for the different splice variants was controlled by the ORF and in a tissue-specific manner. In addition, choice of poly-adenylation signal appears to also occur in a tissue-dependent manner, such that neurons express principally the short transcript, whereas both lengths of transcript are found in muscle (Legay et al., 1993b;Rachinsky et al., 1990). These observations, and the existence of multiple molecular forms and transcripts specific to given tissues, indicate that *AChE* gene expression is highly regulated by defined intrinsic and extrinsic factors. Accordingly, tissue-specific expression, and the diverse factors effecting the abundance and regulation of protein and transcript levels in various excitable and non-excitable tissues, has been the subject of numerous studies.

1.2 AChE expression in skeletal muscle

AChE is one of the key proteins located within the NMJ that is essential for normal nerve-stimulated muscular activity, as evidenced by muscle weakness and NMJ abnormalities observed when AChE is overexpressed or absent (Adler et al., 2004;Andres et al., 1997;Andres et al., 1998 and see for recent review Rotundo, 2003). Consequently, it comes as no surprise that expression of this enzyme has been extensively studied and characterized in skeletal muscle of numerous species. In higher vertebrates, such as birds and mammals, the molecular forms expressed in muscle derive solely from the T transcript, while in *Torpedo* and Xenopus the H transcript is also used to produce GPI-anchored AChE (Inestrosa et al., 1988). The molecular forms expressed in adult muscle of most mammals, therefore, include asymmetric collagen-tailed forms, mostly A12 with a minor portion of A8, and globular G4 and G1 forms (Hall, 1973;Vigny et al., 1976b;Younkin et al., 1982;Weinberg and Hall, 1979).

Depending on the muscle fiber type, AChE transcripts and molecular forms are preferentially expressed at the junctional domain in adult muscle. Generally though, throughout the muscle fiber more AChE catalytic activity is found intracellularly, due to the presence of G1 and G4 molecular forms, than in the cell-associated extracellular space (Younkin et al., 1982;Hall, 1973). At the NMJ, AChE activity is found predominantly in the junctional infoldings (Donoso and Fernandez, 1985) and corresponds to A12 and G4 molecular forms in equal proportion (Younkin et al., 1982;Hall, 1973;Vigny et al., 1976b and see for review Aldunate et al., 2004;Rotundo, 2003;Rotundo et al., 2005). The A12 molecular form is associated with the basal lamina

in the extracellular space and not with the cell membrane, as was demonstrated by McMahan *et al.* (1978), who found that AChE activity persisted in the basal lamina after degeneration of both nerve and muscle (McMahan et al., 1978). Specifically, ColQ associates with the basal lamina through interactions with perlecan (Arikawa-Hirasawa et al., 2002;Peng et al., 1999), as exhibited by release of AChE from the endplate region by heparin (Torres et al., 1983;Torres and Inestrosa, 1983), and by the absence of AChE in the NMJ of perlecan-deficient mice (Arikawa-Hirasawa et al., 2002). There are, however, other possible anchors for AChE at the NMJ, such as muscle specific kinase (MuSK) (Cartaud et al., 2004). In comparison, the G4 molecular form present at the extracellular surface was proposed to associate with PRiMA and the sarcolemma (Perrier et al., 2002).

Expression and localization of these different molecular forms varies depending on the developmental stage of the muscle, the region studied (synaptic or extra-synaptic) and the muscle fiber-type. In particular, expression levels of AChE mRNAs and molecular forms are highly variable during maturation of the muscle. During development, there are two separate events that influence expression of AChE, the first occurs during myogenic differentiation of myoblasts into multinucleated myotubes, and the second occurs during the initial contact of nerve and muscle and synaptogenesis. In mature muscle, AChE transcript and protein expression are further influenced by activity of the nerve, and by circulating and nerve-derived trophic factors. Consequently, expression of AChE in muscle is modulated by a variety of factors both intrinsic and extrinsic.

1.2.1 Expression during myogenesis

AChE expression during myogenic differentiation has been studied predominantly in cultured muscle cell lines or primary cultures of embryonic muscle. These studies have demonstrated that prior to myogenic differentiation myoblasts express very little AChE transcript or protein and the molecular forms expressed are restricted to G1, similarly to what is observed *in vivo* during skeletal muscle development (Rotundo and Fambrough, 1979). During the course of differentiation, as myoblasts fuse to form contracting myotubes, levels of AChE T transcript increase dramatically by 4- to 10-fold depending on the study (Angus et al., 2001;Fuentes and Taylor, 1993). Expression of G4 followed by A12 molecular forms, also increases as myoblasts start to fuse (Inestrosa et al., 1983;Legay et al., 1995). Undifferentiated cells express all three AChE splice variants, albeit, at significantly different levels. As differentiation proceeds, alternative splicing is specifically directed to the T transcript and formation of G4 and asymmetric forms as was demonstrated both by RT-PCR and transfection of a plasmid construct, containing the R, H, and T exons, into myoblasts (Legay et al., 1999;Legay et al., 1995).

Notably, expression of the A12 molecular form coincides temporally with development of spontaneous contractions (fibrillations) (Fernandez-Valle and Rotundo, 1989). This electromechanical activity is important to the increase in AChE enzyme activity and expression of globular and asymmetric forms of AChE because treatment with Tetrodotoxin (TTX), which blocks the sodium channels involved in propagation of action potentials, decreased the level of cell-associated extracellular AChE activity and A12 molecular form (Brockman et al., 1984;Fernandez-Valle and Rotundo, 1989). Maintaining the sodium channels in an open state had the opposite effect, such that total

AChE activity and the amount of asymmetric forms expressed were increased (De La Porte et al., 1984). Therefore, these differentiation studies provided the first indication that AChE expression, specifically of A12, is regulated by intrinsic mechanisms, including contractile activity (Fernandez-Valle and Rotundo, 1989).

1.2.2 Expression and compartmentalization in developing muscle

Both co-culture and *in vivo* studies have examined AChE transcript and protein expression in muscle following myogenic differentiation and during synaptogenesis to demonstrate the specific roles and importance of the nerve and muscle in expression, localization, and clustering of AChE mRNA and protein during muscle development. Studies performed *in vivo* revealed that during early embryonic development (embryonic day 10-14), muscle expresses predominantly G1 and G4 globular forms and was only beginning to express asymmetric forms, which represented ~25% of total AChE activity (Vigny et al., 1976b;Koenig and Vigny, 1978;Tennyson et al., 1971). As well, the ratio of globular to asymmetric forms changes as the muscle develops, such that at postnatal day 2, when myotubes are spontaneously contracting, AChE expression in muscle consists of 50% globular forms and 50% asymmetric forms found in both endplate and non-endplate regions of the muscle (Chapron et al., 1997;Grubic et al., 1995). Increasing expression of A12, therefore, results from the intrinsic capacity of the myotubes to contract prior to formation of functional synapses, inasmuch as fully functional synapses are not established until ~14-days postnatal (Koenig et al., 1982).

As skeletal muscle matures and synapses are formed, from 7-days postnatal to ~60-days postnatal (adult), whole muscle total AChE activity levels were demonstrated to

decrease by ~7-fold (Fernandez and Seiter, 1984). Specifically, studies have shown that total AChE activity levels decrease to a greater extent in non-endplate regions than in endplate regions, such that AChE activity is detected equally in the endplate and non-endplate regions at 7-days postnatal; whereas in adult muscle a greater proportion of AChE activity is measured in the endplate region (78% of total activity) than in the non-endplate region (22% of total activity) (Chapron et al., 1997;Grubic et al., 1995;Koenig et al., 1982;Fernandez and Seiter, 1984).

Expression of A12 follows the same pattern of change as total AChE activity in whole muscle during this developmental phase. For example, at the developing endplate, subcellular localization of A12 changes during maturation, such that in young muscle (postnatal day 7) this form is found predominantly intracellularly and as the animal ages the pool of intracellular A12 decreases and the extracellular pool increases (Fernandez and Seiter, 1984). In non-endplate regions of the muscle intracellular levels of A12 also decrease, but there is no concomitant increase in the extracellular pool (Brzin et al., 1981;Fernandez and Seiter, 1984;Rieger et al., 1984). Notably, the motor nerve also affects localization of extracellularly expressed molecular forms, such that during synaptogenesis AChE migrates in the basal lamina from non-endplate regions to the site of nerve-muscle contact. Clustering and lateral migration of pre-existing AChE molecules at the site of nerve-muscle contact was demonstrated using fasciculin, the mamba snake venom toxin that binds specifically to AChE (Karlsson et al., 1984), to pre-label AChE molecules expressed on the extracellular surface of the cell (Peng et al., 1999). Together, these studies have demonstrated that not only does AChE molecular form expression in skeletal muscle decrease, particularly in the non-endplate regions of

the muscle, but it also becomes specialized during establishment of nerve-muscle contact. Indicating, therefore, that the motor nerve is able to drive expression of AChE at the synapse while directly or indirectly decreasing its expression outside of the junctional region, as well as cluster or attract pre-existing protein to the developing synapse (see for review Gaspersic et al., 1999).

During muscle development, expression of AChE transcripts also becomes compartmentalized to specific domains within the muscle fiber. Within differentiated cultured myotubes, prior to development of spontaneous contractions, AChE mRNA is widely distributed throughout the myotube and co-localized with the majority of nuclei (Tsim et al., 1992). At the subcellular level, Rotundo et al. (1990) demonstrated that AChE transcripts were preferentially translated and protein assembled in the endoplasmic reticulum and Golgi apparatus associated with the nucleus where the transcripts were transcribed (Rotundo, 1990). This was further demonstrated in multinucleated fused myotubes of quail and mouse origin, in which quail AChE protein was specifically localized to the extracellular matrix directly above the quail nuclei or within a 20-μm radius from the centre of the quail nucleus (Rossi and Rotundo, 1992). Accordingly, these studies advanced that in multinucleated muscle cells individual nuclei are functionally compartmentalized with respect to transcription, translation, and protein assembly.

Studies performed with co-cultured muscle and neurons demonstrated that subsequent to onset of contractions, AChE mRNA is specifically localized to nuclei below the site of nerve-muscle contact (Grubic et al., 1995). This observation was supported by *in vivo* studies demonstrating by *in situ* hybridization for the T, R, and H transcripts at early

embryonic ages (E14 and E16) that during establishment of nerve-muscle contacts the T transcript begins to accumulate at endplates in diaphragm and leg muscles (Legay et al., 1995). As well, AChE mRNA is compartmentalized in innervated adult muscle fibers as demonstrated by significantly higher levels of transcripts in junctional domains than in extrajunctional regions of muscle fibers (Jasmin et al., 1993;Michel et al., 1994). These studies, therefore, suggest that the abundance of AChE transcripts is functionally compartmentalized in multinucleated muscle fibers such that the nerve or signals from the overlying extracellular region (Rossi et al., 2000) directly influence the adjacent nuclei and AChE expression, resulting in preferential expression by the nuclei underlying the nerve terminal.

1.2.3 Fiber-type specific expression

Although the A12 molecular form is preferentially expressed in innervated over non-innervated regions of the muscle, the relative level of expression depends on the muscle fiber-type. Measurement of AChE molecular form profiles in differentiated cultured satellite cells from fast-twitch and slow-twitch muscle revealed that both types of muscle display a similar pattern of AChE expression in the non-innervated state (Boudreau-Lariviere et al., 1997;Barjot et al., 1993;Boudreau-Lariviere et al., 2000b). However, as development proceeds, expression of AChE activity and molecular forms between fast-twitch and slow-twitch muscles begins to differ. Specifically, developing slow-twitch muscles, as compared to fast-twitch muscles, express higher levels of asymmetric forms for a longer period of time before they decline to adult levels of expression (Sketelj et al., 1991); which are greater in adult fast-twitch muscles at the mRNA and activity levels

than slow-twitch muscles (Cresnar et al., 1994;Boudreau-Lariviere et al., 1997;Sketelj et al., 1998;Pregelj and Sketelj, 2002;Gisiger and Stephens, 1982). Importantly, fast-contracting and slow-contracting muscles have different complements of molecular forms, such that fast muscles have a higher content of G4, whereas slow muscles have more modest levels of G4 and an increased proportion of A8 relative to A12 (Boudreau-Lariviere et al., 1997;Bacou et al., 1982;Gisiger and Stephens, 1982;Gisiger and Stephens, 1983;Jasmin and Gisiger, 1990) and see for review (Fernandez et al., 1996). Fast-twitch muscles also show a preferred accumulation of A12 in their endplates (Sketelj et al., 1998), whereas slow-twitch muscles have more extrajunctional A12 and AChE accumulates in the myotendinous junctions (Sketelj et al., 1991). These differences are driven in part by intrinsic differences between the muscle fiber-types, but mostly by the influence of the motor nerve. This was demonstrated *in vivo* using various approaches, including extrinsic nerve stimulation of a fast nerve with a slow pattern of electrical activity and vice versa, and denervation of one muscle fiber-type followed by reinnervation with the other type of nerve that induce a nerve-dependent fiber-type switch (Cresnar et al., 1994;Sketelj et al., 1991;Sketelj et al., 1998). Thus, a typically slow-twitch muscle was induced to express a fast-twitch profile of AChE transcript and protein, and vice-versa; suggesting, therefore, that nerve-evoked activity has a greater influence on AChE muscle expression than the intrinsic properties of the muscle.

1.2.4 Activity-dependent modulation of expression

Denervation studies have further demonstrated the important role of nerve-induced muscular activity on the overall expression of AChE transcripts and molecular forms.

Specifically, transection of the sciatic nerve, which results in denervation of all the hindlimb muscles downstream of the injury, results in dramatic decreases in both AChE asymmetric forms and mRNA levels in all muscle types examined (Boudreau-Lariviere et al., 1997;Sketelj et al., 1998;Boudreau-Lariviere et al., 2000a). However, fast-twitch muscles respond more severely than the slower counterparts, showing a greater decrease in G4 and total activity levels and, thus, exhibit profiles characteristic of slow-contracting muscles. Similarly, paralysis of skeletal muscle by chronic TTX treatment results in dramatic decreases of both AChE activity and mRNA levels at the site of nerve muscle contact (Michel et al., 1994). Notably, denervation has a more dramatic effect on AChE expression levels than TTX treatment, suggesting that other factors, such as integrity of the synaptic structure and nerve-derived trophic factors exert an equally important regulatory role in maintaining AChE expression in muscle. By contrast, enhanced motor activity, resulting from exogenous stimulation of the motor nerve or from exercise, increases AChE activity levels, transcripts, and, specifically, levels of G4 (Fernandez and Donoso, 1988;Gisiger et al., 1991;Jasmin and Gisiger, 1990;Jasmin et al., 1991;Sveistrup et al., 1995 and see for review Fernandez et al., 1996).

Interestingly, although mammalian skeletal muscles respond to decreased nerve-evoked activity with a concomitant decrease in AChE expression levels and vice-versa for increased activity, avian skeletal muscles respond in the opposite manner. Accordingly, denervation of chick muscle results in increased total AChE activity, globular forms and mRNA levels (Linkhart and Wilson, 1975;Rimer and Randall, 1999). The reason for these species differences remains to be explored. In addition, the existence of an inactive pool of AChE proteins has predominantly been characterized in

avian tissues (muscle and brain) and chick primary culture systems (Chatel et al., 1993;Rotundo, 1988). The functional significance of the inactive pool is still being explored; however, one hypothesis suggests that in response to physiological demand or stresses the inactive molecules could shift to the active pool (Lazar et al., 1984;Rotundo, 1988;Chatel et al., 1993;Eichler and Silman, 1995).

1.3 AChE expression in neurons

In the CNS and PNS, AChE expression is principally associated with neurons that are either cholinergic, those that use acetylcholine as a neurotransmitter, or cholinoceptive, those that receive cholinergic input. AChE expression has also been shown in neurons with no apparent association to the cholinergic system (see further) and in glial cells such as oligodendrocytes and astrocytes (Gisiger et al., 1978;Koenig and Koelle, 1961;Razon et al., 1984;Darvesh and Hopkins, 2003;Wright et al., 1993). This atypical expression has led to the identification of non-cholinergic functions for AChE (see *1.6 Non-cholinergic functions of AChE*). At the subcellular level, AChE protein and activity are found diffusely throughout the cytoplasm, as well as in dendrites of cholinoceptive neurons and axons of cholinergic neurons (Thullbery et al., 2005;Hosli and Hosli, 1970;Mesulam and Geula, 1991). Because AChE protein is transported along axons or dendrites to cholinergic synapses distant from the cell body where it is translated, AChE activity and mRNA do not always perfectly co-localize in the brain.

RT-PCR and *in situ* hybridization experiments demonstrated that neurons predominantly express the T transcript, but a minor proportion of H and R transcripts is also detected and constitutes less than 1% of total AChE mRNA found in the brain (Li et

al., 1991;Perrier et al., 2003;Hammond et al., 1994). Within the brain, G4 is the principal molecular form observed in neurons, whereas a significant amount of asymmetric form is measurable in neurons of the spinal cord and PNS (Perrier et al., 2003;Marchand et al., 1977;Rieger and Vigny, 1976;Razon et al., 1984;Vigny et al., 1976a;Rieger et al., 1980a;Di Giamberardino and Couraud, 1978;Fernandez et al., 1979;Gruber and Zenker, 1973;Koelle, 1955;Schlaepfer and Torack, 1967 and see for review Massoulie and Bon, 1982). G4 is localized to the extracellular membrane in association with PRiMA, loosely bound in the extracellular space, and secreted into the cerebral spinal fluid (CSF) (Perrier et al., 2003;Atack et al., 1987 and see for review Greenfield, 1984). Notably, the membrane bound G4 form represents greater than 85% of AChE expressed in the brain (Perrier et al., 2003). Similarly to muscle, AChE expression patterns vary during neuronal differentiation and development of the nervous system, as well as in response to neuronal injury.

1.3.1 Expression during neuronal differentiation and CNS development

Many of the studies that have characterized cellular AChE expression during neuronal differentiation have used cultured cell lines that mimic fairly closely the process of neuronal differentiation when cultured in appropriate conditions, such as PC12 pheochromocytoma and P19 neuroblastoma cell lines (Jones-Villeneuve et al., 1982;Greene and Tischler, 1976). Regardless of the cell line or the differentiation stimulus, AChE T transcript levels, specifically, increase considerably during the process of differentiation (Coleman and Taylor, 1996;Lebel et al., 1994). With the exception of P19 cells, these cultured cell lines all express AChE globular forms (G1, G2 and G4) and

activity prior to neuronal differentiation (Coleman and Taylor, 1996;Inestrosa et al., 1981;Lazar and Vigny, 1980;Rieger et al., 1980b). Generally, AChE activity and protein levels increase as the cells proliferate (Thullbery et al., 2005;Rieger et al., 1980b). In PC12 cells specifically, expression of the molecular forms varies depending on the density of undifferentiated cells such that at a higher cell density smaller molecular forms predominate (G1 and G2) and during the logarithmic phase of growth the larger globular form are predominant (G4) (Rieger et al., 1980b). When the different cell lines are induced to differentiate into neurons, AChE activity increases exponentially. In PC12 cells, in particular, differentiation induces expression of asymmetric molecular forms (Rieger et al., 1980b). Together, these studies demonstrate that AChE expression paradigms during differentiation are similar between muscle and neurons, in that transcript and the order and pattern of molecular form appearance are comparable. Notably, excitable cells express an elementary combination of AChE proteins and mRNA prior to functional activation.

AChE expression has also been characterized *in vivo* during the early stages of embryonic development and organization of the CNS (Layer, 1983 and see for review Layer and Willbold, 1995). The earliest detection of AChE activity was found in cells that were leaving the cell cycle, migrating and beginning to differentiate (Stage 13 of chick development) (Layer and Willbold, 1995;Miki et al., 1983;Ravin et al., 1952). As brain development progresses from embryonic to early postnatal, the overall level of AChE activity increases up to ~15-fold (Marchand et al., 1977;Layer and Sporns, 1987;Inestrosa et al., 1994). Generally, the increase in activity is due to increased abundance of G4, such that G4 represents ~10% of the activity at E10, 50% at P0 and

90% in adult brains (Inestrosa et al., 1994;Rakonczay et al., 1981). Notably, the developmental age during which peak levels of AChE activity are observed correlates with the end of cell proliferation (Layer and Sporns, 1987). As the nervous system matures, and connections increase and become specialized, AChE activity slowly decreases in most regions of the brain.

In this regard, some regions of the brain express AChE only transiently during development. For example, in rat early postnatal life (~P9-P10), AChE activity was found in cell bodies of pyramidal cortical neurons of layers II, III and V. By P15, however, the level of AChE activity observed in cell bodies decreased dramatically and was almost undetectable; whereas activity localized to axons that arise from the basal forebrain area, a cholinergic area of the brain, was increased, and maintained during adulthood (Geula et al., 1993;Geula et al., 1995). Similarly, during early postnatal life (beginning around P5), AChE activity from innervating fibers of the septal region, another cholinergic domain, progressively accumulate in regions surrounding pyramidal and granular cells of the hippocampus and dentate gyrus, and slowly disappear from interneurons of the hippocampus and hilar region of the dentate gyrus (Vijayan, 1979). Appropriately, AChE expression levels in distinct regions of the brain are modulated during development by specialization of the different domains and connections formed.

In addition, other research groups have observed transient expression of AChE activity in axons and terminals derived from thalamic nuclei, a structure that is not normally cholinergic in the adult, and extending to various sensory cortical areas (Robertson, 1987;Kristt, 1989;Robertson et al., 1989;Robertson et al., 1991). Specifically, dorsal thalamic nuclei, the principal sensory thalamic nuclei, and their

axonal projections to the somatosensory, visual and auditory primary sensory cortical areas all show transient AChE reactivity early during development (E12-13) (Schlaggar et al., 1993). Once the thalamus is developed and the neuronal projections are contacting their targets, at ~P19, AChE expression decreases. For instance, there is very little AChE activity in the adult ventral medial geniculate nucleus, ventral lateral geniculate nucleus and ventral posterior nucleus in comparison with the developing animal (~P7-P10) (Robertson et al., 1989;Robertson, 1987). Transient expression of AChE in these sensory thalamic nuclei coincides with development of the corresponding cortical regions (layer IV) and thalamocortical axonal ingrowth (Robertson et al., 1989). Transient AChE expression in specific brain regions during development, suggests that some neurons are transiently cholinergic, that the enzyme is expressed to signal or attract cholinergic innervation, or that AChE is acting in a non-cholinergic manner and is involved in the morphogenesis and targeting of the developing neurons and projections (see section *1.6 Non-cholinergic functions of AChE* for more details). Most importantly, these studies reveal that AChE is expressed prior to synaptogenesis and suggest, therefore, that AChE has significant non-cholinergic functions and plays a key role in development.

In situ hybridization and immunological and histochemical techniques have been used to localize AChE transcript, protein and activity in different regions of the adult brain. As in the developing brain, these studies showed that AChE activity and transcripts are diffusely expressed in cholinergic and cholinoceptive neurons, as well as in the neuropil and CSF of the adult brain (Bernard et al., 1995;Hammond et al., 1994;Landwehrmeyer et al., 1993). Generally, the level of expression of AChE mRNA corresponds with the relative amount of AChE activity in most regions of the brain

(Geula and Mesulam, 1989;Mesulam et al., 1991;Landwehrmeyer et al., 1993;Kostovic and Rakic, 1980;Kostovic et al., 1988;Mesulam and Geula, 1988). There are, however, some exceptions and irregularities observed, such as exceptionally high levels of AChE activity in some regions of the brain that are not significantly cholinergic or cholinoceptive, striatum versus somatosensory cortex for example. Discordance between the level of AChE protein and mRNA has also been reported. For instance, the cerebellum has low AChE activity but high mRNA levels (Barmack et al., 1992;Hammond et al., 1994). In addition to these discrepancies, some significant differences between the level of expression of AChE, acetylcholine, choline acetyltransferase (ChAT), and cholinergic innervation were also observed. Most notably, the substantia nigra, cerebellum, globus pallidus and hypothalamus all have disproportionately low levels of ChAT in comparison to AChE activity (see for review Greenfield, 1991). Thus, imbalances in AChE expression levels that are not associated with development or the projection of cholinergic axons into non-cholinergic domains denote the possibility of additional non-catalytic functions for AChE in the adult (see *1.6 Non-cholinergic functions of AChE*).

1.3.2 Expression in motoneurons and neurons of the PNS

Developing motoneurons, and sensory and sympathetic neurons are the first cells to express AChE protein and transcripts during embryonic development (Bernard et al., 1995;Fairman et al., 1976;Layer et al., 1988;Koenigsberger et al., 1998). Accordingly, AChE is expressed in these neurons prior to synaptogenesis, similarly to developing muscle and neurons in the brain (Biagioni et al., 1989;Fairman et al., 1976;Eranko,

1972;Klinar and Brzin, 1978). Several studies have shown that within the spinal cord, AChE activity is localized to the cell bodies and white matter early in embryogenesis; however, in the adult spinal cord, AChE activity and transcripts are found almost exclusively in the grey matter and mostly in the ventral and intermediolateral horns where motoneurons reside (Navaratnam and Lewis, 1970;Tsim et al., 1997;Mis et al., 2003;Yew and Chan, 1999). Nevertheless, in mature spinal cords some AChE histochemical stain is found in thick white matter fibers, located mostly in the thoracic and sacral regions of the spinal cord, that are extending out from the grey matter towards the ventral roots (Tsim et al., 1997). As with developing neurons of the brain, levels of AChE expression decrease following synaptogenesis and during the later postnatal phases (Tsim et al., 1997).

Although motoneurons anatomically belong to the CNS and express predominantly the T transcript (Mis et al., 2003), they express a different complement of molecular forms from neurons in the brain. Specifically, mammalian and avian motoneurons express four different AChE molecular forms, the three globular forms G1, G2 and G4 and the asymmetric form A12 (Bon et al., 1979;Lewis and Shute, 1966). All four molecular forms of AChE are also found within the sciatic nerve, albeit in variable amounts, such that G4 accounts for the greatest proportion of AChE (63% of total AChE activity) and A12 the smallest (1.6% of total activity) (Di Giamberardino and Couraud, 1978;Inestrosa and Alvarez, 1988). Accordingly, AChE protein is transported from the cell bodies in the spinal cord via the axons to the periphery. Transport was initially demonstrated using cut or ligated sciatic nerves, in which AChE accumulated at the proximal end of the nerve stump (Lubinska and Niemierko, 1971;Brimijoin and Wiermaa,

1978;Kasa et al., 1973;Sjostrand et al., 1970;Kreutzberg, 1969). These studies showed that G4 and A12 molecular forms are the predominant isoforms that accumulate at the nerve stump, while the lower molecular weight forms either remain unchanged or accumulate more slowly (Di Giamberardino and Couraud, 1978;Inestrosa and Alvarez, 1988). Consequently, it was proposed that the different molecular forms use distinct transport mechanisms along the axons, such that A12 and G4 are transported by fast-axonal flow, while G1 and G2 are transported much more slowly by slow-axonal transport (Couraud and Di Giamberardino, 1980;De Repentigny et al., 2003 and see for review Brimijoin, 1983). AChE activity also accumulates at the distal end of the ligated nerve, albeit to a lesser extent, indicating that AChE is transported bi-directionally in axons (Lubinska and Niemierko, 1971;De Repentigny et al., 2003;Brimijoin and Wiermaa, 1978;Ochs, 1972). Transported AChE is subsequently secreted from the motor nerve at the neuromuscular junction, where it participates in terminating neurotransmission or in synaptogenesis (see *1.6 Non-cholinergic functions of AChE*) (Anglister, 1991;Brimijoin et al., 1978;Skau and Brimijoin, 1978).

AChE activity has also been measured in sensory neurons of the dorsal root ganglia (DRG) and sympathetic neurons of sympathetic ganglia. Although these types of neurons are both part of the PNS, they have unique patterns of AChE expression. For instance, sympathetic neurons contain globular and asymmetric forms of AChE and, in this context, are more analogous to motoneurons and muscle (Viana and Kauffman, 1984;Gisiger et al., 1978). In addition, studies performed with primary cultures of superior cervical ganglion (SCG), demonstrated that expression of the A12 molecular form is dependent on presence of nerve growth factor (NGF) in the culture medium and

is strongly influenced by formation of cell-cell contacts (Verdiere et al., 1982). This study suggests that expression of AChE protein and molecular forms is regulated during development by contact with target tissues and NGF, which is one of the principal growth factors secreted from target tissues that is essential to sympathetic neuron survival (Belliveau et al., 1997;Federoff et al., 1992;Sofroniew et al., 2001). This form of regulation is also comparable to the regulation described in muscle.

Sensory neurons, in comparison, express only globular forms of AChE and, thus, have a greater similarity to CNS neurons (Gisiger et al., 1978). Interestingly, sensory neurons are not cholinergic or cholinoceptive and do not form synapses in the DRG body (Biagioni et al., 2000). Presence of ChAT and AChE in the neuronal cell bodies, therefore, denotes non-classical functions for this neurotransmitter system and enzyme in these neurons (Biagioni et al., 2000). For example, recent reviews have proposed that ACh acts as a neuromodulator in neurons, including sensory neurons, and has a role in neurite elongation (Biagioni et al., 2000;Cousin et al., 2005). AChE, therefore, is indirectly implicated in these functions, as is suggested by the developmental defects in sensory neuron extension in Zebrafish mutants that contain either null or activity blocking mutations in the *AChE* gene (Behra et al., 2002). In addition, AChE knockout mice also display sensory-motor defects, such as delayed development of the righting reflex and abnormal gait (Xie et al., 2000). These observations indicate that in sensory neurons AChE is implicated in neuronal development, differentiation and survival.

1.3.3 Expression following neuronal decentralization and axotomy

As in skeletal muscle, AChE expression in neurons is modified by extrinsic factors, such as activity of the pre-synaptic nerve and factors derived from the post-synaptic target. Consequently, any injury to the pre-synaptic nerve or to axons that leads to disrupted synaptic activity, such as a crush or a lesion, results in modifications to the level of expression of AChE. The effects on AChE expression of nerve crush or lesion have been well described for motoneurons and sympathetic neurons.

The downstream effects of nerve injury on AChE expression in motor neurons has predominantly been examined using the facial motor nerve (FMN) and nuclei as a model system in earlier and more recent studies. Initial studies demonstrated that AChE activity in facial nuclei decreased to approximately 60% of control values within the first 2-weeks following FMN axotomy and recovered to ~80% of control values 120-days post-axotomy (Tetzlaff and Kreutzberg, 1984;Engel and Kreutzberg, 1986). A later study showed that decreased AChE protein and activity was paralleled by a similar decrease in AChE transcript level (Fernandes et al., 1998). Furthermore, downregulation of AChE mRNA and activity was prevented by infusion of muscle-derived ligands of the tyrosine kinase B (trkB) receptor, neurotrophin 4/5 (NT4/5) and brain derived neurotrophic factor (BDNF), specifically, (Fernandes et al., 1998). Therefore, AChE expression in motoneurons is influenced by the muscle and muscle-derived trophic factors, just as AChE expression in muscle is influenced by the nerve.

Very similar results were obtained following axotomy of sympathetic nerves. One of the best studied models of peripheral nervous system damage and repair is the SCG found along the carotid arch. Axotomy of the rat SCG results in an ~50% decrease in AChE

activity in the ganglion within the first week following injury (Viana and Kauffman, 1984;Klingman and Klingman, 1969). Deafferentation or decentralization of the SCG, achieved by sectioning the pre-ganglionic nerve, also results in rapid and significant loss of AChE activity to ~50% of control values (Chang, 1977;Gisiger et al., 1978). In comparison, preganglionic sympathetic denervation of newborn (1- or 2-days old) rat SCG does not have any significant effect on the level of AChE expression (Klinar and Brzin, 1979), whereas axotomy of early postnatal animals results in large decreases in AChE expression and cell death due to loss of target-derived trophic factors (Gilad and Gilad, 1988;Hendry, 1975b;Hendry, 1975a). These observations indicate that AChE expression during development is independent of pre-synaptic contacts, but is completely dependent on post-synaptic contacts and trophic factors; whereas in fully developed sympathetic neurons AChE expression is equally influenced by pre- and post-synaptic contacts. Notably, when allowed to recover and repair, AChE expression levels return to ~80-100% of control levels in all systems studied (Dupree and Bigbee, 1994;Gisiger et al., 1978;Farris et al., 1993), suggesting that perhaps some neurons expressing AChE have the capacity to recover from injury and even regenerate. Regenerative capacity of these neurons is, therefore, considered to result from non-cholinergic functions of AChE as experienced in the developing CNS (see *1.6 Non-cholinergic functions of AChE*).

1.4 AChE expression in disorders of the nervous system

As evidenced in the preceding sections, AChE is widely expressed in the CNS, PNS, and muscle, and exhibits complex patterns of expression and regulation. Consequently, significant alterations in AChE expression are often associated with or the cause of

various disease states, including Alzheimer's disease, brain cancer, stress, and congenital myasthenic syndromes.

1.4.1 Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder marked by cognitive and memory deteriorations. Pathology of the disease includes the presence of senile plaques, consisting of amyloid-β (Aβ) peptide complexes, neurofibrillary tangles (NFT) and dysfunction of the cholinergic neurotransmission system. AD is characterized in part by structural changes in cholinergic synapses, such that there is loss of acetylcholine receptors (AChR) and death of ACh producing neurons, resulting in decreased cholinergic neurotransmission and increased relative content of AChE. Currently, the only available treatment that decreases the rate of progression of symptoms, but does not offer a cure, is the use of AChE inhibitors to increase the availability of ACh for neurotransmission.

In recent years, though, it has become apparent that AChE has other detrimental effects in AD. In this regard, histochemical and immunohistochemical staining for AChE in the brains of AD patients revealed a shift in AChE expression from cell bodies to senile plaques (Mesulam et al., 1987;Ulrich et al., 1990). Importantly, this shift was suggested to occur early in the development of senile plaques and disease progression. Consequently, several *in vitro* and *in vivo* studies were undertaken to examine the putative role for AChE in the appearance, formation and stability of senile plaques. Initial *in vitro* studies demonstrated that incubation of purified AChE with Aβ peptides increased the formation and stability of amyloid fibrils (Alvarez et al., 1995;Alvarez et al.,

1998;Alvarez et al., 1997;Campos et al., 1998;Inestrosa et al., 1996a;Inestrosa et al., 1996b). Using different and complementary techniques, these studies also demonstrated that AChE binds to Aβ peptides. The aggregating ability of AChE proteins involves the PAS, but not the active site, as demonstrated using specific inhibitors and blocking antibodies (Reyes et al., 1997;Alvarez et al., 1997). Notably, these studies showed that AChE has greater aggregate forming capabilities on Aβ peptides that are less amyloidogenic or less likely to form fibrils, suggesting that AChE can increase the amyloid properties of Aβ peptides. Finally, the fibril promoting activity of AChE was further demonstrated *in vivo* in transgenic mice that are a cross between AChE overexpressing mice and human amyloid precursor protein expressing mice (Rees et al., 2003). These doubly transgenic mice display early onset of plaque deposition in the regions of the brain that are normally affected in AD.

1.4.2 Brain cancers

Tumors in the brain originate from immature or proliferative neuronal, glial or fibroblast cells. Regardless of the origin, several studies have shown that most brain tumors express AChE activity and transcripts and often to a greater extent than in normal tissues (Barbosa et al., 2001;Wollemann and Zoltan, 1962;Razon et al., 1984;Perry et al., 2002). Recent studies have demonstrated that the intensity of cell labeling for AChE protein, activity and mRNA (T and R transcripts) is markedly different depending on the tumor type and rate of growth (Barbosa et al., 2001;Karpel et al., 1994;Perry et al., 2002). For instance, type II gliomas that present a moderate rate of growth have considerably lower AChE levels than type IV gliomas that have rapid rates of growth and are

considered to be more aggressive and result in poorer prognosis. In addition, a shift in the ratio of R:T transcripts was noted in these cells, such that as the severity of the tumor increases expression of AChE transcripts shifts towards the R transcript (Perry et al., 2002). Together, these results advance that AChE activity levels and expression of the R transcript correlates with tumor grade and prognosis. However, since the protein product derived from the R transcript has not been detected in these types of tumors (Perry et al., 2002), it is unclear whether there is in fact a relationship between this splice variant and pathology. Nevertheless, the correlation between tumor agressiveness and AChE expression suggests a role for the enzyme in undifferentiated developing cells.

1.4.3 Stress and post-traumatic stress disorder.

Post-traumatic stress disorder (PTSD) is an anxiety disorder that is characterized by psychological and physiological problems, including depression, difficulty sleeping and concentrating, and impairment in social and occupational performance (see for review Vieweg et al., 2006). The mechanism by which the brain responds to stress is often compared to the cognitive deficits and psychiatric symptoms that result from exposure to AChE inhibitors (Kamel and Hoppin, 2004). Given this association, the specific role of AChE in PTSD was further examined (Kaufer et al., 1998). In initial studies, expression of AChE protein and transcripts was examined in mice experiencing either forced swim-induced stress, immobilization-induced stress or exposed to AChE inhibitors (Kaufer et al., 1998;Nijholt et al., 2004;Meshorer et al., 2002). In all situations, increased levels of AChE mRNA but not protein were measured in the brain. Closer examination of the AChE transcripts expressed in the brains of the stressed or cholinesterase inhibitor treated

mice demonstrated increased levels of R transcript with no apparent change in the expression level of the other transcripts. Thus, this group suggested a model in which stress modulates gene expression in the cholinergic system to downregulate cholinergic signaling. Specifically, it is the authors' postulation that increased expression of R transcripts has a very important role in protecting the brain against neurodegeneration induced by chronic exposure to stress (Sternfeld et al., 2000).

In a comparative study, non-lethal organophosphate intoxication of mice also resulted in a three-fold increase in AChE-R transcript level, but only in the striatum (Perrier et al., 2005). AChE-R transcript levels, however, are generally extremely low in the brain and represent only ~1% of total AChE transcripts (Li et al., 1991;Perrier et al., 2005). Consequently a modest increase in the amount of R transcript may not have any significant effect to the physiology or cognitive abilities of the animal. Notably, AChE-R derived molecular form has not been observed in any of the brain regions examined at any time period following stress. As a result, it is still unclear as to whether and what possible role AChE may play in PTSD.

1.4.4 Congenital myasthenic syndromes

Congenital myasthenic syndromes (CMS) are a group of disorders in which there is a defect with proteins in the pre-synaptic domain, basal lamina or post-synaptic domain of the NMJ (see for review Engel et al., 1999;Engel et al., 2003a;Engel et al., 2003b). In one rare form of the disease, ~15% of cases, there is a defect in the accumulation of asymmetric forms of AChE at the endplate (endplate AChE deficiency) (Engel et al., 1977). Reduced levels of AChE at the NMJ result in generally disabling weakness that is

increased by exertion. This physiological effect is a consequence of prolonged synaptic currents that evoke repetitive compound muscle action potentials. In addition, the NMJ presents morphological abnormalities, including abnormally small pre-synaptic nerve terminals, simplified post-synaptic folds and a reduced number of AChR. As compared to the predominant forms of CMS, which result from mutations in the AChR, this form of the disease is caused by mutations in ColQ (Donger et al., 1998). ColQ mutations of the PRAD prevent attachment of AChE G4 subunits, while mutations in the collagen domain or C-terminal domain block assemply of the triple-helical structure, or insertion into the basal lamina. Accordingly, ColQ mutations result in a significant decrease in AChE levels at the NMJ. Given the necessary function of AChE at the NMJ, any deficiency in its expression is detrimental.

1.5 AChE expression in non-excitable tissues

AChE expression is not limited to cholinergic tissues and synapses. It is also found in a number of tissues that are non-excitable and tissues that will not continue to express this enzyme in the mature state (Drews, 1975) including, thrombocytic precursor cells in bone marrow and the spleen; megakaryocytes, platelets, and erythrocytes (Lev-Lehman et al., 1997;Paulus et al., 1981;Soreq et al., 1994;Grisaru et al., 2001); fetal chondrocytes (Grisaru et al., 1999a); and tissues such as oocytes and sperm (Malinger et al., 1989;Sastry et al., 1981;Mor et al., 2001 and see for review Grisaru et al., 1999b). AChE expression by the lymphatic and hematopoietic systems, to date, is the best studied example of non-cholinergic AChE function outside of the nervous system. Descriptive studies showed that spleen, lymph nodes, and circulating cells, express all three AChE

transcripts, and monomers and dimers, which were secreted or linked to the membrane by GPI (Ott and Brodbeck, 1984;Gomez et al., 2003;Nieto-Ceron et al., 2004).

AChE expression in myeloid and lymphatic tissues coincides with periods of proliferation and development. For example, during induced differentiation of cultured murine erythroleukemia (MEL) cells, which resemble bone marrow erythroid precursors, expression of AChE activity and T and R transcripts increases significantly (Chan et al., 1998). In several studies, AChE expression was functionally associated with the processes of megakaryocyte proliferation and differentiation (Burstein et al., 1980;Burstein and Harker, 1983;Burstein et al., 1985;Paulus et al., 1981;Chan et al., 1998). In this context, exogenous application of AChE to cultured erythroleukemic cells inhibits cell proliferation and, treatment with AChE inhibitors increases cellular proliferation (Burstein et al., 1980;Paoletti et al., 1992;Burstein and Harker, 1983;Dutta-Choudhury and Rosenberry, 1984;Lev-Lehman et al., 1997;Zajicek, 1954).

Although, the exact role of AChE in the hematopoietic and lymphatic systems is still unclear, numerous studies point to a role in regulating proliferation, differentiation and maturation of the different cell types (Nieto-Ceron et al., 2004). As the cells proliferate AChE expression, but not release, increases; whereas AChE secretion coincides with the process of differentiation and results in subsequent inhibition of proliferation (Paoletti et al., 1992;Soreq et al., 1994). Accordingly, deletions or mutations of the *AChE* gene locus are associated with myelodysplastic syndromes and acute myeloid leukemia, characterized by increased proliferation of myeloid precursor cells (Ehrlich et al., 1992;Stephenson et al., 1996 and see for review Soreq et al., 1991).

1.6 Non-cholinergic functions of AChE

AChE expression in typically non-cholinergic tissues is just one example of the multifaceted character of this enzyme that has resulted in the study of non-catalytic functions for AChE. Several studies examining the developmental expression of AChE have found that AChE is expressed in many different tissues during embryonic development that do not or never will show any evidence of cholinergic neurotransmission (see *1.3.1 Expression during neuronal differentiation and CNS development*) (Drews, 1975;Falugi and Raineri, 1985;Fitzpatrick-McElligott and Stent, 1981;Pannese et al., 1971). In addition, AChE is highly expressed in cholinergic tissues very early and transiently in development, and often prior to expression of ChAT, ACh, and synaptogenesis. In some regions of the brain, there is also a discrepancy between levels of AChE expression in cholinoceptive neurons and cholinergic innervation of these cells. For example, AChE protein density in some cells of the cortex is greater than the expected cholinergic transmission (Mesulam and Geula, 1991), and certain regions of the brain, including substantia nigra, cerebellum and hypothalamus, have disproportionately low levels of ChAT in comparison to AChE activity. Other supporting arguments include, secretion of a soluble form of AChE in the same regions of the brain that express AChE outside of its cholinergic context (substantia nigra) (Chubb and Smith, 1975b;Chubb and Smith, 1975a and see for review Appleyard, 1992). Aside from these circumstantial observations, several different neurotransmitters are known to influence neurite outgrowth (see for review van Kesteren and Spencer, 2003), including ACh, which can decrease neurite length and outgrowth (Small et al., 1995). As a result, some of the best characterized non-cholinergic functions of AChE include stimulation of

neurite outgrowth, which most likely results from its cell adhesive properties, and synaptogenesis (see for review Soreq and Seidman, 2001).

1.6.1 Neurite outgrowth

Some of the first indications that AChE has a role in neurite outgrowth were based on observations of AChE expression during development (see *1.3.1 Expression during neuronal differentiation and CNS development*). In particular, during emigration of neural crest cells in the developing chick brain, AChE is highly expressed and decreases as the cells near their destination (Layer and Kaulich, 1991). Other research groups observed transient expression of AChE activity in axons and terminals derived from thalamic nuclei and extending to various sensory cortical areas (Robertson, 1987;Kristt, 1989;Robertson et al., 1989;Robertson et al., 1991;Brimijoin and Hammond, 1996). AChE expression in very early phases of thalamic nuclei development suggests that it may have a specific role in the differentiation process, but most importantly in axon tract development (Schlaggar et al., 1993). In this context, Kristt *et al.* (1989) observed AChE in points of non-synaptic contact between distal processes of developing thalamic nuclei, suggesting that there is a spatial and temporal association between expression of AChE, process outgrowth and early cell contacts (Kristt, 1989).

Several different culture systems and approaches have been used in order to more clearly demonstrate that AChE has a significant role in stimulating or facilitating neurite outgrowth. In one instance, a greater number of cultured primary neuronal cells extended neurites and displayed significantly longer neurites when grown in the presence of soluble AChE or on an AChE-HSPG substrate (Small et al., 1995;Whyte and Greenfield,

2003;Holmes et al., 1997). In another approach, overexpression of AChE in cultured cells, using either adenoviral vectors consisting of full-length AChE cDNA or by transfection with sense AChE cDNAs, resulted in an increased number of cells extending neurites and a 2- to 3-fold increase in neurite outgrowth (Koenigsberger et al., 1997;Sternfeld et al., 1998;Karpel et al., 1996;De Jaco et al., 2002;Bigbee et al., 2000;Koenigsberger et al., 1997). By contrast, expression of antisense cDNAs to AChE decreases the overall length of extended neurites; and, remarkably, in one case even a small decrease in AChE expression (60% of control values) resulted in a 2-fold decrease in neurite length (Bigbee et al., 2000). Although most studies used cDNAs that encode the T transcript in overexpression studies, AChE overexpression, regardless of the splice variant encoded by the cDNA, can increase neurite outgrowth (De Jaco et al., 2002). However, there are some inconsistencies in the literature since other studies have suggested that the R transcript when overexpressed in either cultured glioma cells or cultured *Xenopus leavis* embryonic neurons is unable to stimulate neurite outgrowth (Karpel et al., 1996;Sternfeld et al., 1998). Nevertheless, results from these studies taken as a whole indicate that AChE promotes neurite outgrowth via the invariable domain by either decreasing the levels of extracellular ACh, by other non-catalytic mechanisms or both. Furthermore, the decreased extension of sensory neuron dendrites exhibited in mutant Zebrafish, in which AChE was absent or catalytically inactive, suggests that AChE has a role in neurite outgrowth (Behra et al., 2002). Note, however, that the authors of this last study have more recently proposed alternative models to explain the defects observed in AChE knockout models, which focus on the additional neuromodulatory roles of ACh (Cousin et al., 2005).

In attempts to identify which AChE protein domain is responsible for these trophic effects, several researchers have used AChE inhibitors with distinct site preferences to treat cells. Initial studies using catalytic site inhibitors have suggested that catalytic activity is involved in the growth promoting activities of AChE (Dupree and Bigbee, 1994;Koenigsberger et al., 1997;Jones et al., 1995;Layer et al., 1993). However, the amount of inhibitor used in these studies was several-fold greater than the amount needed to block enzymatic activity and considered to be toxic to the cell; therefore, growth inhibition could be attributed to the secondary effects of excess drug and not to their primary function (Postuma et al., 1999). In addition, not all catalytic site inhibitors influenced AChE's trophic activity (Koenigsberger et al., 1997;Blasina et al., 2000). Consequently, other sites of action were examined, with a particular focus on the PAS since it was demonstrated to share electrostatic properties with known cell-adhesion molecules (Botti et al., 1998). For instance, inhibition or blockage of the PAS by pharmacological agents or antibodies results in decreased neurite outgrowth (Small et al., 1995;Dupree and Bigbee, 1994;Sharma and Bigbee, 1998;Johnson and Moore, 2004;Johnson and Moore, 2000). More specifically, a recent study used a combination of approaches to identify the specific region of the protein involved in stimulating cell-growth and concluded that particular structural loops associated with the PAS are responsible for the neuritogenic capacities of AChE (Johnson and Moore, 2004). Together these studies support a role for AChE in stimulating and facilitating neurite outgrowth of developing neurons in a catalytically-independent manner.

1.6.2 Cell adhesion

Given that AChE's neuritogenic activity is suggested to be independent of its hydrolyzing activity; several researchers have proposed that AChE has cell adhesive properties. A number of cell adhesion proteins, termed cholinesterase-domain proteins (see Table 1), including neurotactin and glutactin in *Drosophila*, and the vertebrate gliotactin and neuroligin, all share significant sequence and structural similarities with AChE (Auld et al., 1995;Darboux et al., 1996;De La Escalera S. et al., 1990;Ichtchenko et al., 1995;Olson et al., 1990 see for review Scholl and Scheiffele, 2003). In particular, sequence alignment shows that the extracellular domain of these adhesion proteins shares 32-36% sequence identity, depending on which protein, with the AChE catalytic domain, but lacks the catalytic activity (Ichtchenko et al., 1995;Auld et al., 1995;Koenigsberger et al., 1997;Botti et al., 1998).

Various studies that have characterized the functions of cholinesterase-domain proteins also showed that the corresponding domain in AChE can substitute for the endogenous protein domain and interact with the specific receptors; therefore, allowing these hybrid proteins to participate in neurite outgrowth stimulation but perhaps not in activation of the receptors (Darboux et al., 1996;Grifman et al., 1998;Scholl and Scheiffele, 2003;Dean et al., 2003). In this context, transgenic mice that overexpress human AChE in the CNS, have decreased levels of the neuroligin receptor, neurexin 1β, in developing spinal cord motoneurons; suggesting that excess binding of AChE results in receptor downregulation (Andres et al., 1997). The adhesive properties of AChE protein were verified in two independent studies that tested the ability of different cultured cell lines to adhere and extend neurites in the presence of PAS blocking agents

and antibodies directed to this domain (Johnson and Moore, 2000;Sharma et al., 2001). Notably, in both studies adhesion was blocked by both the pharmacological agents and antibodies. Recently, two putative ligands for this binding site, laminin-I and collagen IV, were identified *in vitro*; however, binding has not been confirmed by cell culture or *in vivo* studies (Johnson and Moore, 2003;Paraoanu and Layer, 2004;Johnson and Moore, 2004).

Although the PAS is most often associated with adhesive properties of this enzyme, AChE also contains a carbohydrate HNK-1 epitope, which is a known hallmark of cell-adhesion proteins (Bon et al., 1987;Layer and Kaulich, 1991;Weikert and Layer, 1994). The HNK-1 epitope is a terminal sulphoglucoronyl carbohydrate (Voshol et al., 1996) found on various proteins involved in cell adhesion. A recent study demonstrated that the G4 membrane associated form of AChE, specifically, contains this epitope (Johnson and Moore, 2001;Layer and Kaulich, 1991). Consequently, there are various closely related domains in AChE protein structure which may be involved in cell-adhesion and trophic activity of this enzyme.

1.6.3 Synaptogenesis

In addition to promoting neurite extension, AChE has been associated with formation of cell-cell contacts and synaptogenesis. This theory is once again supported by observations made during studies of AChE expression during development. Specifically, Layer *et al.* (1988) observed AChE expression in myotomes (developing muscle) prior to the arrival of motor axons and, therefore, proposed that AChE participates in formation of the neuromuscular junction (Layer et al., 1988). In addition, cultured rat embryonic

hippocampal neurons express AChE early, within the first day in culture, and expression declines as the neurons continue to differentiate and form functional synapses (Dong et al., 2004). In support of these observations, injection of different human AChE cDNA constructs into blastomeres of two-cell stage *Xenopus leavis* embryos resulted in enlarged NMJ (Sternfeld et al., 1998). This effect depends on the ability of AChE to accumulate in the NMJ and on its catalytic activity. In contrast, Zebrafish that are either null for AChE or express a catalytically inactive mutant form displayed significantly smaller NMJ and dispersed AChR clusters (Behra et al., 2002).

Of particular interest, in a study examining whether AChE overexpression effects synapse development and maintenance, Andres *et al.* (1997) found that transgenic mice expressing human AChE in the central nervous system, including the spinal cord and motoneurons have larger motor endplates in the diaphragm and quadriceps muscle of the hindlimb than control animals, as well as highly variable NMJ ultrastructure (Andres et al., 1997;Andres et al., 1998). These alterations were suspected to influence neuromotor functioning since transgenic mice display impaired performance in a rope grip test designed to assess coordinated sensorimotor activity of the abdomen, back and leg muscles. In comparison, AChE knockout mice (see next section) have fragmented nerve terminals with several smaller projections occupying one site, as well as shallow, irregular, and less numerous junctional folds (Adler et al., 2004). Taken together, these studies demonstrate that AChE, in addition to having a specific and indispensable role in neurotransmission, also has a significant role in many aspects of development of the nervous system and other non-excitable tissues.

1.7 AChE knockout models

Given the diversity and significance of AChE functions, different species of AChE knockout were created to confirm previous findings and, most importantly, to verify the existence of non-cholinergic functions. Mutations in *Drosophila* and Zebrafish *AChE* genes that abolish enzymatic activity result in embryonic lethality (Behra et al., 2002;Hall and Kankel, 1976). However, creation of AChE mosaic mutant flies, for which only some regions of the fly are AChE-null and others normal, clearly demonstrated morphologically abnormal tissue in the AChE-null patches within the neuropil and CNS (Greenspan et al., 1980). In addition, loss-of-function or null mutations in Zebrafish AChE results in loss of motility in early embryos (27-hours post-fertilization), which is amplified by defects in myofibre arrangement and integrity also associated with gene mutation (Behra et al., 2002). These morphological changes observed in both mutant species further support the relevance of non-classical roles of AChE in development.

Unexpectedly, AChE knockout mice are viable, but fail to thrive and die within weeks of birth (P21) (Xie et al., 2000). With proper care, however, they can persist into adulthood (Duysen et al., 2002). Complete analysis of these mice revealed several significant behavioral, homeostatic and physiological defects associated with AChE absence. For instance, mice that do survive to adulthood, due to increased care, fail to mature and never behave as adults. Notably, AChE -/- mice are hypersensitive to any type of stress, such that even loud noises induce seizures and can lead to death; thereby endorsing a role for AChE in the physiological response to stress. At the physiological level, newborn mice are very weak and display a fine motor tremor at rest that persists into adulthood, which may result from ultrastructural changes at the endplate, including

shallow folds that are less numerous and irregular (Adler et al., 2004). Together, these defects confirm AChE's essential role to normal development and survival; but, the fact that these animals can survive, unlike the other nullizygous models, also indicates that the absence of AChE is compensated for by another protein. In this context, treatment of -/- mice with a specific BChE inhibitor was immediately lethal to the nullizygote but completely harmless to normal and heterozygous littermates (Xie et al., 2000). Appropriately, the authors of these studies proposed that BChE, which is not expressed in *Drosophila* or Zebrafish (Behra et al., 2002;Hall and Kankel, 1976), compensates in many ways for absence of AChE at the NMJ (Xie et al., 2000;Duysen et al., 2002 and see for review Darvesh et al., 2003;Giacobini, 2003;Li et al., 2000).

1.8 Molecular mechanisms regulating AChE expression

To date, there is an abundance of information regarding expression levels of AChE activity, molecular forms, and in some cases of the transcripts, in excitable and non-excitable cells during development, maturation and in response to various stimuli. However, our understanding of the molecular mechanisms regulating the plasticity of AChE expression is still unfolding. Since most studies observed parallel changes in protein and mRNA, transcriptional and post-transcriptional mechanisms are considered as the predominant forms of regulation. For more than a decade, several research groups have examined the specific roles of transcriptional and post-transcriptional mechanisms in both excitable and non-excitable tissues.

1.8.1 Promoter region and tissue-specific expression

AChE's proximal promoter region upstream of the first non-coding exon (exon 1) has been isolated and characterized in different species, including *Torpedo*, mouse, rat and human. Similarly to the ORF, the different promoter regions appear to be well-conserved across species. In particular, none of the species-specific identified promoter regions have discernible TATA- or CAAT-boxes and transcription commences at a conserved initiator element a few nucleotides upstream of exon 1 (Chan et al., 1999;Ekstrom et al., 1993;Getman et al., 1995;Li et al., 1993). In comparison, the predicted promoter regions corresponding to putative upstream exon 1 variants in mouse and human were shown to have TATA-boxes (Meshorer et al., 2004). Whether these alternative upstream elements are relevant to AChE expression in tissues still remains to be determined. Initial characterization of the predominant promoter region revealed that it is G+C rich and contains several conserved AP1, AP2, Egr-1 and SP1 sites upstream of the initiator element. Sequential and individual mutation of these sites revealed that the SP1 sites in particular are essential to gene expression and that AP2 sites act as negative regulators of gene expression (Getman et al., 1995).

Further analysis determined that isolated promoter regions drive AChE expression in a tissue-specific manner, since human AChE promoter activity was greatest in cells that normally express AChE protein (Ekstrom et al., 1993;Getman et al., 1995). Thus, transfection of promoter-reporter constructs, consisting of 5'- and 3'-end truncations of the mouse promoter, into cell lines of different tissue origin demonstrated that discrete regions of the promoter drive AChE expression in a tissue-dependent manner (Li et al., 1993). For example, this study showed that the most 5'-end is more important to

expression in neurons than muscle. Closer examination of some of the identified elements in the promoter revealed that Egr-1 sites, in particular, are important for expression in muscle cells (Li et al., 1993). Similar results were obtained when a complete human AChE construct containing the promoter region was transfected into cell lines of different tissue and species origin (Aziz-Aloya et al., 1993;Karpel et al., 1996;Seidman et al., 1994). Together these results suggest that the promoter region is influential to *AChE* gene expression in a tissue-specific manner in all species.

Previous studies have demonstrated that AChE transcripts accumulate in synaptic regions and are significantly more abundant at the synapse than in non-synaptic regions (see *1.2.2 Expression and compartmentalization in developing muscle*) (Jasmin et al., 1993;Legay et al., 1995;Michel et al., 1994). In order to understand this specific accumulation, Chan *et al.* (1999) examined whether distinct transcriptional mechanisms could be attributed to regulating localized *AChE* gene expression (Chan et al., 1999). In this study, the authors demonstrated that the intronic fragment situated between the first non-coding exon and the second exon is essential to *AChE* gene expression in muscle. Further analysis of the intronic region demonstrated that it has enhancing activity and, importantly, that an N-box motif (5'GGAA/T3', targeted by Ets domain containing transcription factors) within the intronic domain plays a critical role in AChE expression in muscle and, particularly, at synaptic sites. Tissue specificity of this region was further confirmed by removal of the first intron, which did not influence reporter expression in hematopoietic cells but abolished expression in cultured myogenic cells. This result was corroborated by additional studies demonstrating that cDNA plasmids lacking the first intron are not active in transfected myoblasts or myotubes (Luo et al., 1998;Cohen and

Randall, 2006). In addition, transgenic mice that lack the first intron show a trembling phenotype along with loss of AChE activity in muscle; thereby confirming the essential role of this intronic promoter region in muscle specific AChE expression (Camp et al., 2005). The AChE promoter, accordingly, acts to modulate AChE expression in a tissue-specific manner and in response to distinct intrinsic and extrinsic signals.

1.8.2 Transcriptional regulatory mechanisms in tissues

1.8.2.1 Role of AChE gene transcription during myogenesis.

During myogenic differentiation, AChE activity, protein and transcript levels increase considerably from myoblasts to myotubes (see section *1.2.1 Expression during myogenesis*). Several studies have attempted to identify the molecular mechanisms responsible for the dramatic increase in expression. Initial studies performed during myogenesis observed that *AChE* gene transcription rate, as measured by nuclear run-on assays, was already elevated in myoblasts, and did not change in the early phases of differentiation (Fuentes and Taylor, 1993). More recently, *AChE* gene transcription rate and promoter activity were examined at different stages during the differentiation process using complementary approaches of nuclear run-on and promoter-reporter assays (Angus et al., 2001). This study, in comparison, demonstrated that transcription rate increases ~2-fold within the first few days of differentiation and returns to control or basal levels in fully differentiated myotubes. Furthermore, in this and other independent studies, promoter-reporter assays performed during the same time course confirmed that the AChE promoter region is active during differentiation (Angus et al., 2001;Cohen and

Randall, 2006;Siow et al., 2002). Specifically, promoter activity depends on the presence of N- and E-box motifs in the intronic region and their interactions with GA-binding protein alpha and beta (GABPα and β, Ets-related transcription factor) and myogenin, respectively (Angus et al., 2001;Cohen and Randall, 2006). *In vivo* studies using transgenic mice expressing a muscle-specific Ets dominant-negative transgene confirmed the specific role of the N-box in controlling AChE expression in muscle, inasmuch as AChE transcript levels are decreased by greater than 50% in muscles expressing the transgene product (de Kerchove d'Exaerde et al., 2002). Notably, both E- and N-box elements and their trans-acting factors are essential to transcriptional activation and expression of several other NMJ specific genes, including nicotinic AChR (nAChR) and utrophin (de Kerchove d'Exaerde et al., 2002;Duclert et al., 1996;Koike et al., 1995;Gramolini et al., 1999).

Although myogenic factors are clearly involved in regulating *AChE* gene transcription during muscle differentiation, other transcription factors may also have important supportive or accessory roles. For instance, as mentioned above SP1, AP2 and Egr-1 were all shown to bind and/or modulate promoter activity in promoter-reporter assays (Getman et al., 1995;Li et al., 1993). In addition, cAMP-responsive element binding (CREB) protein was also demonstrated to bind the human AChE promoter region in response to elevated cAMP levels, measured during the earliest phases of chick muscle differentiation, and to block promoter-reporter activity (Siow et al., 2002). The unusual downregulatory activity of this trans-acting element and factor are postulated to occur as a result of steric interference with adjacent positive transcriptional activators, similarly to AP2 near the initiator element (Getman et al., 1995). Alternatively, these observations

may stem from the inherent differences between species given that these assays were performed in chick muscle, which appears to regulate AChE expression differently from mammalian muscles (see *1.2.4 Activity-dependent modulation of expression*). Given these distinct results, regulation of AChE expression during myogenesis by the promoter region clearly involves the positive and negative interactions of multiple *cis*-and trans-acting factors.

1.8.2.2 Role of the motor nerve in controlling transcriptional regulation

As was described in the above section (*1.2.2 Expression and compartmentalization in developing muscle*), AChE muscle expression is closely controlled by interactions between muscle and motoneuron. Accordingly, several studies have demonstrated that either nerve-evoked electrical activity or nerve-derived trophic factors influence regulation of AChE expression. In this regard, denervation studies have revealed age-defined regulatory mechanisms in muscle, such that denervation of early postnatal rat hindlimb muscles, which culminates in decreased mRNA levels, results in increased gene transcription as measured by nuclear run-on assays and promoter-reporter transduction studies (Boudreau-Lariviere et al., 2000a). In comparison, neither transcription rate nor promoter activity was modified in adult denervated muscles. The specific involvement of *AChE* gene transcription in the young muscles may reflect, therefore, the developmental status of the muscle and the combined effects of intrinsic and nerve-derived regulatory mechanisms. However, since overall transcript levels are decreased in the denervated muscles of young animals, post-transcriptional mechanisms must also be implicated in

regulating gene expression during development (see *1.8.3.1 Post-transcriptional regulation of AChE expression in skeletal muscle*).

Studies in which nerve-evoked electrical activity at the NMJ was blocked by pharmacological agents, leaving the motoneuron and pre- and post-synaptic domains intact, demonstrated that the nerve influences post-synaptic protein organization and gene expression through secreted factors other than ACh (Apel and Merlie, 1995;Merlie, 1984;Hall and Sanes, 1993). Calcitonin gene-related peptide (CGRP) is one of the identified nerve-derived trophic factors known to have marked effects on AChR expression in muscle (see for review Duclert and Changeux, 1995). In a recent study, CGRP treatment of cultured muscle resulted in increased cAMP levels, which in turn decreased AChE transcript and protein levels (Rossi et al., 2003). As mentioned above, during myogenesis elevated cAMP levels negatively regulated expression of a human AChE promoter-reporter construct through CREB protein and decreased transcription (Siow et al., 2002); consequently Rossi *et al.* (2003) suggested that decreased AChE expression in response to CGRP treatment results from decreased transcription (Rossi et al., 2003). In keeping with the differences noted between chick and mammalian muscle, the opposite effect is observed in cultured chick muscle, such that CGRP treatment increases endogenous chick AChE expression via increased levels of cAMP (Choi et al., 1996;Choi et al., 1998;Choi et al., 2001b). Whether this effect is mediated by CREB and gene transcription remains to be determined since the chicken AChE promoter has not been isolated. However, given the results obtained with the human promoter, elevated gene transcription has been proposed to mediate this effect.

In addition to ACh, vesicles at the synapse also release ATP. Recent studies showed that ATP acts through its receptor to increase AChE activity and transcript levels in cultured chick or mouse muscle cells (Choi et al., 2001a;Choi et al., 2003). Cultured myotubes transfected with AChE promoter-reporter constructs and treated with ATP or ATP receptor (P2Y1) agonists, displayed increased reporter levels, indicating that AChE expression is regulated through transcription (Choi et al., 2001a). Furthermore, the transcription factor Elk-1, which has two binding elements in the AChE promoter, was demonstrated to bind the promoter region and to direct ATP-induced reporter expression (Choi et al., 2003). Similar, albeit less marked effects, were observed through activation of the related P2Y2 receptor by its ligand UTP (Tung et al., 2004). Accordingly, these studies provide some insight into the transcriptional mechanisms and *cis*- and trans-acting factors involved in modulating AChE expression in muscle. Specifically, these observations indicate that AChE expression in muscle is determined by extrinsic signals, such as nerve-evoked activity and trophic factors, and intrinsic signals, such as age and species. In many studies, however, the increase in *AChE* gene transcription does not necessarily correspond with the overall accumulation of AChE mRNAs, suggesting that post-transcriptional mechanisms are also involved (see *1.8.3 Post-transcriptional regulatory mechanisms in tissues*).

1.8.2.3 Transcriptional regulation of AChE expression during neuronal differentiation

Identification of the molecular mechanisms regulating AChE expression in neurons has been greatly influenced by the advances made in muscle, but has progressed more slowly than the progress achieved in muscle. Part of the difficulty arises from the great

heterogeneity of neurons in the CNS and PNS and the variability in expression pattern and regulatory signals. Nevertheless, cultured neuronal systems have allowed individual research groups to identify some of the mechanisms and factors involved during neuronal differentiation.

Initial studies performed with PC12 cells induced to differentiate by NGF-treatment described a significant increase in AChE activity that could be blocked by various transcription inhibitors, including RNA-polymerase II inhibitors actinomycin D and α-amanitin, and the DNA-topoisomerase I inhibitor camptothecin (Greene and Rukenstein, 1981;Lucas and Kreutzberg, 1985). Consequently, increased *AChE* gene transcription was suggested to account for the increasing protein activity. Note, however, that there are limitations to the use of RNA polymerase II inhibitors, such that they inhibit transcription of all genes including those that may be involved in transcript stability (see for review Rajagopalan and Malter, 1997). Transcription inhibitors also have secondary effects on nuclear shuttling of transcription-sensitive heteronuclear ribonucleoproteins (hnRNP) (see for review Dreyfuss et al., 1993), such that they facilitate the cytoplasmic accumulation of these factors. In addition, some transcripts, such as c-fos and granulocyte-macrophage colony-stimulating factor (GM-CSF), were shown to be stabilized in cells treated with actinomycin D (Chen et al., 1995;Shyu et al., 1989). Accordingly, results obtained with this approach are considered suggestive and not definitive. Although these initial studies indirectly showed transcriptional regulation, recent studies have further examined the role of transcription in *AChE* gene expression during differentiation. For instance, in the early stages of neuronal differentiation of either a cultured neuroblastoma cell line or primary embryonic neurons, expression of a

transfected AChE promoter-reporter construct increased approximately 2- to 4-fold (Siow et al., 2005). Since previous studies performed with cultured muscle cells established that ATP in the extracellular space could regulate AChE expression at the transcriptional level, this was also tested during neuronal differentiation. In this investigation, ATP and ATP receptor agonists both increased reporter expression levels in embryonic primary cultures transfected with AChE promoter-reporter constructs (Siow et al., 2005).

Neuronal differentiation of cultured neuroblastoma/glioma hybrid cells was also achieved by neuron-muscle interaction in a co-culture system with primary myotubes (Jiang et al., 2003). In this system, reporter activity of both human and mouse promoter-reporter constructs increased in the neuronal cells in a time-dependent manner during the co-culture, suggesting that increased neuronal AChE expression stimulated by neuron-muscle contact results from increased gene transcription. Furthermore, CREB was identified as a putative trans-acting factor, inasmuch as mutation of the cAMP-response element (CRE) site within the promoter-reporter constructs results in decreased reporter levels in the co-culture system. In addition, transcriptional activation of AChE promoter-reporter constructs has also been demonstrated in cultured neuroblastoma cells treated with cAMP (Wan et al., 2000). Accordingly, the changes in AChE levels occurring during differentiation can be attributed in part to increased *AChE* gene transcription.

1.8.2.4 Transcriptional regulation of AChE in disorders of the nervous system

As mentioned in the above section (*1.4 AChE expression in disorders of the nervous system*), AChE expression varies in accordance with different disease states. One such example is the increased levels of AChE mRNA and protein observed in response to

acute or chronic stress (Kaufer et al., 1998;Nijholt et al., 2004;Meshorer et al., 2002). In a recent study, Meshorer *et al.* (2004) suggested that high levels of glucocorticoids, present in the blood stream in response to stress, activates *AChE* gene transcription in neurons through the glucocorticoid response element (GRE) found in the promoter region (Meshorer et al., 2002;Meshorer et al., 2004). This hypothesis is supported by the increased levels of AChE transcript expressed following treatment of PC12 cells with corticosterone (Meshorer et al., 2002). Transcriptional activation was also implicated in the elevated levels of AChE transcript found in aggressive neuronal tumors (Perry et al., 2002). Specifically, expression of the transcription factor Runx1/AML1, which is important in development of leukemia, was correlated with high levels of AChE in aggressive tumors and was also shown to increase expression of an AChE reporter construct containing the AChE promoter (Perry et al., 2002). Accordingly, these studies suggest that increased *AChE* gene expression is regulated by transcriptional activation in proliferating cancerous cells and stress-induced activated cells.

1.8.3 Post-transcriptional regulatory mechanisms in tissues

AChE gene transcription is decidedly important to AChE expression in muscle and neurons; however, the observed increases in transcription rate can not solely account for the considerable increases in transcript level. For example, during myogenic differentiation, Angus et al. (2001) observed a greater than 50-fold increase in AChE mRNA levels but could only measure a 2-fold increase in gene transcription by nuclear run-on assay or a 5-fold increase using a promoter-reporter construct. Consequently, post-transcriptional regulatory mechanisms must also have significant roles in regulating

AChE expression. Accordingly, a few studies have begun to explore these mechanisms in muscle, neurons and hematopoietic cells.

1.8.3.1 Post-transcriptional regulation of AChE expression in skeletal muscle

In an early study by Fuentes and Taylor (1993), the role of transcript stability during myogenesis was examined using another RNA polymerase II inhibitor, 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), that blocks gene transcription and allowed the investigators to measure transcript levels and decay (Fuentes and Taylor, 1993). Unexpectedly, treatment of myoblasts at early stages of differentiation with DRB actually resulted in increased levels of transcript that were relatively stable. In comparison, treatment of differentiated myotubes resulted in rapidly and significantly decreased transcript levels. The authors, therefore, suggested that AChE mRNA levels are controlled by transcript stability and that in myoblasts DRB treatment blocked the transcription of destabilizing proteins, resulting in increased mRNA levels. As mentioned above, there are disadvantages to the use of pharmacological transcription inhibitors that may explain some of the results obtained with myoblasts. Note that during differentiation, 60-70% of transcripts measured used the first polyadenylation signal and the ratio of short to long transcripts did not change, suggesting that the choice of polyadenylation signal is not involved in regulating AChE expression (Fuentes and Taylor, 1993).

Subsequent studies examined the role of intracellular calcium and calcium channels in *AChE* gene regulation in a search to identify the signaling pathway controlling AChE mRNA stability (Luo et al., 1994;Luo et al., 1999). In a first study, Luo *et al.* (1994)

demonstrated that increased intracellular calcium levels, as mediated by ryanodine receptors and L-type calcium channels, resulted in accumulation AChE transcripts (Luo et al., 1994). This increase was suggested to occur through elevated mRNA stability and not transcription since transcription rate remained unchanged following treatment with calcium channel blockers. In an ensuing study, the specific role of calcineurin, a calcium/calmodulin-dependent phosphatase, in mediating transcript stability was examined (Luo et al., 1999). Transcript stability, as determined using DRB to block gene transcription, was increased in cells treated with a calcineurin inhibitor; suggesting, therefore, that calcineurin is indirectly implicated in regulation of AChE transcript stability. Together these studies propose that transcript stability as controlled by still unknown mechanisms is also important to regulating AChE transcript levels during myogenic differentiation.

In order to establish the role of the nerve in controlling AChE expression in muscle, post-transcriptional regulatory mechanisms were also examined following hindlimb denervation. In this regard, denervation of adult rat hindlimb muscle resulted in a dramatic decline in AChE transcript levels (Boudreau-Lariviere et al., 2000a). This effect was attributed to decreased stability of the transcript, as demonstrated by *in vitro* stability assays, and not to alterations in transcription and promoter activity since neither of these parameters was altered following denervation. Additionally, interactions of the AChE 3'-UTR with suspected destabilizing RNA-binding proteins increased. Similar results were obtained using sternocleidomastoid muscle denervation and *in vitro* stability assays (Grubic et al., 1999). To further assess the role of the nerve, the regulatory mechanisms used by nerve-derived trophic factors, such as CGRP, were also investigated.

Treatment of cultured C2C12 myotubes with CGRP resulted in significant decreases in AChE activity, protein and transcript levels, similarly to other studies (Boudreau-Lariviere and Jasmin, 1999;Rossi et al., 2003). In comparison, though, transcription rate in treated cells did not vary from untreated cells, suggesting that post-transcriptional mechanisms are employed to mediate the effects of CGRP. Accordingly, these studies propose that nerve-evoked electrical activity and trophic factors can control AChE mRNA levels via transcript stability and specific interactions with the 3'-UTR.

1.8.3.2 Post-transcriptional regulation of AChE expression in neurons

As with myogenic differentiation, transcriptional regulation is just one of the identified mechanisms implicated in regulating AChE expression during neuronal differentiation. An initial study performed with pluripotent embryonic carcinoma stem cells (P19 cells) induced to differentiate with retinoic acid demonstrated that AChE transcript and activity levels increased dramatically during neurogenesis (Coleman and Taylor, 1996). Nuclear run-on assays, however, revealed that the already high *AChE* gene transcription rate did not vary during this time period. Interestingly, AChE transcript half-life, as measured in cells treated with the transcription inhibitor DRB, was similar between undifferentiated embryoid bodies and fully differentiated neurons. These observations, therefore, suggest that neither transcription nor transcript stability are responsible for the increased transcript level. Given the limitations of this methodology that are described above, it is possible that a difference between undifferentiated and differentiated neurons was masked by the secondary effects of DRB. Accordingly, the authors concluded that greater mRNA stability was responsible for increased AChE

expression since the AChE mRNA half-life measured in this system was relatively long (12-hours), and transcript stability is suggested to be responsible for AChE expression in other cell systems. In addition, because *AChE* gene transcription is active in the stem cells but transcript levels are undetectable; this provides further support for the role of post-transcriptional mechanisms in regulating AChE expression during neuronal differentiation (Coleman and Taylor, 1996). Complementary results were obtained during thyroid hormone-induced differentiation of PC12 cells, which resulted in elevated AChE transcript levels due primarily to prolonged AChE mRNA half-life with no apparent change in transcription rate (Puymirat et al., 1995). Accordingly, these studies propose that post-transcriptional mechanisms are also acting to regulate AChE expression in neurons.

1.8.3.3 Post-transcriptional regulation of AChE expression in hematopoietic cells

Although most studies have focused on regulation of AChE expression in excitable cells, AChE is also expressed in hematopoietic cells where it has an important role in development and differentiation (see *1.5 AChE expression in non-excitable tissues*). Hence, the molecular mechanisms mediating the significant increase in AChE levels during hematopoiesis have been investigated (Chan et al., 1998). In an initial study, results from *in vitro* mRNA stability assays revealed that AChE transcript stability was elevated in the differentiated cells and there was non appreciable change in transcription rate. These results, therefore, suggest that AChE expression in hematopoietic cells is controlled by post-transcriptional mechanisms.

Although most studies have focused on promoter elements or transcript stability, some recent studies have also suggested that domains in the ORF or splicing can also modulate overall transcript levels (Camp et al., 2005;Morel and Massoulie, 2000;Weill et al., 2002). To date, both transcriptional and post-transcriptional events have been described and proposed as regulatory mechanisms controlling AChE expression in various tissues and species. It is important to note that although in many studies post-transcriptional mechanisms have been suggested, there is relatively little known about the elements and factors mediating this effect.

1.9 Post-transcriptional regulatory mechanisms

Regulated expression of proteins begins at the transcriptional level. However, molecular mechanisms controlling mRNA levels and localization are now considered by some to be more important to directing the flow of genetic information from DNA to protein (Fan et al., 2002 and see for review Keene, 2001;Guhaniyogi and Brewer, 2001). Post-transcriptional regulation of gene expression occurs on many levels and involves alternative splicing in the nucleus, shuttling from the nucleus to cytoplasm, and modulation of transcript stability, intracellular localization and translation. In this regard, levels of expression of some genes, which have a significant impact on cell growth, differentiation, and activity, are controlled by mRNA stability, such that they are finely and promptly up- or downregulated as dictated by the cellular circumstances (Milde-Langosch, 2005). Appropriately, several early response genes have relatively short half-lives (10-60 minutes), while other housekeeping-like proteins have much longer half-

lives (up to 24hrs) (Keene, 2001). Thus, for these and many other essential and highly regulated genes, regulation of transcript turnover is a critical aspect of cellular expression.

1.9.1 Transcript turnover and stability

Regulation of transcript stability is mediated by structures, such as stem-loops and hairpins, and sequences within the mRNA (*cis*-acting elements), such as the 5'-cap structure, 5'-untranslated region (5'-UTR), protein coding region, 3'-UTR, and 3'-polyadenylate [poly(A)] tail, and trans-acting factors that interact with them (see for review Guhaniyogi and Brewer, 2001;Mitchell and Tollervey, 2000;Beelman and Parker, 1995;Decker and Parker, 1994). The 5'-cap and poly(A) tail are the two most important structures that inhibit or slow mRNA decay by 5' to 3' or 3' to 5' exoribonucleases, respectively (see for review Ross, 1995;Sachs, 1993;Shatkin, 1985;Bernstein and Ross, 1989;Sachs, 1990). Transcript degradation is initiated by distinct pathways, although the most common comprises deadenylation as the initial step (see Figure 4). The poly(A) tail of stable transcripts is generally protected by a series of poly(A) binding proteins (PABP) that have a multitude of additional functions in transcript metabolism (see for review Mangus et al., 2003;Shyu et al., 1991;Chen and Shyu, 1995;Decker and Parker, 1994). Removal of PABP after translation, for example, renders the adenylates vulnerable to deadenylation. In this pathway, therefore, degradation begins with shortening of the poly(A) tail by the deadenylases poly(A) nuclease (PAN) or ribonuclease (PARN) (Korner and Wahle, 1997;Uchida et al., 2004). Deadenylation proceeds in either a processive manner, in which the degradation machinery completely degrades the tail of one transcript before moving on to another mRNA, resulting in a pool of transcripts of

heterogeneous length; or in a distributive manner, in which there is simultaneous deadenylation of a group of mRNAs one adenylate at a time, resulting in a pool of transcripts of homogeneous lengths (Xu et al., 1997). Complete deadenylation is followed by transcript body decay.

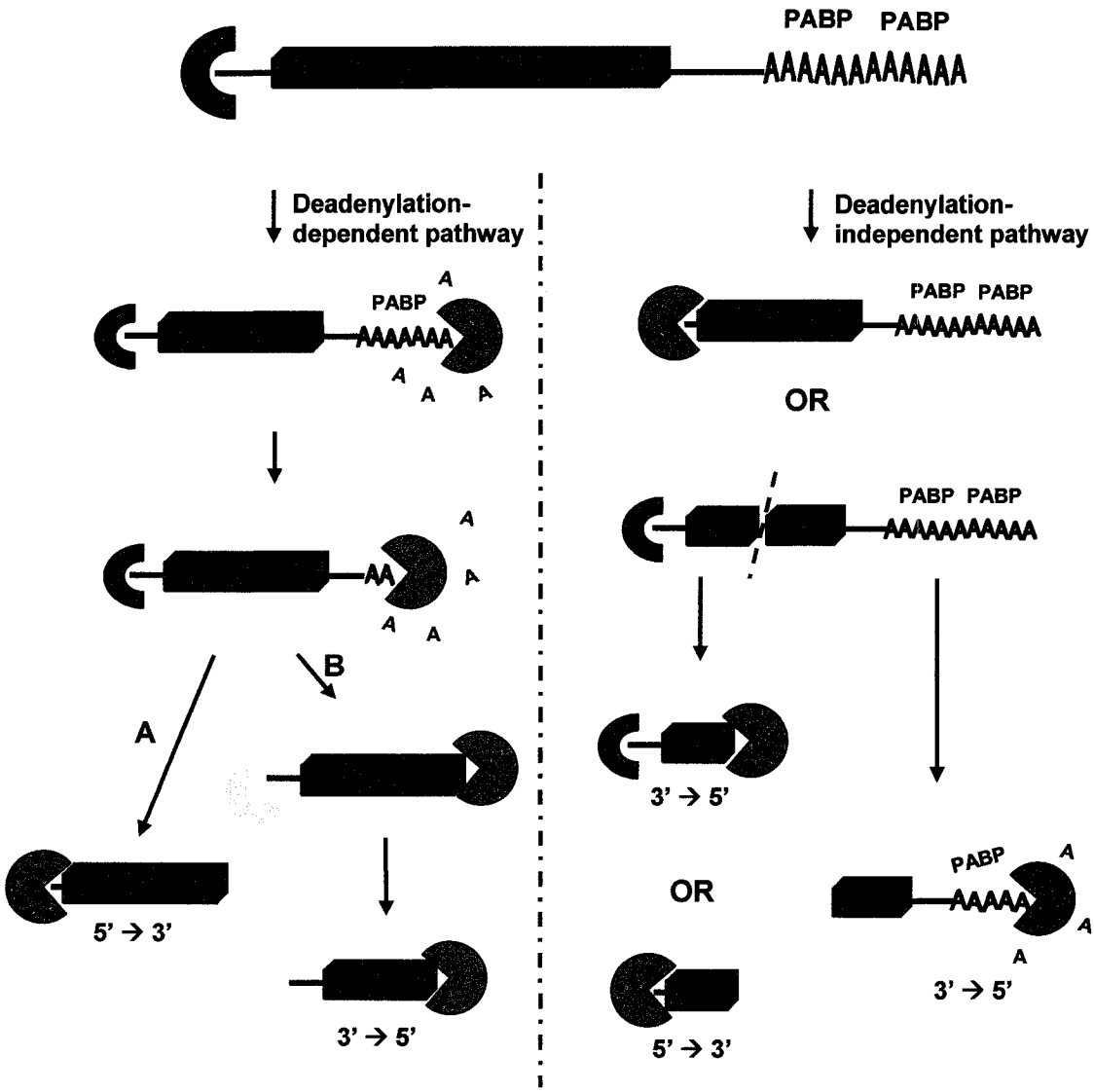

Figure 4. mRNA degradation pathways. mRNA decay can proceed in a deadenylation-dependent or -independent manner. In the deadenylation-dependent manner, the poly(A) tail is shortened followed by either decapping and 5' to 3' decay (A) or decapping and 3' to 5' decay (B). The deadenylation-independent pathway proceeds by 5' to 3' degradation or begins with cleavage within the protein coding region and degradation

proceeds in both 5' to 3' and 3' to 5' orders (see for review Beelman and Parker, 1995; Barreau et al., 2005; Decker and Parker, 1994).

In yeast, deadenylation is followed by removal of the 5'-cap and processive degradation in the 5' to 3' orientation. By contrast, in mammalian cells, nonstop degradation by the exosome typically proceeds in a 3' to 5' order (Wang and Kiledjian, 2001;Jacobs Anderson and Parker, 2000). In addition, it is not clear whether decapping occurs in mammalian cells prior to transcript body degradation or during the degradation process. However, mRNAs lacking the cap are rapidly degraded (Drummond et al., 1985) and enzymes that can remove the cap structure have been described in mammalian cells; thereby indicating that 5'-cap removal is a conserved step in the turnover process between yeast and eukaryotes (Coutts and Brawerman, 1993;Wang and Kiledjian, 2001).

In yeast and eukaryotes additional degradation pathways have been described for non-sense mediated decay (NMD) and sequence specific cleavage and degradation of transcripts (see for review Beelman and Parker, 1995;Decker and Parker, 1994). These pathways are deadenylation-independent and are either initiated by decapping or by endonucleolytic cleavage of the transcript. NMD of transcripts, the turnover mechanism used in the mRNA surveillance process, is initiated by 5'-cap hydrolysis or cleavage within the protein coding region. These pathways are most often used when there are abnormalities in the transcript, such as the presence of introns or premature stop codons (see for review Hilleren and Parker, 1999b;Hilleren and Parker, 1999a). In addition, specific sequences within the 3'-UTR also play an important role in controlling decay events such as deadenylation rate, and in determining the degradation pathway, deadenylation-dependent or initiated by endonucleolytic cleavage (see for example Nielsen and Christiansen, 1992). For example, mRNA half-life correlates with the

presence in the 3'-UTR of an adenylate, uridylate-rich element (AU-rich element, ARE), which can act as an endonucleolytic cleavage site, can increase or decrease transcript stability in a poly(A)-dependent manner or both (Zhao et al., 2000 and see for review Chen and Shyu, 1995).

1.9.2 3'-UTR and AU-rich elements

In recent years, an increasing number of *cis*-acting elements within the 3'-UTR and trans-acting factors have been identified that influence transcript stability, localization and translation (Barreau et al., 2005;Hesketh, 1996;Mitchell and Tollervey, 2000;Wilson and Brewer, 1999). To date, though, AREs are the best characterized. Approximately 1 in 20 human genes contain within their 3'-UTR an ARE (Bakheet et al., 2003;Pascale et al., 2005), which is directly implicated in RNA turnover (Liu et al., 1995;Chung et al., 1996;Bakheet et al., 2006;Bakheet et al., 2003). Thus, this *cis*-acting element is the most common determinant of mRNA turnover (see for review Chen and Shyu, 1995;Xu et al., 1997), and has been identified in many transcripts with short half-lives that are degraded in a deadenylation dependent manner. The presence of an ARE in the 3'-UTR, however, also leads to transcript stabilization, such as for GAP-43 in neurons (Mobarak et al., 2000) and MyoD and myogenin in muscle (Figueroa et al., 2003) (see for review Barreau et al., 2005). AREs are regions of 50-150 nucleotides in the 3'-UTR that are rich in A and U nucleotides and contain the basic AUUUA motif as a single pentamer or as overlapping nonamers (AUUUAUUUA or UUAUUUUAUU) (see for review Barreau et al., 2005). Note, however, that the nonamer motif is the minimal sequence associated with stability of transcripts (Lagnado et al., 1994;Zubiaga et al., 1995).

These elements are separated into three classes based on their sequence and structure. Class I ARE consist of one to five dispersed AUUUA motifs separated by U-rich regions, while class II ARE consist of clusters of three to four AUUUA motifs. Class III ARE, although less-well defined, consist mainly of U-rich sequences and do not contain the AUUUA pentamer (Chen and Shyu, 1995). The different classes of ARE instruct distinct modes of mRNA decay. Class I and III ARE-directed mRNA degradation proceeds in a distributive manner; while, class II ARE-directed mRNA occurs in a processive manner (see above) (Xu et al., 1997). Given, that this element and its different permutations can direct stability and different modes of decay, transcript turnover is largely dependent on the trans-acting factors that bind the ARE.

1.9.3 RNA-binding proteins.

There are several identified RNA-binding proteins (RBP) that bind to AREs (see Table 2). Generally, these proteins contain within their structure RNA-recognition motifs (RRM). RRMs are also found in various proteins involved in RNA processing and turnover, including hnRNP A1 involved in splicing and transport of RNA, PABP involved in transcript stability and translation, and *Drosophila* embryonic lethal abnormal vision (ELAV) protein involved in RNA splicing (see for review Burd and Dreyfuss, 1994;Kenan et al., 1991). The RRM is a conserved structure of ~80-90 residues consisting of two consensus ribonucleoprotein (RNP) motifs separated by 25-35 amino acids that interact directly with the RNA. The RNP motifs, octameric RNP-1 and hexameric RNP-2, of different RBP, including proteins that bind pre-mRNA, mRNA,

pre-rRNA, and small nuclear and heteronuclear RNAs, each contain 3 conserved aromatic residues that are implicated in RNA interactions.

Table 2: RNA-binding Proteins *

RBP	Domains	Function and recognized element
Hu family (HuB, HuC, HuD and HuR)	• 3 RRM	• mRNA stabilization • Cell-cycle regulation and neurite extension • ARE
AUF1 (p37, p40, p42, p45)	• 2 RRM	• mRNA destabilization • ARE
Cytoplasmic polyadenylation element binding (CPEB) protein	• 2 RRM	• Polyadenylation and translation activation (derepression) • mRNA dendritic transport • Synaptic plasticity • CPE (UUUUUAU)
Musashi (Msi1 and 2)	• 2 RRM	• Translation repression • Neuronal development
CUG-binding protein (CUG-BP1 and 2)	• 3 RRM	• mRNA splicing and decay • Implicated in myotonic dystrophy • CUG triplet repeats
Zipcode-binding protein (ZBP1 and 2)	• 1 RRM • 4 KH	• mRNA transport • Translation repression • Growth-cone dynamics • Zipcode
Human K homology-type splicing regulatory protein (KSRP) or rat MARTA-1	• 4 KH	• mRNA dendritic transport • mRNA degradation
NOVA-1 and NOVA-2	• 3 KH	• Splicing regulation • Neuronal survival • UCA(U/C) stem-loop repeats
Fragile X mental retardation protein (FMRP)	• 2 KH • 1 RGG	• Translation repression and mRNA transport • Synaptic structure and plasticity • G quartet structure

Staufen (Stau1 and 2)	5 dsRBD	• Dendritic mRNA targeting • Double-stranded RNA
Tristetraprolin (TTP)	CCCH tandem zinc finger	• mRNA destabilization • ARE
Butyrate response factor 1 (BRF1)	Zinc finger	• mRNA destabilization • ARE

* See for review Antar and Bassell, 2003;Antic and Keene, 1997;Bardoni and Mandel, 2002;Bassell et al., 1999;Deschenes-Furry et al., 2006;Farina and Singer, 2002;Guhaniyogi and Brewer, 2001;Mendez and Richter, 2001;Perrone-Bizzozero and Bolognani, 2002;Roegiers and Jan, 2000;Ueda et al., 2000;Wilson and Brewer, 1999.

The consensus structure of RRM consists of β1-α1-β2-β3-α2-β4 and the location of the RNP motifs in the first and third β-strands of the RRM is highly conserved (Birney et al., 1993;Burd and Dreyfuss, 1994). Although the structure of RRM is well preserved, only a few residues found mostly in the RNP motifs are highly conserved. Consequently, it is the variable regions between the RNP motifs and RRM that tend to lend sequence specificity to the RBP.

Most identified ARE binding proteins act to decrease the stability of the transcript (see Table 2 and for review Barreau et al., 2005), the Hu family of RBP, which consists of four highly similar proteins encoded by different genes, are characterized strictly as mRNA stabilizing. The Hu protein family, whose name derives from the initials of the patient in which the onconeural antibodies were identified, includes HuB (Hel-N1), HuC (Hel-N3), HuD and HuR (HuA) (Szabo et al., 1991 and see for review Deschenes-Furry et al. 2006). Whereas HuR is ubiquitously expressed and HuB is found in neurons and gonads, expression of HuC and HuD is restricted to neurons. Due to the presence of three highly conserved RRM, these proteins share a high degree of homology with the *Drosophila* protein ELAV that is required for the normal development and maintenance

of the nervous system (Szabo et al., 1991;Okano and Darnell, 1997). Alignment of the Hu family protein sequences reveals that Hu proteins show greater variation in the N-terminal domain and in the regions separating the second and third RRM (70 to 85% identity), termed the hinge region (Okano and Darnell, 1997). Although the Hu proteins are highly similar, they have distinct expression patterns in the brain during development and in adults (Clayton et al., 1998;Okano and Darnell, 1997). Importantly, to date these proteins target different transcripts and there is very little overlap in target specificity between the Hu protein family members (Barreau et al., 2005). In this regard, HuB and HuD appear to have similar (Akamatsu et al., 1999;Yano et al., 2005) and opposing (Okano et al., 2005;Akamatsu et al., 2005) functions during neuronal development depending on the context. For example, they can both stimulate neuronal differentiation and neurite outgrowth but have opposing roles in progenitor cell self-renewal, such that HuB positively and HuD negatively affects this capacity. Accordingly, RBP functions and precise targets are highly specific as well as orchestrated during development and activity of cells.

1.10 Statement of the problem and hypothesis

AChE, as a result of its enzymatic and non-cholinergic functions, is an indispensable element of cholinergic synapses, and more broadly of excitable and non-excitable tissues. Given AChE's implication in development and in diverse disorders of the nervous system, it is decidedly important to understand the molecular mechanisms that regulate *AChE* gene expression in these tissues. In this regard, several studies have begun to identify these molecular events in muscle and neurons, and have established a well-defined role for transcriptional regulation, including various *cis*-and trans-acting factors. Importantly, the majority of studies have focused on muscle expression and, thus, less is known about the molecular events in neurons. Although several studies have proposed a role for post-transcriptional regulation of AChE mRNA, there is very little known about the nature of these events and the elements or factors implicated.

Since several studies have indirectly shown that AChE expression can be regulated by post-transcriptional mechanisms, we hypothesize that AChE mRNA stability is an important event in regulation of AChE expression in muscle and neurons. Furthermore, given the conserved nature of the AChE 3'-UTR and elements within it, we propose that the 3'-UTR, specifically the ARE, is implicated in regulating AChE expression in muscle and neurons.

1.11 Objectives

A. Determine the molecular mechanisms regulating AChE expression during neuronal differentiation of PC12 cells. Specifically:

- Determine the specific roles of transcriptional and post-transcriptional mechanisms acting during neuronal differentiation
- Characterize the role of the 3'-UTR and ARE in mediating the post-transcriptional events during neuronal differentiation
- Determine the role of HuD in controlling AChE expression in differentiating neurons

B. Characterize the post-transcriptional mechanisms regulating AChE expression in differentiating C2C12 muscle cells. Specifically:

- Characterize the role of the 3'-UTR and ARE in post-transcriptional regulation of AChE expression during myogenesis
- Determine the role of HuR in controlling AChE expression and transcript stability in differentiating muscle cells

C. Characterize the post-transcriptional mechanisms regulating AChE expression in neurons *in vivo*. Specifically:

- Examine the interaction between HuD and AChE mRNA in neurons *in vivo*

- Determine the role of post-transcriptional mechanisms in regulating AChE expression levels following SCG axotomy
- Identify a role for HuD in mediating AChE expression levels following SCG axotomy

Chapter 2:

Post-transcriptional Regulation of Acetylcholinesterase mRNAs in Nerve Growth Factor-treated PC12 Cells by the RNA-binding Protein HuD

Julie Deschênes-Furry, Guy Bélanger, Nora Perrone-Bizzozero, and Bernard J. Jasmin

Published in The Journal of Biological Chemistry Vol. 278 (8):5710–5717, 2003

Acknowledgements: This work was supported in part by operating grants from the Canadian Institutes of Health Research (CIHR) and the Ontario Neurotrauma Foundation (ONF) (to B. J. J.) and by National Institutes of Health Grant NS-30255 (to N. P. B.).

2.1 Author contribution

Julie Deschênes-Furry:

Unless otherwise indicated all experiments, including preparation of reagents and cell culture, were performed by the first author under the supervision of Dr. Bernard Jasmin. The manuscript was prepared by the first author and revised by Drs. Bernard Jasmin and Nora Perrone-Bizzozero.

Guy Bélanger:

The AChE 3'UTR plasmid constructs and primer sets that were used in this study for transfection experiments and RNA-protein interaction experiments were designed by Dr. Bélanger.

Nora Perrone-Bizzozero:

Dr. Perrone-Bizzozero provided the first author with the different PC12 cell lines and HuD expression plasmids used in this study.

2.2 Abstract

Expression of acetylcholinesterase (AChE) is greatly enhanced during neuronal differentiation, but the nature of the molecular mechanisms remains to be fully defined. In this study, we observed that nerve growth factor treatment of PC12 cells leads to a progressive increase in the expression of AChE transcripts, reaching ~3.5-fold by 72 h. Given that the AChE 3'-untranslated region (UTR) contains an AU-rich element, we focused on the potential role of the RNA-binding protein HuD in mediating the increase in AChE mRNA seen in differentiating neurons. Using PC12 cells engineered to stably express HuD or an antisense to HuD, our studies indicate that HuD can regulate the abundance of AChE transcripts in neuronal cells. Furthermore, transfection of a reporter construct containing the AChE 3'-UTR showed that this 3'-UTR can increase expression of the reporter gene product in cells expressing HuD but not in cells expressing the antisense. RNA gel shifts and Northwestern blots revealed an increase in the binding of several protein complexes in differentiated neurons. Immunoprecipitation experiments demonstrated that HuD can bind directly AChE transcripts. These results show the importance of post-transcriptional mechanisms in regulating AChE expression in differentiating neurons and implicate HuD as a key *trans*-acting factor in these events.

2.3 Introduction

Acetylcholinesterase (AChE) is the enzyme responsible for the rapid hydrolysis of acetylcholine in the central and peripheral nervous systems (see, for review, Refs 1-4). The enzyme exists in multiple molecular forms that differ in their C terminus and mode of anchoring to subcellular structures. The different C termini of the protein are generated through alternative splicing of a single gene, and three different mature mRNAs, referred to as the T (tail), H (hydrophobic), and R (readthrough) transcripts, can be produced. The pattern of expression of these transcripts is known to be tissue-specific because, for example, the T transcript is abundantly expressed in excitable cells such as skeletal muscle and neurons, whereas the H transcript is found predominantly in the hematopoietic lineage. Two polyadenylation signals can be used in the 3'-untranslated region (UTR) to produce a ~2.4- or 3.2-kb transcripts. Choice of the polyadenylation signal is also tissue-specific because neurons appear to preferentially express the shorter form of the transcript whereas skeletal muscle express both species but in different amounts (5-7).

In addition to its role in cholinergic neurotransmission, converging lines of evidence indicate that it likely fulfills additional, non-catalytic functions within the nervous system (see, for review, Ref. 8). For example, Northern blot analysis and *in situ* hybridization experiments have revealed the presence of AChE mRNAs in non-cholinergic areas of the brain such as the cerebellum (9-12). Moreover, AChE expression in the brain is known to precede the establishment of synaptic transmission and coincides with the period of neurite outgrowth (13, 14). Finally, several studies in which AChE levels have been

experimentally manipulated directly support the notion that AChE is indeed involved in neurite outgrowth (see, for example, Refs. 13-15 and 17-22).

In recent years, there have been several studies that have examined the basic molecular events that preside over AChE expression in developing and adult skeletal muscles (see, for example, Refs. 23-30). By contrast, there is relatively little information concerning the mechanisms regulating AChE expression in neurons. In addition, of the few available reports, there also appear to be some contradictory findings. In particular, Greene and Rukenstein (31) have provided evidence indicating that differentiation of PC12 cells, which leads to an increase in AChE expression, induces an increase in AChE gene transcription. Alternatively, embryonic P19 carcinoma cells induced to differentiate into neurons via retinoic acid treatment failed to increase the transcriptional activity of the AChE gene, thereby indicating that post-transcriptional mechanisms represent key events in regulating the abundance of AChE transcripts during neuronal development (32). Given the diverse and key functions of AChE within the nervous system, it appears important to gain a more complete understanding of the molecular mechanisms that control AChE expression in neurons. Accordingly, we have initiated a series of experiments in attempts to characterize some of the molecular events involved in AChE expression during neuronal differentiation. Specifically, we have examined the importance of transcriptional and post-transcriptional mechanisms in the regulation of AChE during NGF-induced differentiation of PC12 cells.

2.4 Experimental procedures

Cell Culture—PC12 cells, a rat pheochromocytoma-derived cell line (33), were cultured on culture dishes coated with type I collagen (Sigma) in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% horse serum, 5% fetal bovine serum, and 100 units/ml penicillin-streptomycin, in a humidified chamber at 37 °C containing 5% CO_2. Stably transfected PC12 cells were maintained as described elsewhere (34). These lines were transfected with the pcDNA3 vector alone (pcDNA) or with plasmids containing the human HuD sequence in sense (pcHuD) or antisense (pDuH) orientation. All cells were plated at a density of $1–2x10^5$ cells/cm^2 and induced to differentiate by adding 100 ng/ml 7S-NGF (Sigma) to the culture medium. Culture media were changed every 72 h.

AChE Enzymatic Assay—Cultures of undifferentiated and differentiated PC12 cells (three 35-mm wells) were washed with cold phosphate- buffered saline (PBS), scraped, and homogenized on ice with a glass Kontes homogenizer in 0.5 ml of a high salt detergent buffer containing anti-proteolytic agents (10 mM Tris-HCl, pH 7.0, 10 mM EDTA, 1 M NaCl, 1% Triton X-100, 1 mg/ml bacitracin (Sigma), and 25 units/ml aprotinin (Sigma)). Following centrifugation of the homogenates (20,000 × *g* for 15 min at 4 °C), the supernatant was removed and stored immediately at –80 °C. AChE activity was measured using a modified version of the spectrophotometric method of Ellman *et al.* (35) as described previously (36). The total amount of protein present in the extracts was determined by the bicinchoninic acid assay (BCA; Pierce).

RNA Extraction and RT-PCR—Total RNA was extracted from undifferentiated and differentiated PC12 cell cultures (three 35-mm wells) using 0.5–1 ml of TRIzol reagent

(Invitrogen) according to the instructions from the manufacturer. Briefly, the cells were scraped from the plates and disrupted by vigorous pipetting, followed by addition of chloroform. The resulting solution was mixed vigorously and centrifuged (12,000 × *g* for 15 min at 4 °C). The aqueous layer was then transferred to a fresh tube and combined with an equal volume of isopropanol. The RNA was precipitated by centrifugation, and the resulting pellet was washed twice with 75% ice-cold ethanol and resuspended in RNase-free water. All samples were stored in –80 °C until used.

RNA from each sample was quantified using the Amersham Biosciences Gene Quant II RNA/DNA spectrophotometer and adjusted to a final concentration of 80 ng/μl. Reverse transcription of RNA was performed using 2 μl of each RNA sample at 42 °C for 45 min, followed by 5 min at 99 °C, as previously described elsewhere (37–39). Negative controls consist of the same RT mixture in which the RNA was replaced with 2 μl of RNase-free water. PCR was used to amplify cDNAs corresponding to AChE and S12 rRNA as described in detail elsewhere (23, 37–39). Primers for AChE (see Ref. 12) and S12 rRNA (used as an internal control; see Ref. 40) were synthesized based on available sequences that have been previously described, and they amplified products of 670 and 368 bp, respectively. PCR cycling parameters for AChE and S12 rRNA consisted of denaturation for 1 min at 94 °C, followed by primer annealing and extension for 3 min at 70 °C for AChE and primer annealing for 1 min at 54 °C and extension for 2 min at 72 °C for S12 rRNA, followed by a 10-min elongation step at 72 °C. PCR products were visualized and quantified on ethidium bromide-stained 1.5% agarose gels. Quantitation of the labeling intensity of the PCR products was performed using the Kodak Digital Science Image Station 440 CF and related Kodak Digital Science 1D

Image Analysis Software (Eastman Kodak Co.). All values obtained for AChE were corrected according to the corresponding level of S12 rRNA present in the sample.

All RT-PCR experiments aimed at determining the relative abundance of AChE transcripts were performed using cycle numbers that fell within the linear range of amplification (37–39). The cycle numbers were between 24 and 27 for AChE and 22 for S12 rRNA. RT-PCR conditions (primer concentration, input RNA, choice of RT primer, cycling conditions) were initially optimized, and these were identical for all experiments. Appropriate precautions (use of sterile filtered tips and gloves) were taken to prevent contamination of the samples and degradation of the RNA. Samples, including the negative control, were always prepared using the same RT and PCR reagents and master mixes, and were run in parallel. In all experiments, PCR products were never detected in the negative controls.

Nuclear Run-on Assays—Nuclear run-on assays were performed as described in detail elsewhere (23, 41, 42). Briefly, nuclei were isolated from undifferentiated and differentiated PC12 cells (two 250-ml flasks) and resuspended in a transcription buffer containing GTP, ATP, CTP, and 25 μCi of [α -^{32}P]UTP. RNA was transcribed for 60 min at 30 °C in the presence of an RNase inhibitor (Promega, Madison, WI). Following a 30-min RQ1 DNase I (Promega) treatment, the nascent radiolabeled RNA was extracted using TRIzol reagent (see above) and hybridized for 48 h to 10 μg of linearized AChE cDNA (2 kb) immobilized on a Protran pure nitrocellulose membrane (Schleicher & Schuell). After hybridization the membranes were washed thoroughly at 42 °C in a 1 × saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) solution and subjected to autoradiography. The intensity of the resulting signals was quantified using a STORM

PhosphorImager and the accompanying ImageQuant software (Amersham Biosciences). The signals corresponding to AChE were standardized relative to the signal obtained from genomic DNA.

Reporter Constructs and Transfection Studies—The recently described 5.3-kb AChE promoter fragment termed GRAP (25) was subcloned into a LacZ reporter vector. In addition, the 3'-UTR from the mouse ~2.4-kb AChE transcript, which contains the shorter 3'-UTR in comparison to the ~3.2-kb AChE mRNA (see Introduction), was amplified by RT-PCR and first inserted into the pGL3 vector. For these experiments, we focused on the short 3'-UTR as opposed to the longer one because (i) previous studies showed that it appears to contain important *cis*-acting regulatory elements (24, 43, 44), and (ii) the ~2.4-kb AChE transcript is considerably more abundant in nervous tissues (12) including PC12 cells (45, 46). The 3'-UTR was subsequently cloned into a luciferase reporter construct driven by the thymidine kinase promoter (phRG-TK) (Promega). Plasmid DNA was prepared using the Mega-Prep procedure (Qiagen, Chatsworth, CA). DNA pellets were resuspended in 10mM Tris-HCl, pH 8.5. Transfections were performed using the LipofectAMINE reagent kit (Invitrogen) according to the instructions from the manufacturer. Undifferentiated cells ($1–2 \times 10^5$ cells/cm^2) were transfected with 0.5 μg of the appropriate reporter gene construct and 0.5 μg of the constitutively expressed chloramphenicol acetyltransferase (CAT) plasmid driven by the SV40 promoter used, in this case, to control for transfection efficiency. Transfected cells were induced to differentiate 24 h later by the addition of NGF. To determine reporter gene activity, undifferentiated and differentiated cells were washed with cold PBS and lysed in Reporter-Lysis buffer (Promega) following two cycles of freezing and thawing. The

extracts were then centrifuged (15,000 × *g* for 2 min at 4 °C), and the resulting supernatants were assayed for β-galactosidase, luciferase, or CAT activities using available kits (Promega). The β-galactosidase and luciferase activities were normalized to CAT levels. Background values, obtained by transfecting promoterless LacZ and luciferase plasmids, were subtracted from the activities obtained with the reporter constructs.

In Vitro Transcription—cDNAs encoding different lengths of the AChE 3'-UTR were obtained by PCR amplification of the plasmid template pGL3–3'-UTR (see above). The PCR primers employed to amplify: 1) the full-length AChE 3'-UTR, 2) a truncated fragment in which the AU-rich element was absent (-ARE), and 3) a small fragment encompassing the ARE (see Fig. 5A), were designed to include a T7 promoter. An *in vitro* T7 transcription system (Promega) was used to synthesize radiolabeled AChE 3'-UTR fragments. Briefly, the transcription reaction containing 0.5 μg of PCR fragment, 5 μCi of [α-^{32}P]UTP, nucleotides, RNase inhibitor, and T7 polymerase, was carried out at 37 °C for 1 h. The template PCR fragments were digested with 1 unit of RQ1 DNase I (Promega) for 30 min at 37 °C. The resulting radiolabeled RNA was purified on an RNase-free G-25 RNA purification column (Roche Diagnostics Corp., Indianapolis, IN). The integrity of the RNA was confirmed by gel electrophoresis. Unlabeled RNA probes were generated by the same method and used in cold competition assays.

Electrophoretic Mobility Shift Assay and Northwestern Analyses—RNA-based electrophoretic mobility shift assays (REMSAs) and Northwestern blots were performed using total protein extracts obtained from undifferentiated and differentiated cells (two

250-ml flasks). The cells were washed with cold PBS, scraped, and lysed in 300 µl of homogenization buffer (0.3 M sucrose, 60 mM NaCl, 15 mM Tris, pH 8.0, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 µg/µl leupeptin, 10 µg/µl pepstatin A, 1 µg/µl aprotinin, pH 7.4). The samples were centrifuged (15,000 × *g* for 15 min at 4 °C), and the resulting supernatant was stored at –80 °C until used. The total amount of protein present in the extracts was determined by the BCA method (see above).

REMSAs were performed as described elsewhere in detail (47–49). Forty µg of protein extract were incubated for 20 min at room temperature with 1 x 10^5 cpm of ^{32}P-labeled AChE 3'-UTR fragments in 2× binding buffer (20 mM Hepes, pH 7.9, 3 mM MgCl2, 50 mM KCl, 1 mM DTT, 5% glycerol, 0.2 µg/µl yeast tRNA) in a total volume of 20 µl. The unbound RNA was digested with ribonuclease T1 (Calbiochem, San Diego, CA) for 20 min at 37 °C, and the samples were then incubated at room temperature for 10 min with heparin (2.5 mg/ml). This mixture was separated by 4 or 6% native polyacrylamide gel electrophoresis with 0.5× TBE (Tris borate-EDTA) running buffer. The gels were subsequently dried under vacuum at 80 °C for 1 h and exposed to x-ray film at –70 °C. Competition assays were performed by incubating a 25 M excess of cold probe with the protein extract for 10 min prior to the incubation with the radiolabeled probe.

Northwestern analyses were performed according to the procedure described elsewhere (50, 51). Fifty µg of protein extract diluted in SDS buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromphenol blue, 10% glycerol) were denatured at 100 °C for 3 min and separated by 8% SDS-PAGE. After separation, proteins were

electroblotted onto a polyvinylidene difluoride membrane (Schleicher & Schuell). The membrane was then incubated in renaturation buffer (15 mM Hepes, pH 7.9, 50 mM KCl, 0.1 mM MnCl2, 0.1 mM ZnCl2, 0.1 mM EDTA, 0.5 μM DTT, 0.1% (w/v) Ficoll 400 D-L, 0.1% (w/v) polyvinylpyrrolidone, and 0.01% (v/v) Igepal CA-630 (a Nonidet P-40 substitute) in RNase-free water) at 4 °C overnight. Following pre-hybridization at room temperature for 1 h in renaturation buffer containing 0.2 mg/ml yeast tRNA, the membrane was incubated for 4 h with 1×10^6 cpm/ml of a probe corresponding to the radiolabeled AChE 3'-UTR RNA dissolved in renaturation buffer containing 0.2 mg/ml yeast tRNA and 5 mg/ml heparin at room temperature. After several 5-min washes, the membrane was put to x-ray film at −70 °C. To ensure that equivalent amounts of proteins were loaded for each sample, membranes were also stained with Ponceau S (Sigma), following exposure to x-ray films.

Immunoprecipitation and AChE mRNA Analysis—The RNA-binding protein HuD was immunoprecipitated from a total protein extract as described (52). Total protein was extracted from PC12 cells stably transfected to express HuD (8 × 100-mm plate). To this end, the cells were pelleted, resuspended in 0.3 ml of immunoprecipitation (IP) buffer (1% Igepal CA-630, 10 mM Tris-HCl, pH 7.5, 1% bovine serum albumin, 150 mM NaCl, 2 mM EDTA, 25 μg/μl pepstatin A, 2.5 μg/μl aprotinin, 10 units of RNase inhibitor), and sonicated (10-s pulse at 50% duty cycle and a power output of 1 using the Branson Sonifier 450). Protein samples (200 μg) were incubated for 1 h at 4 °C, with an affinity purified antibody to HuD previously described (34) or with normal rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) in IP buffer. This reaction mixture was subsequently added to 20 μl of pre-washed A/G chimera Sepharose beads (Pierce) and

incubated by gentle shaking at 4 °C for 1 h. The mixture was centrifuged (10,000 × *g* for 20 s), and the supernatant was removed. After several washes with IP buffer, the RNA was extracted from the pellet using the TRIzol reagent and analyzed by RT-PCR as described above.

Western Blot—Cultured cells were washed in 1× PBS; resuspended in a homogenization buffer containing 0.3 M sucrose, 60 mM NaCl, 15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 mM -mercaptoethanol, 0.01 mM phenylmethylsulfonyl fluoride, 0.01 mM benzamidine, 1 μg of leupeptin, 10 μg of pepstatin A, and 1 μg of aprotinin; and sonicated (see above). Following centrifugation, the supernatant was recovered, aliquoted, and stored at −80 °C. The concentration of proteins in each sample was determined using the BCA method (see above). For Western blotting, 50 μg of protein extracts were denaturated in SDS loading buffer and subjected to SDS-PAGE using a 10% gel. The proteins were then transferred onto a polyvinylidene difluoride membrane (Sigma). Following transfer, the membranes were incubated with antibodies directed against HuD (34) and revealed using a commercially available ECL kit from Pierce.

Statistical Analysis—An analysis of variance was performed to evaluate the effects of NGF-induced neuronal differentiation on AChE expression. The Fisher's Least Square Difference test was used to determine whether the differences seen between group means were significant. The level of significance was set at $p \leq 0.05$. Data are expressed as mean ± S.E. throughout.

2.5 Results

AChE Expression during Neuronal Differentiation—In a first series of experiments, we examined the expression of AChE during the process of NGF-induced neuronal differentiation of PC12 cells. Initially, we compared the level of cell-associated AChE between undifferentiated and 24-, 48-, and 72-h differentiated PC12 cells. Previous studies have demonstrated that undifferentiated PC12 cells express a basal level of AChE activity that increases upon NGF stimulation (31, 53, 54). In agreement with these earlier reports, Fig. 1A shows that AChE activity increases considerably during differentiation. In fact, the cell-associated activity increased significantly by ~2.5-fold ($p < 0.01$) within the first 48 h, and reached a maximal 5-fold induction ($p < 0.0001$) following 72 h of NGF treatment.

We next examined the impact of NGF on the relative abundance of AChE transcripts in PC12 cells. We focused on the level of the T transcript because this transcript is the predominant splice variant found in nervous tissues (12) and PC12 cells (45, 46). As illustrated in Fig. 1B, AChE mRNAs could be detected in undifferentiated cells. However, treatment of PC12 cells with NGF led to a pronounced increase in the levels of AChE mRNA. These increases were highly significant, reaching more than 2-fold ($p < 0.002$) and 3.5-fold ($p < 0.0001$) by 48 and 72 h, respectively (Fig. 1C). The relative amount of S12 rRNA, which was used as an internal control for these assays, did not change during differentiation. The observed increase in transcript level was directly related to the NGF treatment and not to the length of time in culture or cell density because, in separate studies, both of these factors had no effect on AChE expression (data

not shown). In addition, NGF removal from the culture medium 24 h after the initial treatment resulted in a gradual decrease in AChE mRNA levels (data not shown).

Because recent studies demonstrated the importance of transcriptional events in regulating AChE expression at the early stages of muscle differentiation (23, 30), we

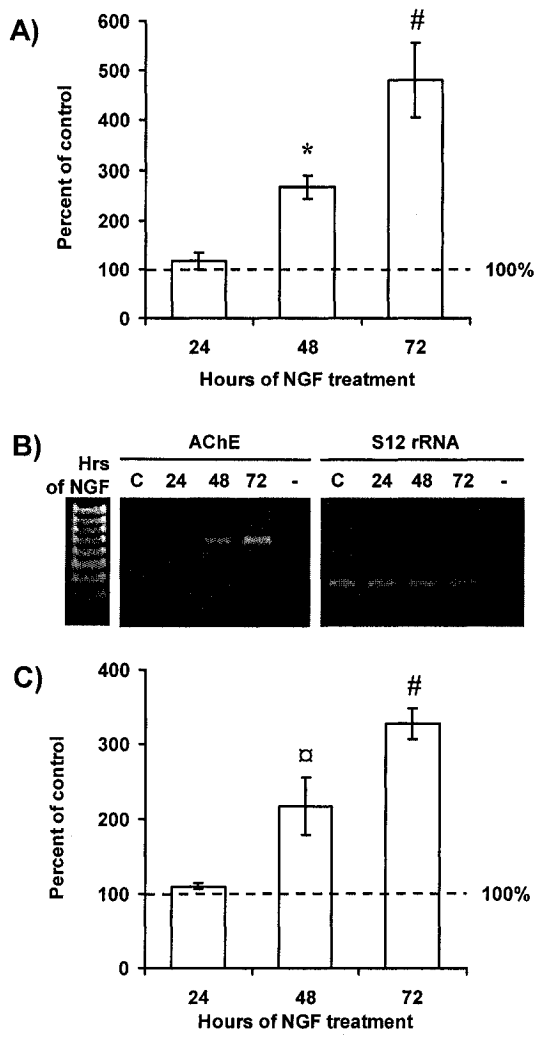

FIG. 1. AChE activity and transcript levels increase in differentiating PC12 cells. *A*, AChE activity was determined from protein extracts obtained from untreated and NGF-treated cells for different time periods. The activity is expressed as a percentage of the activity seen in the untreated (control) cells. *Symbols* indicate significant differences from control cells (*, $p < 0.01$; #, $p < 0.0001$; $n = 5$ independent experiments). *B*, examples of ethidium bromide-stained agarose gels displaying AChE and S12 rRNA PCR products from control (*C*) and NGF-treated (24, 48, or 72 h) PC12 cells. The negative control lane (water, no RNA) is shown with a –. *C*, quantitation of AChE

mRNA levels in NGF-treated (24, 48, and 72 h) PC12 cells expressed as a percentage of untreated (control) cells. *Symbols* indicate significant differences from control cells (¤, $p < 0.002$; #, $p < 0.0001$; $n = 5$ independent experiments).

determined whether the increase in the abundance of AChE transcripts seen in differentiating PC12 cells could be linked to an increase in transcription.

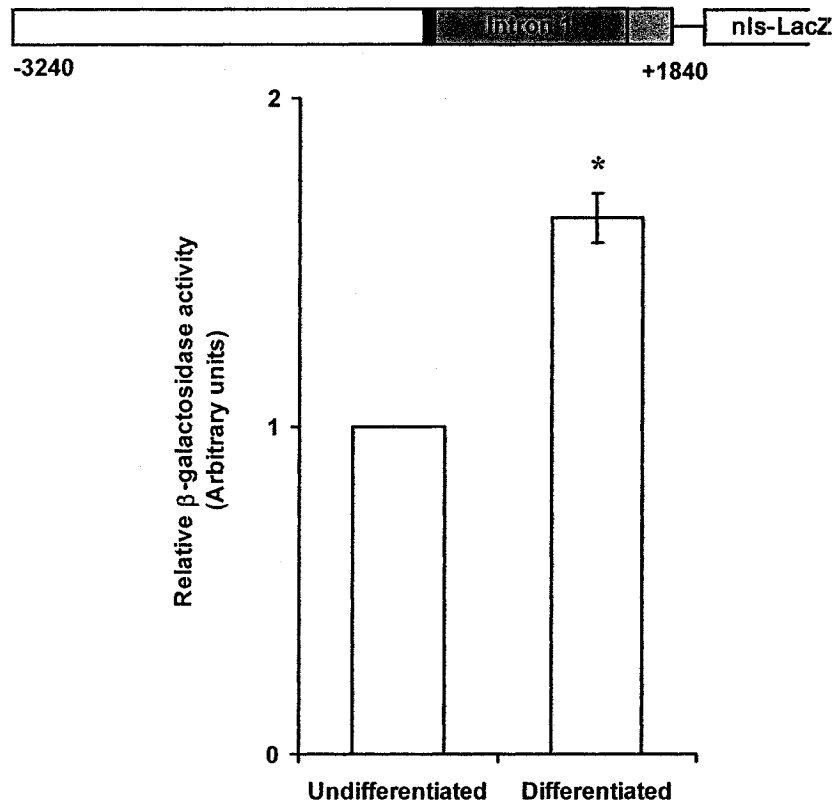

FIG. 2. Transcriptional activity of the ACHE gene increases early in differentiating PC12 cells. Quantitation of normalized β-galactosidase activity expressed in arbitrary units, obtained from undifferentiated and NGF-induced differentiated (24 and 48 h) PC12 cells transfected with the GRAP promoter-reporter construct (*top*). *Symbols* indicate significant differences from undifferentiated cells (*, $p < 0.0001$; $n = 4$ independent experiments).

To this end, we performed transfection studies using an AChE promoter-reporter gene construct that contained a ~5.3-kb promoter fragment termed GRAP (24). This fragment contains, in addition to the basal promoter region, intronic elements previously shown to

be important for muscle expression (23, 25). We observed, using this approach, a small but significant increase (1.5-fold; $p < 0.0001$) in reporter gene expression during the early phases of differentiation of PC12 cells (Fig. 2). Nuclear run-on assays performed with nuclei isolated from undifferentiated and 24-h differentiated PC12 cells confirmed these findings (data not shown). This transcriptional increase parallels the initial 2-fold increase in AChE mRNA levels, which occurs during the initial phase of neuronal differentiation. However, it is important to emphasize that this increase in transcription is transient and that, therefore, it fails to account for the increase in AChE transcript level seen at later time points, *i.e.* 72 and 96 h. Indeed, at these later time points, transcription of the AChE gene had returned to the level seen in undifferentiated cells (data not shown).

Post-transcriptional Mechanisms Regulate AChE Transcript Levels during Neuronal Differentiation—Recent studies have demonstrated that NGF can act indirectly to stabilize neuron-specific transcripts via the ELAV-like RNA-binding protein HuD (52, 55). Interestingly, examination of the sequence of the AChE 3'-UTR revealed the presence of a conserved element known to be recognized by HuD (see Fig. 4*A*). Therefore, to ascertain whether post-transcriptional mechanisms are indeed involved in regulating AChE expression during neuronal differentiation and to explore the possibility that this effect involves HuD, we next examined the expression of AChE transcripts in PC12 cells stably transfected with a HuD construct (pcHuD), with an antisense sequence to HuD (pDuH), or with the empty plasmid (pcDNA) acting in this case, as a control (34). In comparison to the control levels of HuD seen in pcDNA cells, levels of HuD in pcHuD cells are 2–3-fold higher, whereas, in pDuH cells, HuD expression is reduced by ~70% (34).

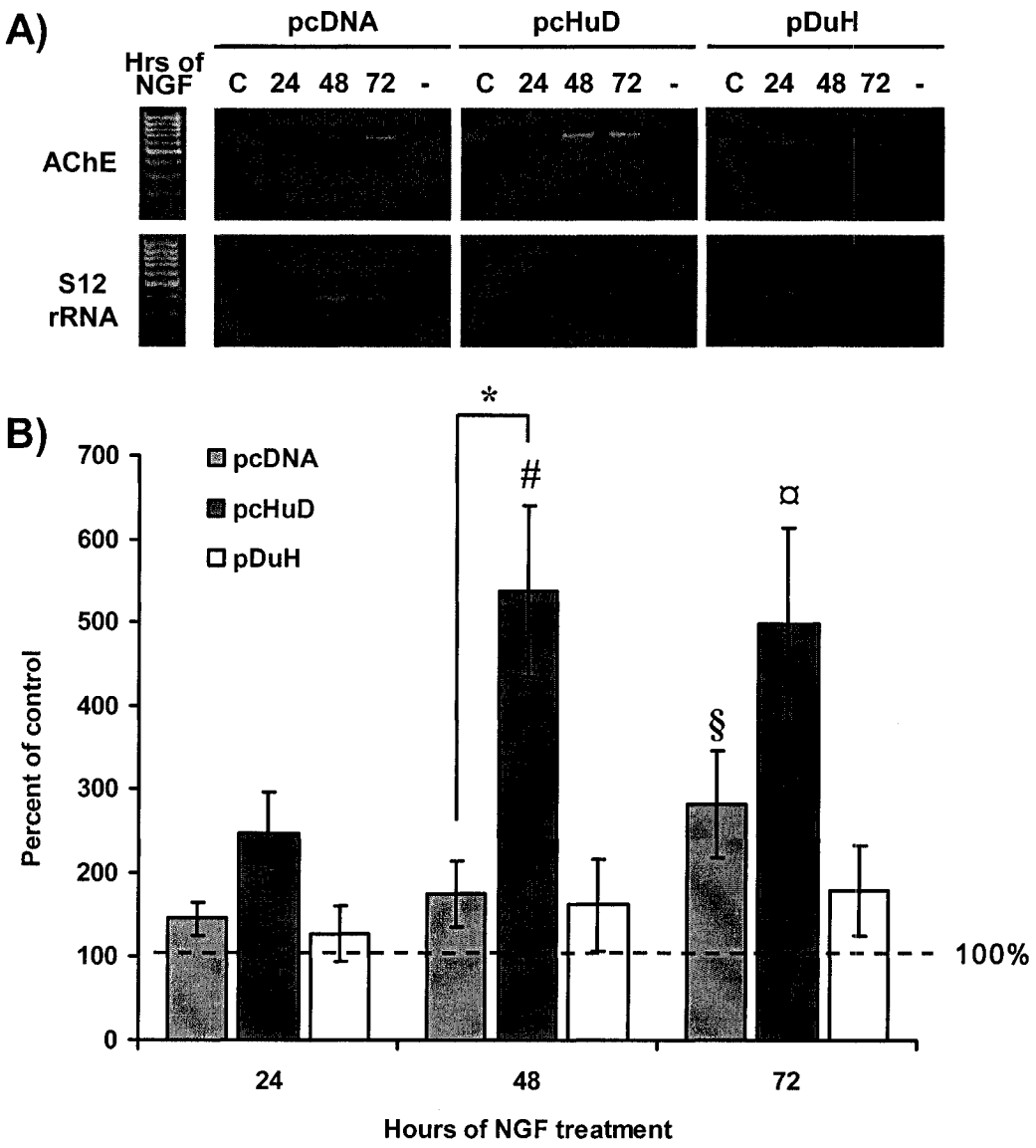

FIG. 3. PC12 cells expressing the RNA-binding protein HuD have higher levels of AChE transcripts. *A*, examples of ethidium bromide-stained agarose gels displaying AChE and S12 rRNA PCR products from control (*C*), NGF-treated (24, 48, or 72 h) PC12 cells stably transfected to express HuD (pcHuD), an antisense to HuD (pDuH), or the empty vector (pcDNA). The negative control lane (water, no RNA) is shown with a –. *B*, quantitation of AChE mRNA levels in NGF-treated (24, 48, and 72 h) PC12 cells expressed as a percentage of the untreated (control) pcDNA cells. *Symbols* indicate significant differences (§, $p < 0.006$; #, $p < 0.01$; ¤, $p < 0.02$; $n = 4$ independent experiments) from control. * indicates a significant difference ($p < 0.006$) from 48-h treated pcDNA cells.

As observed in non-transfected PC12 cells, AChE transcripts are expressed, albeit at different levels, in these undifferentiated stable cells. Specifically, undifferentiated pcHuD cells expressed twice as much AChE transcripts as undifferentiated pcDNA cells, whereas the pDuH cells express ~80% less transcript than the pcDNA cells in the undifferentiated state (Fig. 3*A*). Interestingly, these data parallel the changes in HuD expression seen in the stable cell lines (see above).

In response to NGF, however, AChE mRNA levels showed an increase only in the PC12 cells transfected with pcDNA and pcHuD and not in the cells expressing the antisense sequence to HuD (Fig. 3). In fact, AChE transcript levels in cells transfected with pcDNA increased by a maximum of ~3-fold ($p < 0.006$) following NGF treatment, demonstrating that these cells display a pattern of AChE mRNA expression similar to that seen in non-transfected PC12. NGF treatment of pcHuD cells dramatically increased AChE transcripts to a level greater than that observed in the pcDNA cells. This increase reached more than 2.5- and 5-fold within 24 and 48 h of NGF stimulation ($p < 0.02$), respectively (Fig. 3*B*). Additionally, the maximal increase in AChE mRNA expression in pcHuD cells was reached earlier during differentiation such that, after 48 h of NGF treatment, pcHuD cells contained significantly ($p < 0.006$) more AChE mRNAs than pcDNA cells. By contrast, PC12 cells engineered to express an antisense sequence to HuD (pDuH cells) did not exhibit any significant changes in the relative abundance of AChE mRNA during the course of differentiation.

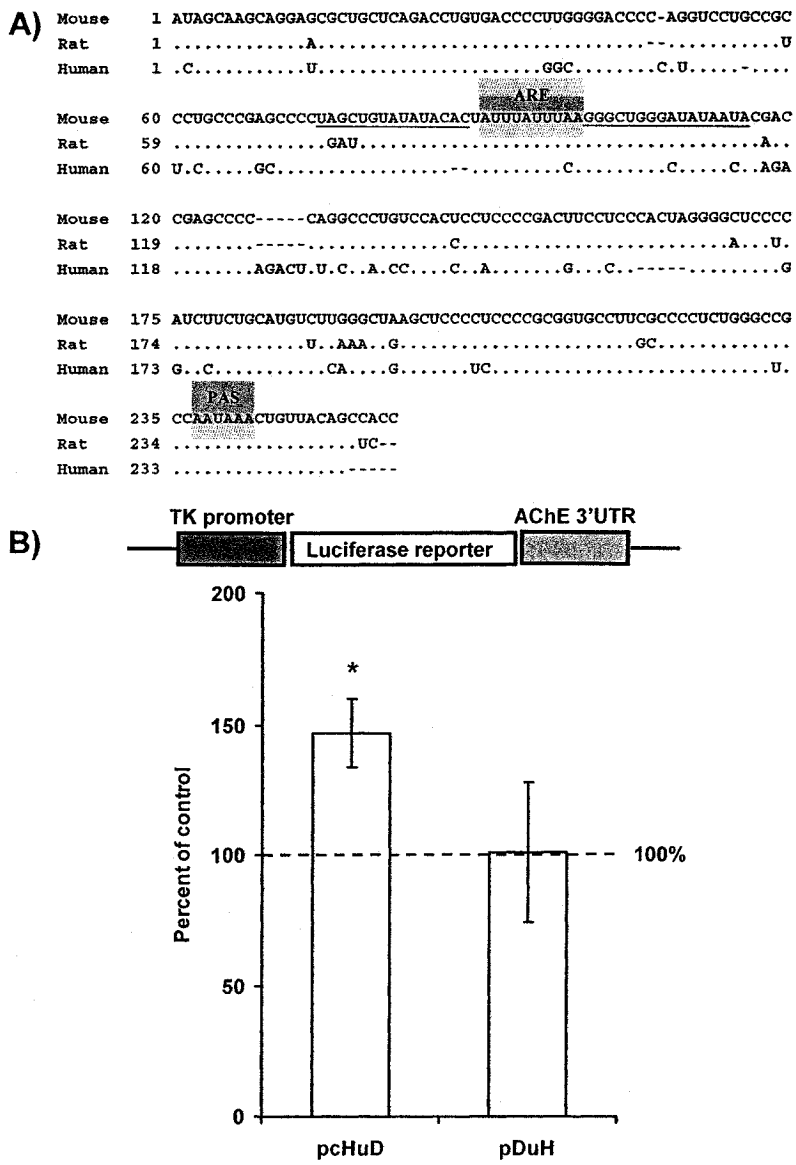

FIG. 4. The AChE 3'-UTR increases expression of a reporter construct in PC12 cells. *A*, alignment of the AChE 3'-UTR from mouse, rat, and human shows the presence of a conserved ARE found within an AU-rich domain (*underlined*) and poly(A) signal (*PAS*). *B*, undifferentiated pcDNA, pcHuD, and pDuH cells were co-transfected with a reporter construct containing the full-length AChE 3'-UTR and with a constitutively expressed CAT plasmid. The luciferase activity was normalized to that seen with CAT and is expressed as a percentage of the activity found in the control pcDNA cells. *Symbol* indicates a significant difference (*, $p < 0.05$; $n = 5$ independent experiments) from control cells.

We next considered whether the effect of HuD occurred directly on AChE transcripts. To this end, we used an approach similar to that used recently by others (56, 57). Therefore, we transfected pcDNA, pcHuD, and pDuH cells with a luciferase reporter construct in which the 3'-UTR of AChE transcript was inserted. This 3'-UTR contains the conserved ARE, which, as mentioned, is known to be a *cis*-acting element recognized by HuD (Fig. 4*A*). These experiments were performed on undifferentiated cells in attempts to minimize the potential confounding impact of an increased level of endogenous AChE caused by NGF stimulation. In comparison to cells stably transfected with pcDNA, the activity of the reporter gene was significantly higher ($p < 0.05$) in cells expressing HuD. Importantly, this effect was completely abolished in pDuH cells, indicating that HuD is indeed an important factor regulating AChE expression via the 3'-UTR.

Changes in the Pattern of RNA-Protein Interactions during Neuronal Differentiation—Based on these findings, we also examined the pattern of proteins that could bind to the AChE 3'-UTR by both REMSA and Northwestern analyses. For these experiments, we used three probes: one corresponding to the full-length AChE 3'-UTR (245 nucleotides); a truncated version of the full-length probe (90 nucleotides) in which the ARE was deleted (–ARE), thereby eliminating the putative HuD binding region; and a smaller probe encompassing the ARE (Fig. 5*A*). In REMSA, four distinct protein complexes could be visualized using the full-length AChE 3'-UTR probe (Fig. 5*B*). Although the relative abundance of all four complexes appeared to increase in PC12 cells stimulated with NGF for 72 h, two, in particular, showed dramatic increases in the binding intensity.

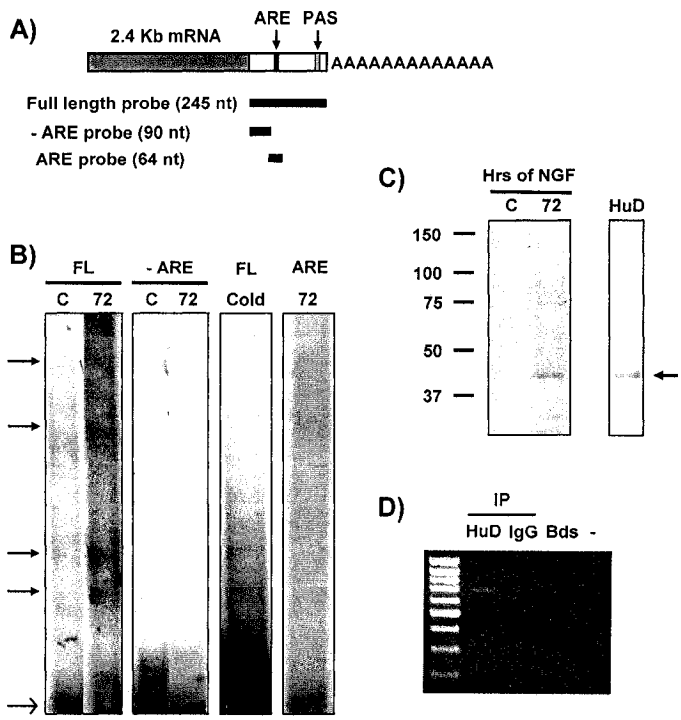

FIG. 5. Changes in the pattern of RNA-protein interactions in the AChE 3'-UTR in differentiating PC12 cells. *A*, schematic displaying the ~2.4-kb AChE transcript depicting important elements within the 3'-UTR, *i.e.* the ARE and poly(A) signal. The locations of the full-length (245 nucleotides (*nt*)), a truncated probe (90 nucleotides) lacking the ARE (*–ARE*), and a probe (64 nucleotides) encompassing the ARE are also shown. *B*, REMSAs were performed using protein extracts from control (*C*) untreated and 72-h NGF-treated (*72*) PC12 cells. Representative autoradiograms demonstrating the interaction between RNA and protein complexes using the full-length (*FL*) probe are shown. *Closed arrows* indicate specific RNA-protein complexes, and *open arrow* points to the free probe. Competition experiments using a 25 M excess of unlabeled full-length (*FL*) probe is also shown (*Cold*). Note the increase in the relative abundance of the protein complexes in differentiated cells and the absence of these complexes in REMSAs performed with the truncated probe (*–ARE*). REMSA performed using the ARE probe shows that a single protein complex can directly interact with the ARE. *C*, Northwestern blots (*left panel*) were performed using protein extracts from control untreated (*C*) and 72-h NGF-treated (*72*) PC12 cells. A representative autoradiogram shows the increase in the amount of proteins interacting with the AChE 3'-UTR and a particular strong induction of a ~42-kDa protein. A Western blot (*right panel*) shows that, indeed, HuD migrates at ~42 kDa (see *arrow*). *D*, example of an ethidium bromide-stained agarose gel displaying AChE PCR product obtained from an immunoprecipitate isolated from differentiated pcHuD cells using an antibody against HuD, normal serum IgG, and A/G chimera Sepharose beads (*Bds*). The negative control lane (water, no RNA) is shown with a –. Note the presence of a PCR product corresponding to AChE only in the immunoprecipitate obtained with the HuD antibody.

The appearance of these complexes began after 48 h of differentiation (data not shown). By contrast, the formation of these complexes could not be observed in REMSA using –ARE probe. Specificity of the protein complexes for the AChE 3'-UTR was demonstrated in competition assays using a 25-fold molar excess of unlabeled, full-length AChE 3'-UTR probe. In addition, REMSA using the small fragment of the AChE 3'-UTR that encompasses the ARE (see Fig. 5*A*) showed that indeed a protein complex could directly interact with this specific region (Fig. 5*B*). This latter complex appeared to correspond to one of the larger complexes seen with the full-length probe. It is important to note that, in all these experiments, both yeast tRNA and heparin were used to eliminate the possibility of nonspecific interactions.

Northwestern analyses were also performed to confirm that the pattern of RNA-protein interactions was indeed altered following NGF stimulation. In comparison to REMSA, Northwestern analyses allow the determination of the molecular mass of specific proteins that interact with the RNA probe. In agreement with our REMSA data, several proteins appeared to interact with the AChE 3'-UTR (Fig. 5*C*). Importantly, NGF stimulation markedly increased the relative abundance of these proteins. The greatest increase appeared at the level of a protein of ~42 kDa. Together with our functional data (see above), the presence of this ~42-kDa protein (approximate mass of HuD) in PC12 cells exposed to NGF for 72 h raised the possibility that this protein may in fact correspond to HuD. Western blot analysis confirmed the molecular mass of HuD at ~42 kDa (Fig. 5*C*). In control experiments, the truncated AChE 3'-UTR probe, *i.e.* –ARE probe, could not bind any proteins in these Northwestern assays (data not shown).

In a final series of experiments, we verified that HuD is able to interact directly and bind with the AChE 3'-UTR in PC12 cells. To this end, we first immunoprecipitated HuD protein from a total protein extract obtained from 48-h differentiated pcHuD cells, isolated total RNA from the immunoprecipitate, and performed RT-PCR to detect AChE transcripts. This procedure has previously been successfully used to show the interaction between other ELAV/Hu family proteins and neuronal transcripts (34, 58). As shown in Fig. 5*D*, we were able to specifically detect the presence of AChE transcripts from the immunoprecipitate obtained using the affinity-purified anti-HuD antibody. By contrast, no AChE PCR product could be visualized in total RNA isolated from immunoprecipitates obtained using either normal rabbit IgG or A/G chimera Sepharose beads alone (34). In these assays, the identity of the AChE PCR product was confirmed by sequencing.

2.6 Discussion

In recent years, there has been significant progress in characterizing some of the transcriptional and post-transcriptional mechanisms involved in the regulation of the AChE gene in skeletal muscle (23, 25, 27, 30, 43, 59, 60). By contrast, there are only a few reports available that have examined the molecular events controlling AChE expression in neurons. Given its central role in terminating neurotransmission at cholinergic synapses, its additional non-catalytic function in the control, for example, of neurite outgrowth (see Introduction), and its involvement in a variety of neurological conditions such as Alzheimer's disease, post-traumatic stress disorder (61), and brain tumors (62), it appears important to gain a better understanding of the molecular

pathways ultimately controlling the expression and localization of AChE in neurons. In the present study, we have examined some of the molecular events involved in regulating expression of the AChE gene during neuronal differentiation. Our findings indicate that, although transcription provides an initial boost necessary to rapidly increase AChE mRNA levels at the onset of neuronal differentiation, post-transcriptional events involving the 3'-UTR and the RNA-binding protein HuD are also involved and largely account for the pronounced and sustained increase in AChE transcripts seen at later stages of differentiation.

Several years ago, a series of experiments focusing on P19 cells induced to differentiate into neurons via retinoic acid treatment led Coleman and Taylor (31) to suggest that changes in mRNA stability was a key mechanism controlling the relative abundance of AChE transcripts during neuronal differentiation. However, this initial study provided little insight into the nature of the *cis*- and *trans*-acting elements that could be involved. In this context, *cis*-elements contained within the 3'-UTR have been shown to be critical for regulating the turnover rate of pre-synthesized mRNAs in a variety of cells. In particular, the element known as the AU-rich element, which is known to exist in different classes (63, 64), has received considerable attention because of its key role in mRNA metabolism. Several studies have shown that this element can either stabilize or destabilize transcripts depending on the number of sequential repeats, variations of the basic sequence, and the identity of the *trans*-acting factor that binds (65, 66). Among the proteins known to interact with the ARE is the ELAV-like family of Hu proteins. This group of RNA-binding proteins consists of HuR, HuB (Hel-N1), HuC, and HuD, of which HuB, -C, and -D are neuron-specific and HuR is more ubiquitously

expressed (65, 67). Several studies from different laboratories have identified HuD as a *trans*-acting factor binding and stabilizing a variety of different cellular transcripts including c-Myc (68), neuroserpin (69), tau (70), and GAP-43 (34).

Because AChE contains in its 3'-UTR a conserved ARE, we examined in the present studies whether HuD plays a functional role in regulating AChE expression in neurons. To this end, we used a series of distinct, yet complementary, approaches to determine whether indeed HuD could affect AChE mRNA expression. Using PC12 cells stably transfected to express HuD or an antisense sequence to its mRNA, we found that the relative abundance of AChE transcripts varied with the amount of HuD expressed by these cells. Moreover, HuD expression induced a faster and greater increase of AChE mRNA levels during NGF-induced differentiation of these cells. Importantly, this differentiating effect of NGF on AChE gene expression was completely blocked in cells expressing the HuD antisense. Furthermore, we also observed that expression of HuD could increase the expression of reporter transcripts engineered to contain the AChE 3'-UTR. Finally, immunoprecipitation experiments revealed that, indeed, HuD can directly interact with AChE transcripts. Taken together, these results strongly implicate HuD and the AChE 3'-UTR in the regulation of AChE mRNA expression during neuronal differentiation.

In comparable studies, HuD has been shown recently to be capable of regulating the stability and localization of transcripts encoding GAP-43 and tau in neurons (34, 55, 70, 71). Interestingly, these proteins are known to be essential for the process of neurite outgrowth during neuronal differentiation. Because AChE has also been implicated in the events regulating neuritogenesis (see Introduction), it appears reasonable to speculate

that HuD may, in fact, directly control neurite outgrowth by regulating the abundance and localization of a variety of transcripts encoding proteins that are key to this process. In agreement with this view, several studies have demonstrated that neurite extension is completely inhibited in PC12 cells stably expressing an antisense to HuD (34, 70–72). Such mechanism would confer HuD a central organizing role and could provide neurons with an efficient post-transcriptional regulatory pathway. In one basic scenario, neurons could therefore simply induce HuD expression to trigger the process of neurite outgrowth, as opposed to activating multiple signaling cascades that would need to culminate in the activation of the transcriptional machinery of important genes via distinct promoter elements.

Although the data presented here strongly indicate that HuD and the AChE 3'-UTR are indeed important elements in controlling AChE mRNA levels in differentiating neurons, our findings do not imply that these elements act alone. It is well established, for example, that the RNA-binding protein AUF1 forms a complex with several other proteins involved in transcript elongation or stability (73). Similarly, it has become increasingly apparent that the precise regulation of mRNA stability, localization, and translation depends on the presence of multiple *cis*-acting elements that interact with several distinct *trans*-acting factors (65, 66). Our analysis of RNA-protein interactions using both REMSA and Northwestern blotting is consistent with this notion because we observed several complexes and proteins that could interact with the AChE 3'-UTR. In future studies, it will be important to characterize these factors to gain a complete understanding of the *cis*- and *trans*-acting factors contributing to the regulation of AChE expression in neurons.

Our experimental approach has also allowed us to examine whether differentiation of PC12 cells was accompanied by a change in the transcriptional activity of the AChE gene. Our promoter-reporter studies and nuclear run-on assays both revealed that NGF initially induces a modest but significant increase in transcription. These results suggest therefore that, during the earlier stages of neuronal differentiation, transcriptional regulation plays a key role in the initial up-regulation of AChE mRNA expression as previously suggested by Greene and Rukenstein (31). In fact, these findings fit nicely with the recent demonstration that transcription, indeed, is involved in regulating AChE expression in neuronal cells (46, 74) and with the transient transcriptional induction of GAP-43 following NGF treatment of PC12 cells (75, 76). Together with the observation that, during the early phases of myogenic differentiation, transcriptional changes can also account for the initial increase in AChE mRNA levels (23), these findings are therefore consistent with a model in which developmental changes in AChE mRNA expression are initially regulated at the transcriptional level with post-transcriptional mechanisms becoming more important at later time points to ensure the adequate supply of AChE to differentiating muscle and neuron.

Because differentiation of PC12 cells is dependent on the continuous presence of NGF in the culture media, it appears reasonable to argue that NGF acts indirectly through HuD to regulate AChE expression in a manner similar to that described previously for the regulation of GAP-43 (see above). In addition to directly affecting transcription of target genes (77, 78), these findings indicate therefore that some of the pleiotropic effects of neurotrophins on various neuronal populations may occur through RNA-binding proteins, which in turn affect, via post-transcriptional mechanisms, the expression of key mRNAs.

In this context, previous animal studies have shown, for example, that treatment of axotomized facial motoneurons and rubrospinal neurons with neurotrophins leads to an increase in the expression of both GAP-43 and AChE transcripts (16, 79–81). Based on the foregoing discussion, we can speculate that some of these neurotrophin-induced changes in gene expression observed in injured neurons may in fact be caused by post-transcriptional mechanisms operating at the level of transcript stability, localization, and translation. Future studies should therefore make an attempt at unraveling the importance of these post-transcriptional events *in vivo* because, ultimately, these could lead to the design of additional therapeutic strategies aimed at promoting neuronal regeneration and survival.

Chapter 3

The RNA-binding Protein HuR Binds to Acetylcholinesterase Transcripts and Regulates Their Expression in Differentiating Skeletal Muscle Cells

Julie Deschênes-Furry, Guy Bélanger, James Mwanjewe, John A. Lunde, Robin J. Parks, Nora Perrone-Bizzozero, and Bernard J. Jasmin

Published in The Journal of Biological Chemistry Vol. 280(27): 25361–25368, 2005

Acknowledgements: This work was supported in part by grants from the Canadian Institutes of Health Research (CIHR) (to B. J. J. and R. J. P.) and by National Institutes of Health Grant NS-30255 (to N. P.-B.). We thank Lauren Smith and Kathy Sargeant for technical assistance with specific portions of this project.

3.1 Author contribution

Julie Deschênes-Furry:

Unless otherwise indicated all experiments were performed by the first author under the supervision of Dr. Bernard Jasmin. The manuscript was prepared by the first author and revised by Dr. Bernard Jasmin.

Guy Bélanger:

All cell culture work, including protein extraction, transfection and analysis of AChE 3'-UTR and HuR plasmid constructs and siRNA studies were performed by Dr. Bélanger. The AChE 3'UTR and complete mouse HuR cDNA plasmid constructs and primer sets that were used in this study for transfection experiments and generation of stable HuR overexpressing C2C12 cells, were designed and prepared by Dr. Bélanger.

James Mwanjewe:

Dr. Mwanjewe performed the *in vitro* stabibility assay.

John A. Lunde:

Mr. Lunde performed some of the RT-PCR reactions for quantification of AChE mRNA levels during differentiation and detection of AChE transcript in the HuR immunoprecipitate.

Robin J. Parks:

Dr. Parks designed and provided the lab with adenoviral constructs to HuD.

Nora Perrone-Bizzozero:

Dr. Perrone-Bizzozero provided the lab with the plasmid construct containing the complete human HuD cDNA. The written manuscript was also revised by this author.

3.2 Abstract

During myogenic differentiation, acetylcholinesterase (AChE) transcript levels are known to increase dramatically. Although this increase can be attributed in part to increased transcriptional activity, posttranscriptional mechanisms have also been implicated in the high levels of AChE mRNA in myotubes. In this study, we observed that transfection of a luciferase reporter construct containing the full-length AChE 3'-untranslated region (UTR) resulted in significantly higher (5- fold) luciferase activity in differentiated myotubes *versus* myoblasts. RNA-electrophoretic mobility shift assays (REMSAs) performed with a full-length AChE 3'-UTR probe and the AU-rich element revealed that the intensity of RNA-binding protein complexes increased as myogenic differentiation proceeded. Using several complementary approaches including supershift REMSA, mRNA-binding protein pull-down assays, and immunoprecipitation followed by reverse transcription-PCR, we found that the mRNA-stabilizing protein HuR interacts directly with AChE transcripts. Stable overexpression of HuR in C2C12 cells increased the expression of endogenous AChE transcripts as well as that of the luciferase reporter construct containing the AChE 3'-UTR. *In vitro* stability assays performed with protein extracts from these cells *versus* controls resulted in a slower rate of AChE mRNA decay. The down-regulation of HuR expression mediated through small interfering RNA further confirmed the role of HuR in the regulation of AChE mRNA levels. Taken together, these studies demonstrate that HuR interacts with the AChE 3'-UTR to regulate posttranscriptionally the expression of AChE mRNA during myogenic differentiation.

3.3 Introduction

Neurotransmission at cholinergic synapses of the central and peripheral nervous systems is promptly terminated by the catalytic activity of the enzyme acetylcholinesterase (AChE) (for review see Refs. 1–4). In addition to this pivotal role in terminating synaptic transmission, several lines of evidence have shown that AChE assumes other functions necessary for the normal operation of the nervous system (for examples see Refs. 5–7). Appropriate expression of AChE is not only important in the brain, but it is also critical for normal skeletal muscle activity as exemplified by the phenotype seen in patients suffering from a myasthenic syndrome linked to a reduction of AChE at the neuromuscular junction (8, 9). Given the functional significance of AChE in the nervous system, it thus becomes important to identify the molecular and cellular mechanisms regulating its expression in developing tissues as well as in neurons and skeletal muscle cells placed under a variety of conditions known to markedly affect AChE expression.

AChE is encoded by a single gene that is alternatively spliced to produce catalytic subunits that can be assembled into different molecular forms. In skeletal muscle, the AChE T transcript is predominantly expressed (10 –13) resulting in the synthesis of a catalytic subunit that can associate with structural subunits encoded by separate genes (14). In particular, the asymmetric forms of AChE as well as AChE transcripts themselves are preferentially expressed at the level of the neuromuscular junction (14 – 17). In mature muscle, AChE expression is tightly controlled by nerve-evoked electrical activity as well as by nerve-derived trophic factors (14, 18–21). However, AChE is also known to be regulated during myogenic differentiation, at times preceding the occurrence

of nerve-muscle interactions (22–24). Under these conditions, however, there is relatively little information available concerning the nature of the molecular mechanisms that control AChE expression.

In a recent study, we have shown that an increase in the transcriptional activity of the *AChE* gene could partially account for the initial increase in AChE transcript levels observed in differentiating muscle cells grown in culture (25). In this latter study, the contribution of E- and N-box motifs located within the first intronic region, along with their respective transcription factors, myogenin and GA-binding protein α and -β, was shown to be involved in mediating this transcriptional induction. More recent studies performed by others have also highlighted the contribution of specific 5'-regulatory regions in the *AChE* gene during myogenesis, including Sp1, Egr-1, and cAMP response elements (26, 27). In our earlier study, however, we noted a clear discrepancy between the observed increase in transcription and the overall induction in AChE transcripts, thereby suggesting that transcription alone could not fully account for the increase in AChE expression seen at later stages of differentiation (25). In agreement with a previous report (23), our results indicated that posttranscriptional events also participate in regulating the abundance of AChE mRNAs in differentiating muscle cells (19). Despite the implication of posttranscriptional mechanisms, the nature of the specific trans- and cis-acting elements involved in these regulatory events remains unclear. With this in mind, we have therefore initiated a series of experiments in an attempt to characterize the posttranscriptional mechanisms that regulate AChE transcripts in differentiating muscle cells.

3.4 Experimental procedures

Cell Culture—Mouse C2C12 cells (ATCC, Manassas, VA) were cultured on culture dishes (6-well and 100-mm) coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 20% fetal bovine serum, 292 ng/ml L-glutamine, and 100 units/ml penicillin-streptomycin in a humidified chamber at 37 °C with 5% CO_2. Myogenic differentiation and fusion was induced by replacing the growth medium on confluent myoblasts with differentiation medium containing low serum (2% horse serum). Culture medium was changed every 48 h.

Plasmid Construction and Transfection Studies—The 3'-untranslated region (UTR) of the mouse ~2.4-kb AChE mRNA was isolated and subcloned into a *Renilla* luciferase reporter construct driven by the thymidine kinase promoter (phRG-TK from Promega, Madison, WI) as described previously (19, 28). The shorter 3'-UTR was chosen for these studies because the first polyadenylation site is predominantly used in differentiating C2C12 cells (23). To generate the AChE 3'-UTR fragment without the AU-rich element (ARE), we employed a PCR-based protocol described elsewhere and used to delete the ARE from the GAP-43 mRNA (29). Briefly, the AChE 3'-UTR cDNA was used as a template, and two rounds of PCR amplification were first performed with the following primers: 5'-CGAGCCCCTAGCAGGGCTGGGATATAATACGACCGA-3' and the flanking primer 5'-AGGTCTCGGATCCTTTATTGGCGGCCCAGAGGGGCGAAGG-3' (reaction 1); and the flanking primer 5'-AGGTCTCTCTAGACCCCTTGGGGAC CCCAG-3' and primer 5'-ATCCCAGCCCTGCTAGGGGCTCGGGCAGGGCGGCA-3' (reaction 2). To remove the internal ARE region (21 nucleotides), we performed a third

round of PCR with the reaction 1 and reaction 2 PCR products using the two flanking primers listed above to yield a product containing the AChE 3'-UTR without the ARE region as confirmed by sequencing.

The mouse HuR coding region was amplified by PCR from mouse muscle cDNA using primers designed from the available mouse HuR sequence that spans the entire coding region. The resultant PCR product was subcloned into the pCI-neo vector (Promega) driven by the cytomegalovirus enhancer/promoter. The resulting vector (pHuR) product was sequenced and used to stably transfect C2C12 cells that were selected for G418 resistance resulting in a mouse HuR overexpressing C2C12 stable cell line pool.

Plasmid DNA was prepared using the Mega-Prep procedure (Qiagen, Mississauga, Ontario, Canada). DNA pellets were resuspended in 10 mM Tris-HCl, pH 8.5. Transfections were performed with the Lipofectamine reagent kit (Invitrogen) according to the manufacturer's instructions. Briefly, C2C12 myoblasts at 80% confluency were transfected with a total of 2 μg of plasmid DNA (1.0 μg of either the reporter construct or the parental vector and 1.0 μg of the vector used for transfection efficiency). Transfection efficiency was monitored by cotransfecting the basic firefly luciferase construct (pGL3) or the basic LacZ reporter vector (30). Transfected myoblasts were collected 24–48h after transfection or were induced to differentiate and collected 48–72h later.

To determine reporter gene activity, transfected myoblasts and myotubes were washed with cold 1× PBS, scraped, and lysed in 1× reporter lysis buffer (Invitrogen) followed by several freeze-thaw cycles. The extracts were centrifuged (12,000 × *g* for 10

min at 4 °C), and the resulting supernatant was used for the luciferase assays (*Renilla* and firefly) or for β-galactosidase assays using the appropriate kits and according to the manufacturer's instructions (Invitrogen). Values obtained for the AChE reporter luciferase and control parental (phRG-TK) constructs were standardized to the values obtained with firefly luciferase or with β-galactosidase, thereby controlling for transfection efficiency.

HuR siRNA and Transfection Studies—The HuR normal and mutant siRNA duplexes (target sequence, 5'-AAAAGUCUGUUCAGCAGCAUUGG-3', and mutant sequence, 5'-AAAAGUCAAUUCAUCAGCAAUGG-3') were obtained from Dharmacon RNA Technologies. C2C12 myoblasts were transfected with siRNA duplexes designed to mouse HuR sequence using the RNAiFect transfection kit (Qiagen) according to the manufacturer's instructions. Transfected myoblasts were harvested within 24–48 h, and RNA was extracted (see below) and used for reverse transcription (RT)-PCR (see below).

Adenoviral Construct and Infection Studies—An adenoviral construct containing the human HuD sequence (AdLS5a) was generated using the HuD sequence subcloned from the previously described pcHuD vector (31). AdLS5a contains the human HuD cDNA under the regulation of the cytomegalovirus promoter and bovine growth hormone polyadenylation sequence. In these viruses, the HuD expression cassette replaces the early region 1, and transcription is directed leftward, relative to the conventional human adenovirus serotype 5 map. A control virus, Ad-lacZ, contains the *Escherichia coli* β-galactosidase gene under the regulation of the murine cytomegalovirus promoter and simian virus 40 polyadenylation sequence, and transcription from the expression cassette is directed rightward. The early region 1-deleted, first generation adenoviral vectors used

in these studies were constructed using a combination of conventional cloning techniques and RecA mediated recombination (32, 33). Recombinant adenoviral helper viruses were grown and titered on 293 cells as described previously (34). 293 cells (35) were grown in monolayer in minimum essential medium supplemented with 100 units of penicillin/ml, 100 mg of streptomycin/ml, 2.5 mg of fungizone/ml, and 10% fetal bovine serum (complete medium). All cell culture media and reagents were obtained from Invitrogen.

Confluent C2C12 myoblasts were infected with 9.5×10^7 particles of the adenoviral construct in a volume of 100 μl of culture medium, with a multiplicity of infection of 475. At this multiplicity of infection, almost all of the cells on the plate were infected.[2] The cells were placed in a humidified incubator at 37 °C and 5% CO_2 for 1 h before the addition of a supplementary growth medium. During this period, the cell culture plates were agitated every 15 min to ensure that the HuD adenoviral construct was in contact with all of the cells in the dish. The culture medium was changed 24 h later, and myogenic differentiation was induced with a low serum culture medium. Infected myoblasts and myotubes were washed with 1× PBS 24–72 h following infection, and RNA was extracted and used for RT-PCR as described below.

RNA Extraction and RT-PCR—Total RNA was extracted from myoblasts and differentiating myotubes using 1.0 ml of TRIzol reagent (Invitrogen) according to the manufacturer's instructions and as described previously (25, 28). Precipitated RNA was resuspended in RNase-free water and stored at −80 °C until used. RNA from each sample was quantified using the Amersham Biosciences Gene Quant II RNA/DNA spectrophotometer and adjusted to a final concentration of 50 ng/μl.

[2] R.J. Parks, unpublished observations.

Reverse transcription of 4 μl of RNA (200 ng) was performed using the Omniscript reverse transcription kit (Qiagen) as recommended by the manufacturer. Briefly, the RNA was reverse transcribed in a final volume of 20 μl of RT master mix, 0.5 mM concentration of each dNTP, 10 units of RNase inhibitor (Applied Biosystems, Foster City, CA), 1 μM random hexamers (Applied Biosystems), and 4 units of Omniscript reverse transcriptase. RT was carried out at 37 °C for 60 min. Negative controls consisted of the same RT mixture in which the RNA was replaced with RNase-free water. All of the samples were prepared using the same RT master mix.

PCR was used to amplify the cDNAs corresponding to AChE, HuR, and S12 ribosomal protein (S12 hereafter) mRNAs. The primers for AChE, S12 (used as an internal control), and HuR (5'-primer CAGAGGTCATCAAAGATGC and 3'-primer ATCCCACTCATGTGATCTAC) were synthesized based on available sequences and amplified products of 670, 368, and 394 bp, respectively (16, 17, 25, 36–38). PCR was performed using HotStarTaq DNA polymerase (Qiagen) according to the manufacturer's instructions. All of the samples were prepared using the same PCR master mix. PCR cycling parameters consisted of an initial activation step at 95 °C for 15 min followed by denaturation at 94 °C for 1 min; annealing at 70 °C (for AChE), 54 °C (for S12), and 57.5 °C (for HuR) for 1 min each; and extension at 70 °C (for AChE) for 2 min and 72 °C (for S12 and HuR) for 1 min followed by a 10-min elongation step at 72 °C. PCR amplification was stopped during the linear range of amplification (16, 17). Typically, the cycle numbers were 35 cycles for AChE, 24 cycles for S12, and 24 cycles for HuR. PCR products were visualized on ethidium bromide-stained 1.5% agarose gels and quantified using the fluorescent dye VistraGreen (Amersham Biosciences) in 1.5%

agarose gels. Quantification was performed using the Storm PhosphorImager and the accompanying ImageQuant software (Amersham Biosciences). The values obtained for AChE and HuR were standardized relative to the corresponding level of S12 in the same sample.

In Vitro Stability Assay—Poly(A)$^+$ RNA was isolated from a total RNA extract obtained from mouse brain using Oligotex mRNA mini kit (Qiagen) according to the manufacturer's instructions. Cytoplasmic protein fractions were prepared as described previously (39) from C2C12 cells stably transfected with the HuR cDNA (pHuR cells) or from cells stably transfected with the empty vector (pCI-neo) as control. Briefly, C2C12 cell pellets were homogenized in MOPS buffer (10 mM MOPS-NaOH, pH 7.2, 200 mM sodium chloride, 2.5 mM magnesium acetate) with 100 μM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, and 1 Complete Mini, EDTA-free Protease Inhibitor Cocktail tablet as per the manufacturer's recommendations (Roche Applied Science). Homogenates were centrifuged (10,000 × *g* for 15 min at 4 °C), and the resulting supernatants were stored until further use. After optimization of protein and RNA concentrations, 2.5 ng/μl poly(A)$^+$ RNA were incubated with 0.2 μg/μl protein extracts in MOPS buffer containing 1 mM ATP, 0.1 mM spermine, 2 mM dithiothreitol, and 1 unit/μl SUPERasin (Ambion, Austin, TX) in a final volume of 180 μl and incubated at 37 °C. At intervals of 0, 15, and 30 min, aliquots of 40 μl were removed, and the reaction stopped by adding 200 μl of ice-cold phenol/chloroform. De-proteination and precipitation of the RNA was then carried out in the presence of yeast tRNA (10 μg) as a carrier. AChE mRNA was detected by RT-PCR as described above.

In Vitro Transcription—cDNAs encoding for the AChE full-length 3'-UTR, the ARE region alone, and the AChE 3'-UTR minus the ARE were obtained by PCR amplification of the pGL3–3'-UTR plasmid as described previously (19, 28). This cDNA was used as the template for the *in vitro* transcription of the 3'-UTR. *In vitro* transcription was performed with the T7 *in vitro* transcription system (Promega) as recommended by the manufacturer. Briefly 0.5 μg of cDNA, 5 μCi of [α-^{32}P]UTP, nucleotides, RNase inhibitor, and T7 polymerase were used to synthesize radiolabeled transcripts. The template PCR was digested with DNase I for 20 min at 37 °C. The resulting radiolabeled RNA was purified using the RNase-free G-25 RNA purification column (Roche Applied Science). The integrity of the RNA was verified by gel electrophoresis. Biotin-labeled transcripts (AChE 3'-UTR with or without the ARE, and with the ARE region alone) were obtained using the same method except that 0.5 μM bio-11-CTP (Sigma) was used instead of the radiolabeled UTP. Unlabeled probes were generated by the same method and used in competition assays.

Electrophoretic Mobility Shift Assay and Supershift—RNA-based electrophoretic mobility shift assays (REMSAs) and supershift assays were performed with total protein extracts obtained from myoblasts and myotubes. The cells were washed with 1× PBS, scraped, and lysed in 500 μl of homogenization buffer (0.3 M sucrose, 60 mM NaCl, 15 mM Tris, pH 8.0, 10 mM EDTA) supplemented with protease inhibitors (mini-complete protease inhibitor, Roche Applied Science) followed by several freeze-thaw cycles. The samples were centrifuged (15,000 × *g* for 15 min at 4 °C), and the resulting supernatants were stored at −80 °C in 20–100-μg aliquots to be used for REMSAs and other assays.

The total amount of protein present in the extracts was determined with the Coomassie protein assay kit (Pierce).

REMSAs were performed as described previously (28). In brief, 20 μg of protein extract were incubated with 2×10^5 cpm of ^{32}P-labeled AChE 3'-UTR or the ARE region in the presence of yeast tRNA (0.2 μg/μl) and heparin (2.5 mg/ml) for 20 min at room temperature. The mixture was separated by 5% native PAGE with 0.5× Tris borate-EDTA running buffer. The gels were subsequently dried and exposed to film at −80 °C. Competition assays were performed by incubating an excess of cold probe with the protein for 10 min prior to the incubation with the radiolabeled RNA. Supershift assays were performed by incubating the protein in the binding buffer with 0.5 μg of mouse monoclonal antibody directed against HuR (Molecular Probes, Eugene, OR) or a nonspecific antibody at room temperature for 1 h prior to the addition of the radiolabeled transcripts.

Immunoprecipitation and AChE mRNA Analysis—Immunoprecipitation of HuR from myoblasts and myotubes was performed as previously described with some modifications (28, 40). Myoblast and myotube protein extracts obtained as described above were precleared with 5 μg of normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and 30 μl of paramagnetic protein G Dynabeads (Dynal Biotech, Oslo, Norway) in 300 μl of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 1% Igepal CA-630, 1% bovine serum albumin fraction V, 2 mM EDTA, 3 mM magnesium acetate, 100 μM $ZnCl_2$, 1 μg/ml yeast tRNA) for 1 h at room temperature with constant mixing. The precleared protein extracts were then incubated with 2 μg of a mouse monoclonal antibody directed to HuR (Molecular Probes) or normal mouse IgG (Santa Cruz Biotechnology) for 1 h at

room temperature with constant mixing. The protein antibody solution was incubated with 30 μl of prewashed paramagnetic protein G Dynabeads for 1 h at room temperature with constant mixing. The protein G Dynabeads were washed with immunoprecipitation buffer three times, and the protein and mRNA were extracted with 0.5 ml of TRIzol according to the manufacturer's instructions. The mRNA was precipitated in the presence of 10 μg of RNase-free glycogen (GenHunter, Nashville, TN) and subsequently used for RT-PCR. The protein pellet was solubilized in 1× PBS with 1% SDS and used for Western blotting (see below).

mRNA-binding Protein Pull-down Assay—Myoblast and myotube protein extracts (100 μg) were incubated with 2 μg of biotin-labeled AChE 3'-UTR with and without the ARE or with the ARE region alone, in 1× binding buffer as used in REMSAs (20 mM Hepes, pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol, 0.1 mM $ZnCl_2$, 5% glycerol, 0.2 mg/ml yeast tRNA) for 30 min at room temperature with intermittent mixing. The biotin-labeled RNA and associated mRNA-binding proteins were incubated with 50 μl of paramagnetic streptavidin Dynabeads (Dynal Biotech), prewashed in RNase-free buffers and 1× binding buffer for 1 h at room temperature with constant mixing, and washed three times with 1× binding buffer. The protein was solubilized in PBS and subjected to Western blot analysis for the detection of HuR.

Western Blot—For Western blotting, 25 μg of myoblast and myotube protein extracts or proteins extracted from the mRNA-binding pulldown assays were denatured in SDS loading buffer and subjected to 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose Trans-Blot transfer medium (Bio-Rad, Hercules, CA). Following transfer the membranes were blocked in either 10% nonfat skim milk or 10%

bovine serum albumin in Tris-buffered saline and Tween 20 for 1 h at room temperature with constant agitation. The membranes were incubated with mouse monoclonal antibodies directed against HuR (1:1,000) or α-tubulin (1:5,000) (Sigma) followed by the appropriate secondary antibody (1:10,000 or 1:25,000). The proteins were revealed using the SuperSignal West Dura extended duration substrate enhanced chemiluminescence kit (Pierce).

Statistical Analysis—An analysis of variance was performed to evaluate the effect of the AChE 3'-UTR on luciferase activity in transfected C2C12 cells. The Fisher's least squares difference test was used to determine whether the differences seen between group means were significant. The level of significance was set at $p < 0.05$. Data are expressed as mean ± S.E. throughout.

3.5 Results

AChE mRNA Expression during Myogenic Differentiation— In an initial series of experiments, we examined the levels of AChE mRNA during myogenic differentiation by RT-PCR. As shown in Fig. 1*A*, AChE mRNA levels are barely detectable in 50% confluent myoblasts and increase considerably as the cells become more confluent and begin to differentiate. Upon the second day of differentiation, AChE transcript levels increased by ~10-fold over the levels seen in 50% confluent myoblasts. The extent of this increase reached more than 50-fold in 4-day-old myotubes (Fig. 1*B*). This pattern of expression of AChE mRNA during myogenic differentiation is in agreement with earlier reports (23, 25).

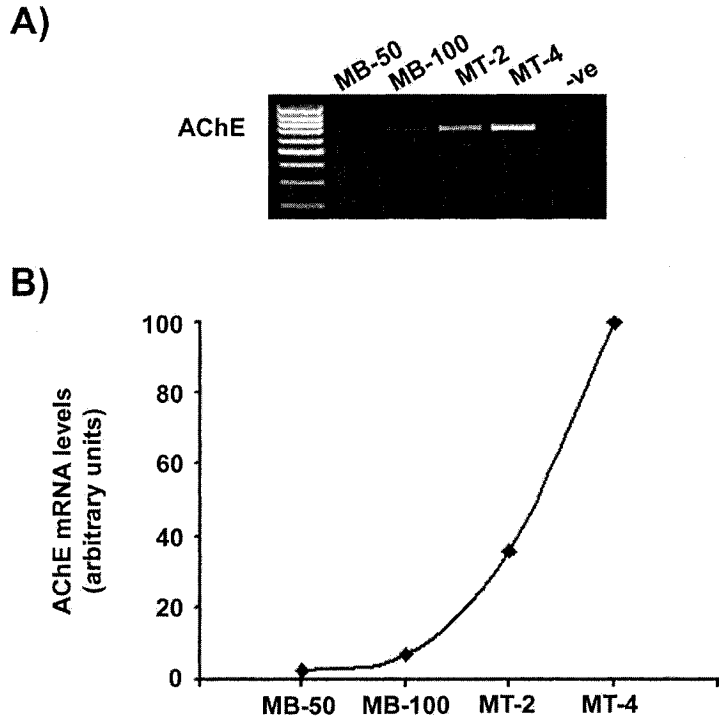

FIG. 1. AChE transcript levels increase during myogenic differentiation. *A*, an example of an ethidium bromide-stained agarose gel displaying AChE PCR products from 50% confluent myoblasts (*MB- 50*), from 100% confluent myoblasts (*MB-100*), and from myotubes cultured in differentiation medium for 2 or 4 days (*MT-2* and *MT-4*, respectively). The negative control lane (no RNA) is indicated (*-ve*). *B*, quantitation of AChE mRNA levels in 50 and 100% confluent myoblasts and in myotubes differentiated for 2 or 4 days) expressed in arbitrary units. AChE mRNA levels were standardized to the corresponding S12 mRNA levels.

Previous studies have also shown that changes in the transcriptional activity of the *AChE* gene cannot solely account for the large increase in transcript levels seen in myotubes, therefore implicating posttranscriptional mechanisms such as those regulating transcript stability. Because elements located in the 3'-UTR are often implicated in the control of mRNA turnover (41, 42), we next sought to determine whether the AChE 3'-UTR plays a role in regulating the relative abundance of AChE transcripts in differentiating muscle cells. To this end, we engineered a luciferase reporter construct in

which the endogenous 3'-UTR was replaced with the mouse AChE 3'-UTR terminating at the first polyadenylation signal (see Fig. 2*A*). The short 3'-UTR was chosen over the longer 3'-UTR because previous studies have demonstrated that the first polyadenylation signal is used preferentially over the second signal (23, 43).

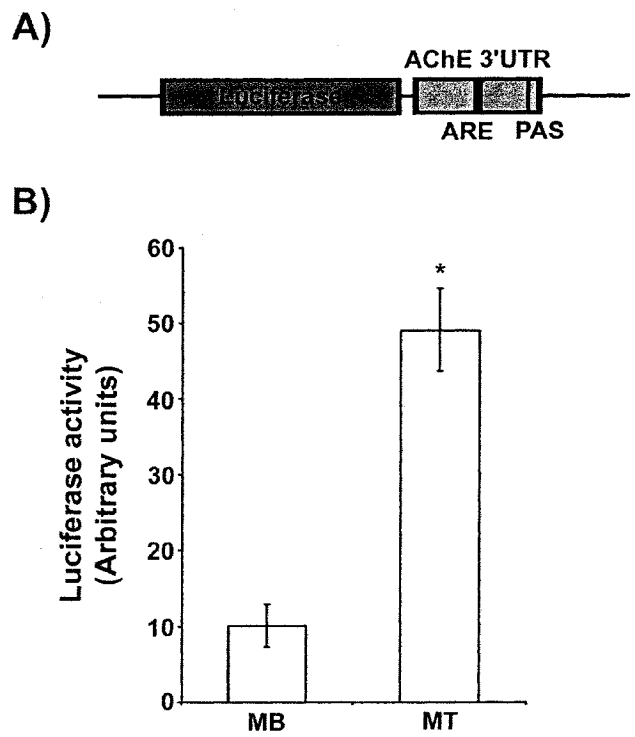

FIG. 2. The AChE 3'-UTR increases the expression of a reporter construct in differentiated myotubes. *A*, schematic diagram displaying the FL downstream of the *Renilla* luciferase reporter construct used in our studies and the location of important elements found within the 3'-UTR such as the ARE and the poly(A)$^+$ signal (*PAS*). *B*, myoblasts were co-transfected with the FL reporter construct and a control vector expressing firefly luciferase. The cells were harvested when they were still myoblasts (*MB*) or following differentiation into myotubes (*MT*). *Renilla* luciferase activity was measured and standardized to the activity obtained with the parental vector and the firefly luciferase activity and expressed in arbitrary units. The *asterisk* indicates a significant difference from myoblasts ($p < 0.0032$; $n = 3$ independent experiments).

As a result, there is significantly more of the ~2.4-kb AChE mRNA in C2C12 cells than the ~3.2-kb transcript. In addition, the short 3'-UTR was shown to be important in the regulation of AChE mRNA expression during neuronal differentiation of PC12 cells (28, 44).

Transfection of this construct into myoblasts resulted in a low level of expression of the luciferase reporter (Fig. 2*B*). In 4-day-old myotubes, however, the activity level of the luciferase reporter increased nearly ~ 5-fold. To show the specificity of this increase, we transfected a reporter construct containing the 3'-UTR of utrophin. As expected on the basis of recent studies showing a lack of posttranscriptional regulation of utrophin transcripts during myogenic differentiation (45, 46), no change in reporter expression was observed between myoblasts and myotubes (data not shown). These results highlight the specificity of the increase in reporter expression obtained with the AChE 3'-UTR. The increased expression of the reporter gene under these conditions supports the notion that the 3'-UTR is involved in regulating AChE transcript levels during myogenic differentiation.

Pattern of Interactions between the AChE 3'-UTR and RNA-binding Proteins—We next examined the intensity and binding pattern of proteins found in myoblasts and myotubes that interact with the AChE 3'-UTR using REMSA. For these experiments, we used *in vitro* transcribed RNA that corresponded to either the full-length AChE 3'-UTR (FL) or to ARE. AREs are well characterized cis-acting elements found in UTRs that mediate transcript stability through interactions with destabilizing trans-acting factors such as AUF1 or with stabilizing trans-acting factors including those of the Hu family (41, 47). REMSA performed with the FL probe revealed the presence of two major protein-

RNA complexes (Fig. 3, indicated with *black arrows*) that formed in myoblasts and myotubes.

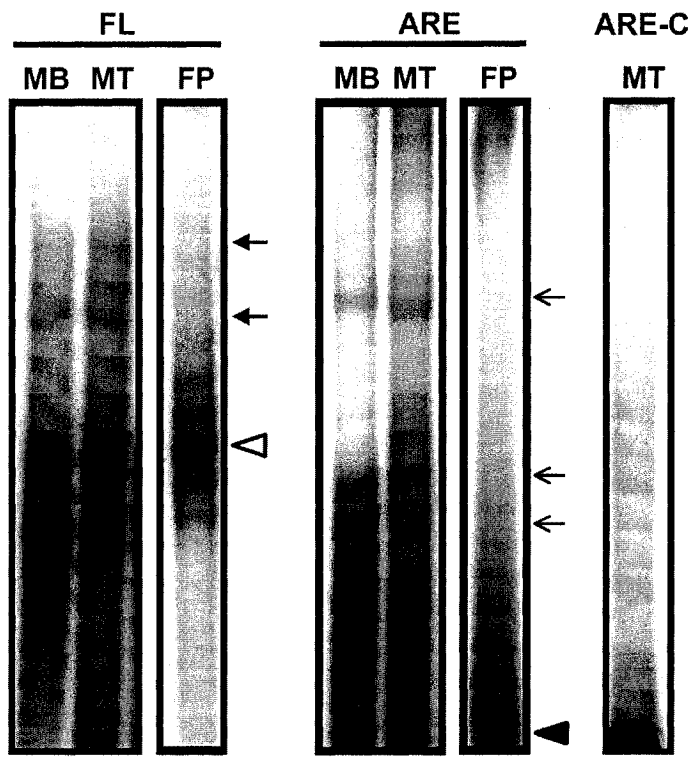

FIG. 3. The binding intensity of RNA-protein complexes formed with the AChE 3'-UTR increases during myogenic differentiation. REMSAs were performed using total protein extracts from myoblasts (*MB*) or myotubes (*MT*). Representative autoradiograms demonstrating the interaction between *in vitro* transcribed FL RNA or *in vitro* transcribed RNA corresponding to the ARE and protein complexes are shown. *Closed arrows* indicate protein complexes formed with the FL RNA probe, and *Open arrows* point to protein complexes formed with the ARE RNA probe. *Arrowheads* point to the FL and ARE free probe (*FP*). Competition experiments using a 50-fold excess of unlabeled ARE RNA are also shown (*ARE-C*).

Although there is no apparent change in the mobility of these bands, the binding intensity of both complexes is greatly increased in myotubes, suggesting that the abundance of mRNA-binding proteins is increased in myotubes and/or that their binding characteristics to the AChE 3'-UTR are affected. This apparent increase in binding activity in myotubes

was observed in several independent experiments using different protein extracts ($n = 4$). REMSA performed using the ARE probe also showed some important changes in the pattern of RNA-protein interactions in myoblasts *versus* myotubes (Fig. 3). In particular, there was a clear increase in the band intensity using proteins extracted from 4-day-old myotubes (Fig. 3, indicated with *arrows*). Here, again, the increased banding intensity obtained with differentiated myotubes was consistently observed ($n = 3$). The specificity of the binding was demonstrated in competition experiments using a cold probe to the ARE (Fig. 3, *AREC*). Taken together, these results suggest that there are specific RNA-binding complexes that interact with the AChE 3'-UTR in which the ability to bind to the 3'-UTR increases according to the state of myogenic differentiation.

HuR Binds the AChE 3'-UTR—Our findings using REMSA illustrate the importance of ARE in the binding of cytoplasmic factors to the AChE 3'-UTR. Members of the ELAV-like family of mRNA-binding proteins, known to interact with ARE, have recently received an increasing amount of attention because of their ability to stabilize a variety of transcripts in cells placed under different conditions. In our studies, we focused on one family member, HuR, because it is expressed in skeletal muscle (48, 49).

In an initial experiment, we performed supershift REMSA with an antibody directed to HuR and protein extracts from myoblasts and myotubes, to determine whether HuR was present in the protein complexes interacting with the AChE 3'- UTR. As shown in Fig. 4*A*, the addition of the HuR antibody to the RNA/protein extract mixture resulted in the supershift of an RNA-protein complex formed using myotube protein extracts. This supershift was mostly evident using the myotube extract. As a control, no supershift was

apparent when a nonspecific antibody was used instead of the antibody directed against HuR.

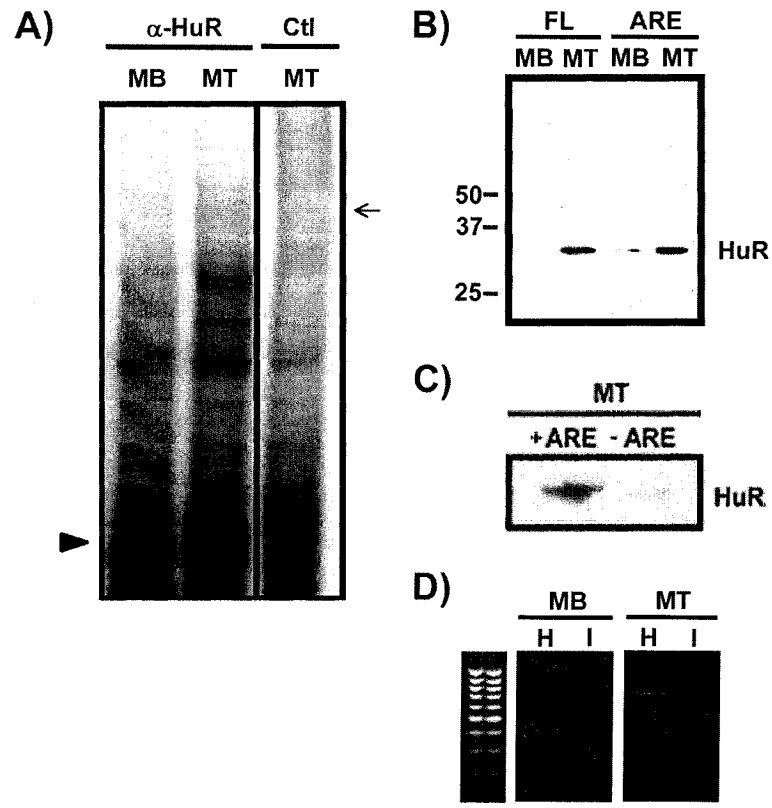

FIG. 4. The mRNA-binding protein HuR binds to the AChE 3'-UTR in myotubes. *A*, supershift REMSAs were performed with total protein extracts from myoblasts (*MB*) and myotubes (*MT*) and a monoclonal antibody directed to HuR (α-*HuR*) or a nonspecific antibody (control (*Ctl*)). Representative autoradiograms demonstrating the interaction between *in vitro* transcribed FL RNA and protein complexes are shown. *Arrow* points to the supershifted RNA-protein complex obtained with the antibody directed to HuR and the myotube protein extract. *Arrowhead* points to the free probe. *B*, a Western blot for HuR performed with myoblast and myotube proteins pulled down by *in vitro* transcribed biotin-labeled FL or ARE transcripts demonstrates that HuR from these extracts can bind to the AChE 3'-UTR. *C*, a Western blot for HuR performed with myotube proteins pulled down using *in vitro* transcribed biotin-labeled AChE 3'-UTR transcripts with (+) ARE or without (–) the ARE demonstrates that HuR binds to the AChE 3'-UTR via the ARE. *D*, example of an ethidium bromide-stained agarose gel displaying AChE PCR product obtained from immunoprecipitates isolated from myoblasts and myotubes using an antibody directed against HuR (*H*) or normal mouse IgG (*I*). Note the presence of a PCR product corresponding to AChE only in the immunoprecipitate obtained with the HuR antibody and myotube protein extract.

The interaction between HuR and the AChE 3'-UTR was further confirmed using an mRNA-binding pull-down assay. In this assay, excess *in vitro* transcribed biotin-labeled AChE 3'-UTR probes (both FL and ARE) were incubated with protein extracts from myoblasts or myotubes and subsequently pulled out of solution along with any proteins that might bind to the probes with streptavidin-coated paramagnetic beads. The pulled-down proteins were then resolved by SDS-PAGE and used for Western blotting. A Western blot to detect the presence of HuR (Fig. 4*B*) showed that indeed HuR could be pulled down with both the AChE full-length 3'-UTR and the ARE in experiments using myotube extracts. As observed with the supershift REMSAs, the FL probe was not able to bind HuR proteins from myoblast extracts, although a small amount of HuR could be detected in pull-down assays using the ARE probe. To further examine the binding of HuR to the ARE, we performed an additional set of pull-down experiments using AChE 3'-UTR probes that included (Fig. 4*C*, *+ARE*) or not (*–ARE*) the ARE and myotube extracts. As shown in Fig. 4*C*, the ARE is essential for the binding of HuR to the AChE 3'-UTR because we observed a significant decrease in the binding of HuR to the mutant AChE 3'-UTR probe (*–ARE*). In these assays, we confirmed that HuR was still present in the protein extract following incubation of the extract with the mutant AChE 3'-UTR probe that did not contain the ARE (data not shown). Together, the results of the supershift and pull-down assays clearly indicate that HuR expressed in myotubes can interact with the AChE 3'-UTR via the ARE.

In complementary experiments, we determined whether HuR could bind to endogenous AChE transcripts in living cells. For these assays, we immunoprecipitated HuR from myoblasts and myotubes, extracted total RNA, and performed RT-PCR to

amplify AChE. As shown in Fig. 4*D*, AChE mRNAs were present in immunoprecipitates obtained from myotubes but not in those from myoblast extracts. As a control, AChE could not be amplified from RNA isolated following immunoprecipitation with normal mouse IgG (Fig. 4*D*) or protein G paramagnetic beads alone (data not shown).

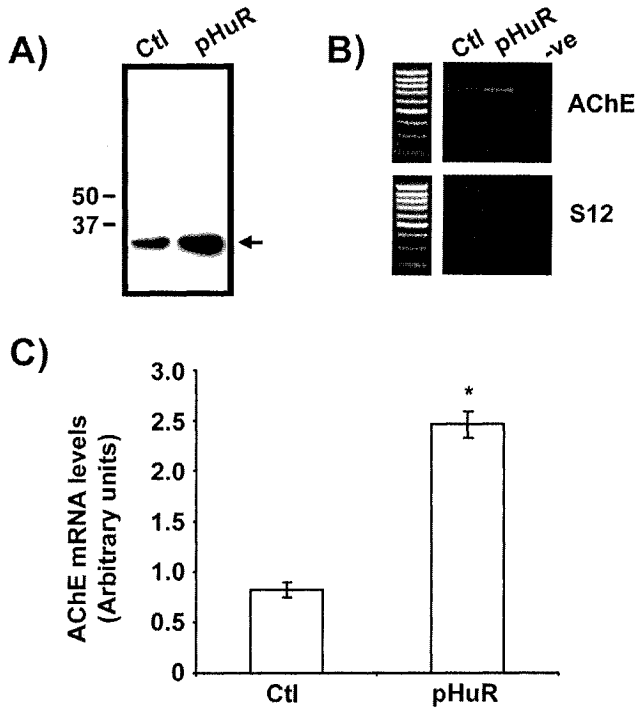

FIG. 5. AChE mRNA levels are modulated by the level of expression of HuR. *A*, example of a Western blot for HuR depicting endogenous HuR levels in the pCI-neo control (*Ctl*) and HuR overexpressing stable C2C12 cells (*pHuR*). *B*, example of an ethidium bromide- stained agarose gel displaying AChE and S12 PCR products obtained from two-day differentiated stable cells. *C*, quantification of AChE mRNA levels in the control and HuR-overexpressing myotubes expressed in arbitrary units following standardization to S12. The *asterisk* indicates a significant difference from control ($p < 0.0004$; $n = 3$ independent experiments).

HuR Increases the Expression of AChE mRNA—To further demonstrate that HuR has a direct effect on the level of AChE mRNA, we created stable C2C12 cells that overexpressed HuR (pHuR). As shown in Fig. 5*A*, endogenous HuR protein levels are

increased by severalfold in HuR-overexpressing muscle cells. Stable transfections with the empty vector (pCI-neo), which was used as a control in these experiments, showed no effect on endogenous HuR levels. We next measured endogenous levels of AChE mRNA in these cells by RT-PCR. In undifferentiated myoblasts, we did not observe any significant changes in AChE mRNA levels between control and pHuR cells (data not shown, see "Discussion"). In 2-day differentiated myotubes, however, we observed an ~3-fold increase in the levels of AChE mRNA (Fig. 5, *B* and *C*). Similarly, siRNAs directed against HuR resulted, as seen previously (50), in a decrease in HuR that in our experiments was accompanied by an ~30% decrease in the levels of endogenous AChE transcripts. Finally, infection of C2C12 cells with an adenoviral vector encoding the highly related HuD family member resulted in a further induction of AChE mRNA in myotubes (data not shown).

We next performed *in vitro* stability assays using protein extracts from control and pHuR cells and used poly(A)$^+$ RNA from mouse brain to determine whether overexpression of HuR affected AChE mRNA stability. As shown in Fig. 6, *A* and *B*, the rate of AChE transcript decay was slower in the presence of protein extracts from the pHuR cells, thereby confirming the importance of posttranscriptional regulation in mediating the changes seen in steady-state AChE mRNA levels in these cells. Using these stably transfected cells, we also determined whether HuR, through its interaction with the AChE 3'-UTR, could increase the expression of the luciferase-AChE 3'-UTR reporter construct. For these experiments, the luciferase-AChE 3'-UTR reporter construct was transfected into control and pHuR stable C2C12 cells. As shown in Fig.

6*C*, expression of the reporter construct was increased by ~ 2–3-fold in C2C12 cells that stably overexpressed HuR.

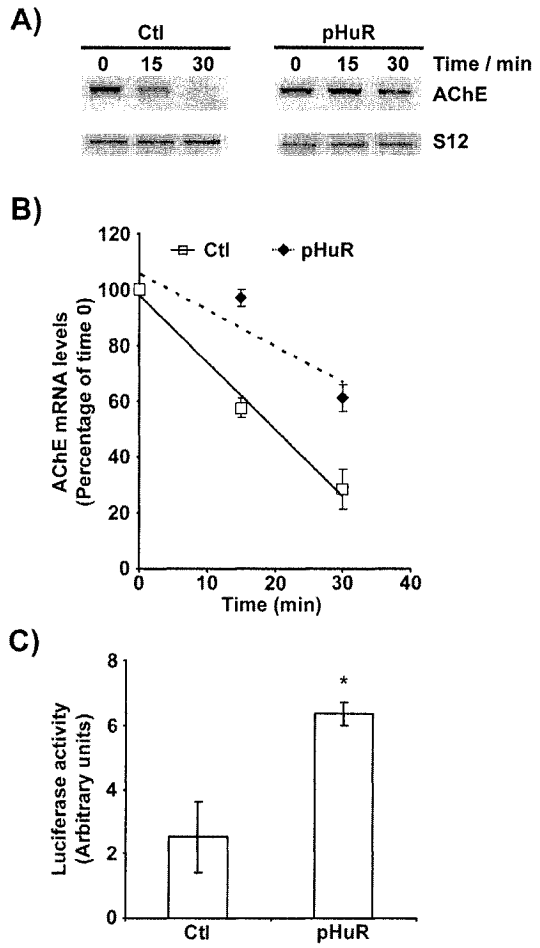

FIG. 6. HuR overexpression increases AChE mRNA stability. *In vitro* stability assays were performed with protein extracts from pCI-neo control (*Ctl*) and HuR-overexpressing (*pHuR*) C2C12 cells and poly(A)$^+$ RNA from mouse brain. *A*, example of ethidium bromide-stained agarose gels displaying AChE and S12 PCR products following 0, 15, and 30 min of incubation with protein extracts. Note that there is a greater amount of AChE PCR product remaining after the 30-min incubation with the pHuR protein extract than with the control extract. *B*, quantitation of the PCR products remaining following incubations and expressed as a percentage of the initial amount found at time 0. *C*, control and pHuR cells were co-transfected with AChE 3'-UTR reporter or parental constructs and a firefly luciferase construct (to measure transfection efficiency). Luciferase activity obtained in control and pHuR cells was quantified and standardized to the activity obtained with the parental vector and the firefly luciferase construct and expressed in arbitrary units. The *asterisk* indicates a significant difference from control ($p < 0.0081$; $n = 2$ independent experiments done in triplicate).

In additional control experiments, we verified that the ARE plays an important role in this process because transfection of a reporter construct containing the mutant AChE 3'-UTR fragment without the ARE decreased significantly expression of the reporter. These results indicate that through its interaction with the ARE located in the 3'-UTR, HuR increases the expression of AChE transcripts in differentiating skeletal muscle cells.

3.6 Discussion

In recent years, there has been considerable interest in characterizing the transcriptional and posttranscriptional mechanisms that regulate AChE expression during myogenic differentiation (23, 25–27, 51, 52). In contrast to the recent progress made in the elucidation of some of the transcriptional events (see the Introduction), there is essentially no information available concerning the nature of the specific trans- and cis-acting factors that participate in the posttranscriptional regulation of AChE in differentiating skeletal muscle cells. In the present study, we have therefore sought to begin the characterization of these posttranscriptional events that act to stimulate the production and maintenance of high levels of AChE transcripts during myogenic differentiation. Transfection experiments performed using a luciferase reporter construct in C2C12 cells showed the important contribution of the 3'-UTR in controlling AChE transcript levels. Furthermore, we found that the AChE 3'-UTR interacts with several distinct complexes using muscle protein extracts and that the intensity of the binding clearly increases during differentiation. Using a combination of different assays, we established that the stabilizing mRNA-binding protein HuR binds to the AChE 3'-UTR.

Finally, we have shown a functional role for HuR in increasing AChE mRNA levels in muscle cells via stabilization through its 3'-UTR.

The Hu Family of Proteins Regulates AChE Expression at the Posttranscriptional Level—The role of posttranscriptional regulatory mechanisms has recently been implicated as an important component of the molecular events regulating AChE transcript levels (23, 25). In recent work, we observed for example a discrepancy between the increase in transcription and the extent of AChE mRNA induction during muscle differentiation (25). Here, we show that the presence of the AChE 3'-UTR resulted in an increase in the expression of a reporter construct in differentiating muscle cells, thereby demonstrating that posttranscriptional mechanisms indeed regulate the expression of AChE during myogenic differentiation through the 3'-UTR.

It is well established that transcript stability is governed by the presence of the 5'-cap, the 3'-poly(A)$^+$ tail, and mRNA-binding proteins that bind to specific sequences or structures in the 3'-UTR. In skeletal muscle, recent studies have found that in response to different stimuli, the stability of certain transcripts, including vascular endothelial growth factor and cytochrome *c* oxidase (COX), is altered (49, 53). During myogenic differentiation, transcripts encoding the myogenic factors myoD and myogenin display increased stability that results, at least in part, from the specific binding of the mRNA-binding protein HuR (50, 54). The increase in the stability of these transcripts is important in dictating, in turn, the overall levels of proteins encoded by these mRNAs during myogenic differentiation.

HuR is a member of the ELAV (embryonic lethal abnormal vision)-like family of mRNA-binding proteins that includes the neuronally expressed HuB (HelN1), HuC, and

HuD proteins (48). These proteins contain three RNA recognition motifs that are known to bind AREs: the AUUUA general sequence, U-rich sequences, and CU-rich motifs (55, 56). The function of this family of mRNA-binding proteins is to stabilize transcripts by inhibiting the de-adenylation and targeting of the transcripts to the exosome or by blocking the activity of specific endonucleases that recognize the AREs (57–59). Using several complementary approaches, we have shown here that HuR can directly interact with AChE transcripts via their 3'-UTR. Additionally, overexpression of HuR in stably transfected C2C12 cells, as well as down-regulation of HuR via siRNA, clearly affected the endogenous levels of AChE mRNA through the 3'-UTR. In parallel experiments, we have also found that HuD, although not normally expressed in muscle, can increase AChE transcript levels in differentiated cells. This indicates that the Hu proteins share similar target preferences. Thus, AChE joins a growing family of transcripts that contain varying types of AREs that are stabilized by the Hu family of proteins in several distinct tissues (40, 41, 49, 60, 61).

Transcriptional and Posttranscriptional Regulation of AChE—Myogenic differentiation is a complex process involving the expression of several genes including those encoding proteins found at the neuromuscular junction (for review see Refs. 62–65). Previous studies have established that the expression of genes encoding synaptic proteins in muscle is most often induced by myogenic regulatory factors such as myoD and myogenin (63). As mentioned above, recent studies have demonstrated that HuR binds to elements in the 3'-UTR of myoD and myogenin during myogenic differentiation, resulting in an increase in the stability of these transcripts (50, 54). Thus, transcription

factors that are key to the process of myogenic differentiation are themselves regulated posttranscriptionally by HuR (50, 54).

In the present study, we found that AChE, for which transcriptional regulation during myogenic differentiation is known to involve myogenin and an E-box (25, 26), is also a direct posttranscriptional target for the stabilizing effects of HuR. Together, the results of these and other studies show that AChE expression is regulated by increases in transcription as well as by increases in transcript stability during myogenic differentiation (28). In this context, our preliminary studies performed with pHuR stable C2 cells have shown that overexpression of HuR does increase the expression of an AChE promoter-reporter construct.[3] Accordingly, combined with earlier studies that focused on the role of HuR in myogenic differentiation, our findings further indicate therefore that HuR assumes a key regulatory function: (i) by controlling the stability of mRNAs encoding transcription factors known to modulate the transcriptional activity of the *AChE* gene during myogenic differentiation; and (ii) by also directly controlling the stability of AChE transcripts. Such dual roles for HuR in differentiating myogenic cells would confer to this RNA-binding protein a master regulatory function necessary to optimize gene expression via both transcriptional and posttranscriptional events (see further discussion in Refs. 60 and 66–68).

Additional Factors Regulate AChE Expression Posttranscriptionally— On the basis of our studies, however, it appears that HuR is not the sole factor important for modulating the abundance of AChE transcripts in differentiating myotubes at the posttranscriptional level, as suggested by the 50–100-fold increase in AChE mRNA *versus* the ~ 3-fold increase caused by HuR. Indeed, we observed the formation of

[3] G. Bélanger and B.J. Jasmin, unpublished observation.

different protein complexes with both the full-length and the ARE probes using extracts from myoblasts and myotubes. The 3'-UTR of transcripts often behave in a fashion similar to 5'-regulatory regions of genes because several trans-acting factors with different properties may bind with different affinities to form active complexes that affect the stability of specific transcripts. For instance, the 3'-UTR of cyclooxygenase-2, GAP-43, and BC1 are all known to associate with a number of different mRNA-binding proteins (31, 61, 69–71). In addition, the same cis-acting elements within the 3'-UTR, including the ARE, can also interact with different trans-acting factors that have contrary effects such as the predominantly destabilizing factors AUF1 and tristetraprolin and the stabilizing factor HuR (61, 72, 73). In some cases, the level of expression of the different factors and their binding affinity may determine the preferential level of interaction of one factor *versus* another that can result in dramatically opposite effects (74). Therefore, HuR may represent only one of the several RNA-binding proteins that can interact with AChE transcripts to modulate their expression during myogenic differentiation.

In addition to the myriad of trans-acting factors that are expressed in cells under specific conditions, there are other elements that also need to be considered because they may regulate the binding ability and specificity of these RNA-binding proteins. In this context, one intriguing finding in our study was the inability of HuR from myoblasts to bind exogenous AChE 3'-UTR, despite the presence of HuR in these cells (data not shown). In fact, RT-PCR assays and Western blotting experiments revealed that HuR is equally abundant in myoblasts and myotubes (data not shown) as was also observed by others (see also Ref. 50). This indicates therefore that additional regulatory events favor HuR-RNA interactions in myotubes. One possibility involves the cellular localization of

HuR in myoblasts *versus* myotubes. Previous studies have demonstrated that HuR is predominantly localized in nuclei in myoblasts and that it shuttles out to the cytoplasm during myogenesis where it can interact with and stabilize specific transcripts (50, 54). Because in our study we used total protein extracts containing equivalent amounts of HuR protein and found that HuR from myoblast extracts was unable to bind the AChE 3'-UTR, we speculate that there must be some modifications or other factors that affect the ability of HuR to bind to exogenous, *in vitro* transcribed, AChE transcripts or to those already present in the cytoplasm. The regulation of HuR binding could indeed be mediated by posttranslational modifications, such as phosphorylation or methylation. In this context, metabolic stress was recently shown to regulate the cytoplasmic localization of HuR and its binding through activation of the AMP-activated kinase (75). In addition, HuR has been shown recently to be methylated by the arginine methyltransferase CARM1 (co-activatorassociated arginine methyltransferase) in the hinge region adjacent to the novel nuclear localization signal, resulting in a nuclear-cytoplasmic shuttling ability and/or enhanced interactions with other proteins or transcripts (76, 77). Therefore, although our study clearly highlights the important role of HuR in the regulation of AChE mRNA in differentiating myogenic cells, there are several aspects relevant for our complete understanding of the nature of these posttranscriptional regulatory events that are obviously complex and that will hence necessitate further experimentation.

Chapter 4

The RNA-binding Protein HuD Binds Acetylcholinesterase mRNA in Neurons and Regulates its Expression Following Axotomy

Julie Deschênes-Furry, Kambiz Mousavi, Federico Bolognani, Rachael L. Neve, Robin J. Parks, Nora I. Perrone-Bizzozero and Bernard J. Jasmin

Manuscript accepted to the Journal of Neuroscience.

Acknowledgements: We would like to thank Dr. Victor Gisiger for teaching Julie Deschênes-Furry the surgical techniques (SCG axotomy) used in this paper. Work in the Jasmin lab is supported by the Canadian Institutes of Health Research, the Muscular Dystrophy Association of America and the Association Française contre les Myopathies. Work in the Perrone-Bizzozero lab is supported by the National Institutes of Health (NS30255).

4.1 Author contribution

Julie Deschênes-Furry:

Unless otherwise indicated all experiments were performed by the first author under the supervision of Dr. Bernard Jasmin. The manuscript was prepared by the first author and revised by Dr. Bernard Jasmin and Dr. Nora Perrone-Bizzozero.

Kambiz Mousavi:

The *in situ* hybridizations and RT-PCR for AChE performed with brains from HuD transgenic mice were carried out by Dr. Mousavi.

Federico Bolognani:

Dr. Bolognani produced and characterized the HuD transgenic mice used in these studies under the supervision of Dr. Perrone-Bizzozero. Dr. Bolognani also removed and prepared the brains for *in situ* hybridization experiments.

Rachael L. Neve:

Dr. Neve prepared the HSV-HuD and HSV-LacZ viruses that were used in these studies.

Robin J. Parks:

Dr. Parks designed and provided the lab with adenoviral constructs to HuD.

Nora Perrone-Bizzozero:

Dr. Perrone-Bizzozero provided the lab with the HuD plasmid construct containing the complete human HuD cDNA and HuD transgenic mice. The written manuscript was also revised by this author.

4.2 Abstract

Following axotomy, expression of acetylcholinesterase (AChE) is greatly reduced in the superior cervical ganglion (SCG), however the molecular events involved in this response remain unknown. Here, we first examined AChE mRNA levels in the brain of transgenic mice that overexpress human HuD. Both *in situ* hybridization and RT-PCR demonstrated that AChE transcript levels were increased by more than two-fold in the hippocampus of HuD transgenic mice. Additionally, direct interaction between the HuD transgene product and AChE mRNA was observed. Next, we examined the role of HuD in regulating AChE expression in intact and axotomized rat SCG neurons. Following axotomy of the adult rat SCG neurons, AChE transcript levels decreased by 50% and 85% by the first and fourth day, respectively. *In vitro* mRNA decay assays indicated that the decrease in AChE mRNA levels resulted from changes in the stability of pre-synthesized transcripts. A combination of approaches performed using the region that directly encompasses an AU-rich element within AChE 3'-UTR, demonstrated a decrease in RNA-protein complexes in response to axotomy of the SCG and, specifically, a decrease in HuD binding. Following axotomy, HuD transcript and protein levels also decreased. Using a herpes simplex virus construct containing human HuD sequence to infect SCG neurons *in vivo*, we found that AChE and GAP-43 mRNA levels were maintained in the SCG following axotomy. Taken together, the results of this study demonstrate that AChE expression in neurons of the rat SCG is regulated via post-transcriptional mechanisms that involve the AU-rich element and HuD.

4.3 Introduction

Acetylcholinesterase (AChE) is the enzyme responsible for terminating cholinergic neurotransmission in the central and peripheral nervous systems (CNS and PNS), by rapid hydrolysis of acetylcholine (see for review (Legay, 2000;Rotundo, 2003;Massoulie et al., 1993;Soreq and Seidman, 2001). AChE is predominantly expressed in cholinergic tissues (muscle and neurons) however, it is also expressed in some non-cholinergic neurons and non-excitable cells, such as hematopoietic cells (Lev-Lehman et al., 1997;Hammond et al., 1994;Brimijoin and Hammond, 1996;Bernard et al., 1995;Chan et al., 1998). As a result, several non-cholinergic roles have been described for AChE including in neurite elongation, cell adhesion and synaptogenesis ((Brimijoin and Hammond, 1996;Grifman et al., 1998;Koenigsberger et al., 1997;Lev-Lehman et al., 2000;Sharma et al., 2001;Sternfeld et al., 1998) and see for review (Soreq and Seidman, 2001)). In addition to having these non-classical roles, AChE and its different molecular forms have been implicated in development of neuronal tumors, in Alzheimer's disease and post-traumatic stress disorder (Kaufer et al., 1998;Karpel et al., 1994;Perry et al., 2002). Although AChE is implicated in many different aspects of neuronal development, function and diseases, the molecular mechanisms involved in regulating its expression are still relatively poorly defined, especially *in vivo*.

Nevertheless, a small number of distinct studies mostly performed with cultured neuronal cell lines, have begun to address the specific roles of transcriptional and post-transcriptional mechanisms in AChE regulation. For instance, during differentiation of cultured neuronal cell lines, initial studies have suggested that increased AChE activity

is a downstream consequence of up-regulated gene transcription (Greene and Rukenstein, 1981). More recent studies have confirmed that AChE promoter activity is significantly increased during neuronal differentiation (Jiang et al., 2003;Siow et al., 2002;Siow et al., 2005;Wan et al., 2000). Furthermore, transcriptional activation of the AChE gene was reported to occur in response to stress and in aggressive tumor cells (Meshorer et al., 2002;Meshorer et al., 2004;Perry et al., 2002).

By contrast, an early study performed with pluripotent P19 cells implicated post-transcriptional mechanisms as the principal mechanism involved in AChE mRNA regulation during neuronal differentiation (Coleman and Taylor, 1996). Recently, we reported that following a transient increase in AChE gene transcription, post-transcriptional events are indeed predominantly responsible for regulating AChE mRNA levels during neuronal differentiation. Specifically, we demonstrated that the RNA-binding protein HuD (name based on the initials of the patient in which the onconeural antibodies were discovered, see for review (Deschenes-Furry et al., 2006)), a neuronal member of the Hu family, through binding with the AChE 3'UTR, could increase AChE mRNA levels via its stabilizing activity (Deschenes-Furry et al., 2003). It remains to be determined, however, whether similar regulatory mechanisms are acting *in vivo* to mediate AChE expression in neurons. To this end, we initiated a series of experiments aimed at characterizing the post-transcriptional mechanisms regulating AChE expression *in vivo*. Specifically, we have examined the interaction of HuD with AChE mRNA in neurons of the CNS and the importance of this interaction in regulating AChE mRNA levels following PNS neuronal injury.

4.4 Methods and Materials

Animal Care and Surgical Procedures. Production and characterization of human HuD overexpressing transgenic mice has been described in detail elsewhere (Bolognani et al., 2006). Briefly, the complete human HuD cDNA sequence (Szabo et al., 1991) with an N-terminal myc tag inserted downstream of the Ca^{2+}/Calmodulin Kinase II α-subunit (α-CaMKII) promoter was used to generate the transgenic mice. Two lines were used in these studies, a low expresser (HuD2), which was used as a control for some experiments, and a high expresser (HuD4).

Female Sprague-Dawley rats weighing 150-200 g were obtained from Charles River Laboratories (Québec, Canada) and housed in a 12 hr light/dark cycle with access to standard food and water *ad libitum.* Rats were anesthetized by gas inhalation with halothane, and superior cervical ganglion (SCG) axotomy was performed by cutting the internal and external carotid nerves ~2-5 mm from the ganglion body. In sham-operated animals, the SCG was exposed but the nerves remained intact and untouched. These SCG were used as controls. Sham-operated and axotomized SCG were removed from anesthetized animals 1-, 2-, and 4-days following axotomy. Success of the axotomy was determined by resulting ptosis of the ipsilateral eyelid. For HuD viral expression studies, 4μl (1 x 10^7 particles/ml) of herpes simplex virus (HSV) containing either the LacZ sequence (HSV-LacZ) (Neve et al., 1997) or the myc-tagged human HuD sequence (HSV-HuD) (Anderson et al., 2001), were injected into SCG using a 27-gauge needle attached to a Hamilton syringe and the Harvard Apparatus PHD 2000 Infuse/Withdraw syringe pump (Holliston, MA) at a rate of 1.5 μl/minute. Axotomy was performed on the SCG four-days following viral infection. HSV were prepared and tittered as previously

described (Anderson et al., 2001). All tissues were stored at –80 °C until used. Animal care and surgical procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

RNA Extraction and RT-PCR. Total RNA was isolated from 3-4 SCG or from different brain regions of HuD transgenic mice using a Kontes glass tissue homogenizer and 1 mL of TRIzol reagent (Invitrogen, ON, Canada) according to the manufacturer's instructions and as previously described elsewhere (Boudreau-Lariviere et al., 2000;Michel et al., 1994). All RNA samples were stored at –80 °C until used. RNA from each sample was quantified using the Amersham Biosciences Gene Quant II RNA/DNA spectrophotometer and adjusted to a final concentration of 80 ng/µl.

Reverse transcription of RNA and quantitative PCR were performed as described elsewhere (Jasmin et al., 1993;Boudreau-Lariviere et al., 2000;Michel et al., 1994). cDNAs corresponding to AChE, GAP-43, HuD and S12 ribosomal protein (S12, used as an internal control) were amplified as described in detail elsewhere (Jasmin et al., 1993;Mobarak et al., 2000;Angus et al., 2001;Boudreau-Lariviere et al., 1996;Michel et al., 1994). Primers for AChE, GAP-43 and S12 were synthesized based on available sequences and amplified products of 670 bp, 737 bp and 368 bp, respectively (Forster et al., 1993;Mobarak et al., 2000;Legay et al., 1993). The HuD 5' and 3' primers (5', ACGCATCCTGGTTGATCAAG and 3', AGAGGACACTCTCATCAGAATCAG) were designed based on available sequences (GeneBank Accession NM 010488.1) and amplified products corresponded to HuD_{pro} (456 bp) and HuD (411 bp). PCR cycling parameters consisted of an initial denaturation at 94 °C for 1 min, followed by an

annealing step at 54 °C for 1 min for S12, 55 °C for GAP-43, 60 °C for HuD and at 70 °C for 1 min for AChE, and a 2 min extension step at 70 °C for AChE or 1 min at 72 °C for S12, HuD and GAP-43. This was followed by a 10 min elongation step at 72 °C.

PCR products were visualized on ethidium bromide-stained 1.5% agarose gels and quantified using the fluorescent dye VistraGreen (Amersham Biosciences, Piscataway, NJ). Quantitative analysis was performed using a Storm PhosphorImager and the accompanying ImageQuaNT software (Molecular Dynamics, Inc., Sunnyvale, CA). The values obtained for AChE, GAP-43 and HuD (HuD_{pro} and HuD combined) were standardized to those obtained with S12 in the same sample, and expressed as a percentage of the control (sham-operated) sample. All RT-PCR reactions aimed at determining the relative abundance of AChE, GAP-43 and HuD mRNAs were performed during the linear range of amplification. All samples, including the negative control, were prepared using common master mixes containing all the RT and PCR reagents and run in parallel. In all experiments, PCR products were never detected in the negative control.

In Situ Hybridization. *In situ* hybridization (ISH) was performed on 10-µm thick frozen sections of adult mouse brains using a modified version of a detailed previously published protocol (Young et al., 1998). Briefly, sense and antisense cRNA probes were synthesized by *in vitro* transcription using T7 or T3 RNA polymerase (Promega), respectively, and ^{35}S-UTP and ^{35}S-CTP radiolabeled nucleotides (Amersham Biosciences). The cDNA sequence encoding parts of AChE exon 6 and 3'-UTR (nucleotides 1476-1987 of the AChE mRNA sequence, Ascension S50879) were inserted

in frame in the pBS II-SK vector and used as template cDNA for *in vitro* transcription. Radiolabeled cRNA probes were separated from unincorporated nucleotides using NAP-5 Columns (Amersham Biosciences), precipitated and stored in hybridization buffer (50% formamide, 300 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA pH 8.0, 10% dextran sulphate, 1X Denhardt's solution, 50 µg/ml yeast tRNA and 10 mM DTT) at –80 °C until used. The sections were hybridized overnight with 2 x 10^4 cpm/µl of probe in 25-30 µl of hybridization buffer and washed as described elsewhere (Young et al., 1998). Dehydrated and air dried slides were dipped in Kodak NTB2 (Kodak, Rochester, NY) autoradiographic emulsion and exposed for 14 days. The slides were subsequently developed in Kodak Dektol developer. The labeled sections were viewed and photographed using a Zeiss Axiophot microscope.

In vitro mRNA Stability Assay. *In vitro* mRNA stability assay was performed using a protocol adapted from other publications (Ford and Wilusz, 1999;Mobarak et al., 2000;Perrone-Bizzozero et al., 1991). Briefly, total RNA was obtained from PC12 cells differentiated in the presence of NGF for 72-hours as previously described (Deschenes-Furry et al., 2003). Total protein was extracted from sham-operated or 2-day axotomized SCG (see below for procedure). Total RNA from PC12 cells (0.4 µg/µl) was incubated with total SCG proteins (0.1 µg/µl) in a final volume of 200 µl at 37°C in stability buffer (1X MOPS, 1 mM ATP, 0.1 mM Spermine, 2 mM DTT, 1U/µl RNase inhibitor). At intervals of 0-, 5-, 10-, 30- and 60-minutes a 40 µl aliquot was removed and the reaction was stopped by addition of ice-cold phenol/chloroform. The time 0 aliquot was removed promptly after mixture of the protein and RNA. De-proteination and precipitation of the

RNA were then performed in the presence of 2 μg of oyster glycogen. RNA was stored at –80°C until used for RT-PCR and quantified as described above.

Protein Extraction. Total protein was obtained from SCG using a Kontes glass tissue homogenizer and 250-500 μl of homogenization buffer (0.34 M sucrose, 60 mM NaCl, 15 mM Tris-HCl, pH 8.0, 10 mM EDTA and protease inhibitor cocktail). The resulting homogenate was sonicated (10-s pulse at 50% duty cycle and a power output of 1 using the Branson Sonifier 450) and centrifuged (12 000 x g for 15 min at 4 °C). Following centrifugation, the supernatant was recovered and protein concentration was quantified using the Bradford assay (Bio-Rad, Hercules, CA). Aliquots of total protein were stored at –80 °C until used.

GST-HuD Vector and Protein Purification. GST-HuD vector was prepared by inserting the human HuD sequence excised from the pcHuD vector (Mobarak et al., 2000), using the BamH1 sites flanking the sequence, into the pGEX 4T1 vector using the same restriction enzyme sites. The resulting vector was sequenced and insert orientation was verified. GST-tagged HuD was produced and purified using the GST Microspin Purification Module kit (Amersham Biosciences) according to the manufacturer's instructions.

In Vitro Transcription. cDNAs encoding the AChE 3'-UTR were obtained by PCR amplification of the plasmid template pGL3-3'-UTR as previously described (Boudreau-Lariviere et al., 2000;Deschenes-Furry et al., 2003). The primers employed to amplify

the small fragment encompassing the AU-rich element (ARE, 64 nucleotides) and to amplify the region of GAP-43 3'-UTR known to interact with HuD (209 nucleotides) (Chung et al., 1997) were designed to include a T7 promoter. Radiolabeled AChE 3'-UTR transcript fragments were synthesized using α-^{32}P-UTP (Amersham Biosciences) and an *in vitro* T7 transcription system (Promega, Madison, WI) according to the manufacturer's instructions.

Biotin-labeled CTP (Biotin-14-CTP from Invitrogen) was used to synthesize non-radioactive labeled transcripts for mRNA-binding protein pull-down assays. Biotin-labeled CTP consisted of 20% and 40% of the CTP used in the *in vitro* transcription reaction for the ARE, and full-length AChE 3'-UTR and GAP-43 3'-UTR, respectively. *In vitro* transcription with biotin-labeled CTP was carried out using the MEGAscript T7 or SP6 transcription system (Ambion) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay, UV-Crosslinking Assay and mRNA-binding Protein Pull- down Assay. RNA-based electrophoretic mobility shift assays (REMSAs), UV-crosslinking (UV-XL) assays and mRNA-binding protein pull-down assays were performed using total protein extracts obtained from pooled (~14 SCG) sham-operated and 2-day axotomized rat SCG. REMSAs were performed as described elsewhere (Alterio et al., 2001;Hew et al., 2000;Wilson and Brewer, 1999). Briefly, 20 µg of protein extract or 200 mM of GST-HuD construct or GST alone were incubated at 37 °C for 20 minutes with 2 x 10^5 cpm of ^{32}P-labeled AChE 3'-UTR fragment in 2X binding buffer (20 mM Hepes, pH 7.9, 3 mM Mg-Acetate, 50 mM K-Acetate, 1 mM DTT, 5% glycerol, 0.2 µg/µl yeast tRNA and 2.5 µg/µl heparin) in a total volume of 20 µl. The

mixture was separated by 6% native polyacrylamide gel electrophoresis with 0.5X TBE (Tris borate –EDTA) running buffer. The gels were subsequently dried under vacuum at 80 °C for 1 h and exposed to x-ray film at –70 °C.

UV-crosslinking assays were performed similarly to the REMSAs with the exception that the complexes formed between the protein extract and radiolabeled 3'-UTR fragments were crosslinked under 254 nm UV light using the CL-1000 Ultraviolet Crosslinker (UVP Inc., CA, USA). The crosslinked complexes were then treated with 0.5 units RNase T1 (Calbiochem, San Diego, CA) and 1 μg of RNase A (Qiagen, ON, Canada) for 20 min at 37 °C. Following RNase treatment, SDS-loading buffer was added to each sample and the samples were separated by 10% SDS-PAGE. To ensure that equivalent amounts of protein were loaded for each sample, the gels were stained with Coomassie blue prior to being dried. The gels were dried at room temperature between two sheets of cellophane and exposed to x-ray film at –70 °C.

mRNA-binding pull-down assays were performed similarly to the REMSAs. Biotin-labeled mRNA (2 μg) was incubated with protein extracts (100 μg) that had been pre-cleared with streptavidin-coated Dynabeads (Dynal Biotech, Oslo, Norway) for 30 minutes at room temperature in 2X binding buffer (see above). The RNA-protein reaction was incubated for one hour at 4 °C with streptavidin-coated Dynabeads. The supernatant was collected and stored for future use in Western blots. The beads were washed several times with 1X binding buffer and resuspended in 1X binding buffer and SDS-loading dye. The pulled-down proteins were used in Western blots (see below).

Northwestern Analyses. Northwestern analyses were performed as described in detail elsewhere (Erondu et al., 1999;Sagesser et al., 1997). Briefly, 50 μg of total protein extract were separated by SDS-PAGE using a 10% gel and electroblotted onto a nitrocellulose membrane (Bio-Rad). The membrane was then incubated in renaturation buffer (15 mM Hepes, pH 7.9, 50 mM KCl, 0.1 mM $MnCl_2$, 0.1 mM $ZnCl_2$, 0.1 mM EDTA, 0.5 μM DTT, 0.1% (v/v) Igepal CA-630 (a Nonidet P-40 substitute) in RNase-free water) at 4 °C overnight. The membranes were pre-hybridized for 1 h at room temperature in renaturation buffer containing 0.2 mg/ml yeast tRNA and hybridized for 4 hours at 4 °C in renaturation buffer including yeast tRNA, 5 mg/ml heparin, and 1 X 10^6 cpm/ml of RNA probe corresponding to the ARE. The membranes were exposed to x-ray film at –70 °C after several washes in renaturation buffer. To ensure that equivalent amounts of proteins were loaded for each sample, the membranes were stained with Ponceau S (Sigma) following exposure to x-ray film.

Immunoprecipitation and mRNA Analysis. Immunoprecipitation of the HuD transgene protein product from a hippocampal protein extract of the HuD transgenic mice (see above) was performed using protocols adapted from Tenenbaum *et al.* (2002) and Mobarak *et al.* (2000) (Tenenbaum et al., 2002;Mobarak et al., 2000). Proteins were extracted from hippocampal tissues using a polysome lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes, pH 7.0, 0.5% Igepal CA 630, 1 mM DTT, 1 mg/ml pepstatin A, Mini-complete, EDTA-free protease inhibitor cocktail (Roche Applied Science, Québec, Canada) and 100 units/ml of RNasin (Promega)). Protein extracts were centrifuged (12 000 x g for 15 min at 4 °C) and resulting supernatants were aliquoted and kept at –80 °C

until used. Cytoskeletal fractions were obtained from the pellets by resuspending them in 0.1 % SDS containing lysis buffer, followed by addition of excess non-ionic detergent. The solution was centrifuged again and the resulting supernatant aliquoted and stored at – 80 °C until used.

For immunoprecipitations, 500μg of total proteins or 250 μg of the cytoskeletal fraction were first precleared with 1 μg normal mouse IgG (Santa Cruz, Santa Cruz, CA) and 20 μl of protein G Dynabeads (Dynal Biotech) in IP buffer (20 mM Tris, pH 7.0, 50 mM NaCl, 3 mM Mg-acetate, 100 μM $ZnCl_2$, 1% Igepal CA-630, 1% BSA, 10μg/ml yeast tRNA, 5 mM EDTA, 1 mM DTT and 100 units/ml RNasin) for 1 hour at room temperature with constant mixing. The resulting supernatant was incubated with 2 μg of anti-myc antibody (Roche Applied Science) or equivalent amount of normal mouse IgG at room temperature with constant mixing for 2 hours. The reaction mixture was subsequently added to 30 μl of protein G Dynabeads and incubated at room temperature with constant mixing for 1 hour. Following this incubation, the beads were washed several times with IP buffer and total RNA was extracted from the pellet using TRIzol reagent as described above. The extracted RNA was subsequently used for RT-PCR using the Omniscript Reverse Transcription kit (Qiagen) and HotStarTaq DNA polymerase (Qiagen) as recommended by the manufacturer and as described above.

For GST-HuD immunoprecipitation and RT-PCR, 1 μg of GST-HuD or GST was incubated with 5 μg of RNA extracted from sham-operated SCG at room temperature in binding buffer (described above) for 20 minutes. The reaction mixture was precleared and immunoprecipitated using anti-HuD antibody 16C12 (Clonegene, Hartford, CT). Bound RNA was extracted and used for RT-PCR as described above.

Western Blot. For Western blotting, 20-25 μg of protein extract obtained from mouse hippocampus (see above), sham-operated and 2-day axotomized SCG or from the mRNA-binding protein pull-down assays were denatured in SDS-loading buffer and subjected to 10 % SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane (Bio-Rad). Following transfer, the membranes were incubated with antibodies directed to the myc-epitope (Roche Applied Science), HuD (Clonegene), neuronal Hu proteins (Molecular Probes, Eugene, OR) or α-tubulin (Sigma) and revealed using the SuperSignal ECL kit (Pierce).

Statistical Analysis. An analysis of variance was performed to evaluate the effects of SCG axotomy and HuD viral expression on AChE mRNA levels. The Fisher's Least Square Difference test was used to determine whether the differences seen between group means were significant. An unpaired Student's T-test was performed to evaluate the effects of human HuD transgene expression in the hippocampus on the levels of AChE mRNA. The level of significance in both analyses was set at $p < 0.05$. Data are expressed as mean ± S.E. throughout.

4.5 Results

HuD binds AChE transcripts in vivo

In a first series of experiments, we examined whether HuD binds AChE and regulates AChE mRNA levels *in vivo*. To this end, we first examined expression levels

of AChE mRNA in the CNS of the recently described transgenic mice that overexpress human HuD in specific subpopulations of neurons (Bolognani et al., 2006). For these studies, we focused on the HuD4 line of transgenic mice (high-expresser), whose HuD protein levels were two-fold greater than control littermates and whose myc-tagged transgene product was up to four-fold greater than the HuD2 line (low-expresser) that expressed the lowest amount of transgene product (see Figure 2A and (Bolognani et al., 2006)). As well, since the human HuD transgene product was primarily expressed in the forebrain, due to the expression pattern of the promoter (α-CaMKII) driving transgene levels, we concentrated on the hippocampus for these studies.

Figure 1. Expression of AChE mRNA is increased in the hippocampus of HuD overexpressing transgenic mice. *In situ* hybridization for AChE mRNA was performed on coronal brain sections from wildtype (WT) and HuD overexpressing transgenic mice (HuD4) using a radiolabeled anti-sense cRNA probe to AChE encompassing part of exon 6 through to the 3'-UTR. Scale bar (in WT), 500 μm.

As shown in Figure 1, using *in situ* hybridization (ISH) we observed that AChE mRNA was expressed in the hippocampal neurons corresponding to the CA1-CA3 rostrocaudal axis, with very little expression in the dentate gyrus in both wildtype and HuD4 transgenic brains. The AChE mRNA grain density was clearly greater in the

HuD4 transgenic mouse hippocampus than in the wildtype, suggesting that these neurons expressed increased amount of AChE mRNA. This observation was verified by quantitative RT-PCR performed on hippocampal brain regions, demonstrating that there was in fact a significant ($p < 0.05$, $n = 2$ and 3 different brain samples for WT and HuD4, respectively) two to three-fold increase in AChE mRNA levels in the HuD4 transgenic hippocampus (data not shown). In complementary experiments, we examined the mRNA levels in the cerebellum/brainstem areas by RT-PCR and ISH. We found that AChE mRNA levels were also significantly increased by ~four-fold in HuD4 transgenic cerebellum/brainstem regions (data not shown), most likely corresponding to cranial nerve nuclei in brainstem expressing high levels of AChE. Taken together, these results demonstrate that increased expression of HuD is associated with a concomitant increase in AChE transcript levels.

Given these results, we determined whether the transgene product expressed in the hippocampus could directly bind AChE transcripts. To this end, we used an antibody to the N-terminal myc-epitope tag to immunoprecipitate the HuD transgene product from hippocampus cytoskeletal and cytosolic protein extracts of HuD2 and HuD4 transgenic mice, and extracted the mRNA bound by HuD. Immunoblots for the myc-epitope tag demonstrated and confirmed that HuD4 transgenic mice express considerably more HuD transgene product than the HuD2 line (see Figure 2A). As shown in Figure 2B, AChE transcripts were extracted and amplified from the HuD4 cytoskeletal protein immunoprecipitate, but were hardly detectable in the HuD2 immunoprecipitate due to the relatively low level of myc-tagged human HuD present in the HuD2 line (Bolognani et al., 2006). This result serves as an ideal negative control for the immunoprecipitation assay,

in that AChE mRNAs are not binding non-specifically to the antibody or protein G Dynabeads. In addition, we could only successfully extract and amplify AChE mRNA from the HuD4 cytoskeletal immunoprecipitate, which suggests, as previously demonstrated, that HuD and its associated transcripts are predominantly associated with the cytoskeleton (Pascale et al., 2004). These results, together with those from Figure 1, represent the first demonstration that AChE mRNA is associated with HuD *in vivo*.

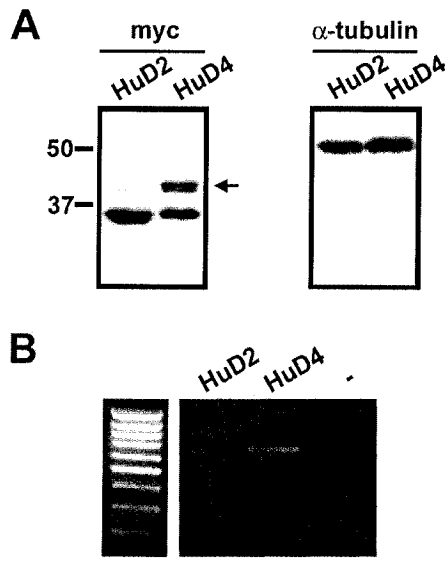

Figure 2. The HuD transgene product in HuD4 transgenic mice binds endogenous AChE mRNA. *A*, immunoblots for myc-tagged HuD (left) and α-tubulin (right, to verify equal loading) were performed on total protein extracts from HuD2 and HuD4 transgenic mice hippocampus. Arrow indicates the bands corresponding to HuD splice variants. The lower band corresponds to non-specific antibody interactions. The myc-tagged HuD transgene product was immunoprecipitated using an antibody directed to the myc epitope from cytoskeletal HuD2 and HuD4 transgenic mice hippocampus protein extracts. The bound mRNA was extracted and RT-PCR for AChE was performed. *B,* shows representative ethidium bromide-stained agarose gel displaying the AChE PCR product. The negative control lane is shown with a –.

Axonal injury results in decreased AChE mRNA levels.

Next, we examined the role of post-transcriptional mechanisms and HuD in regulating AChE transcript levels following neuronal injury. For these experiments, we used the sympathetic neurons from rat SCG.

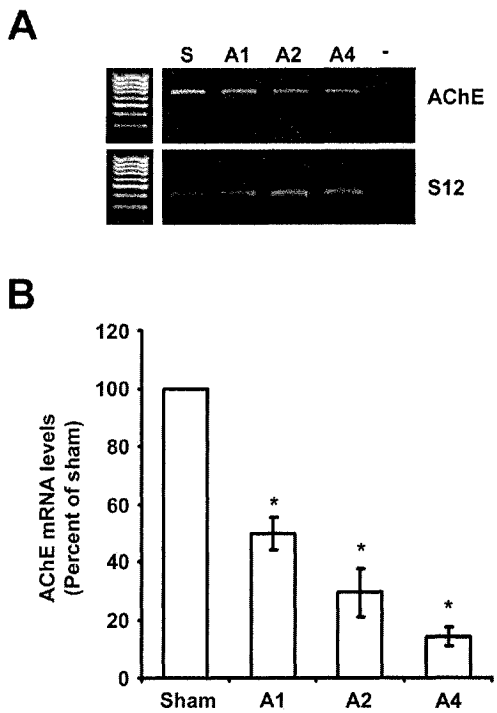

Figure 3. AChE expression levels decrease following axotomy of rat SCG. *A*, example of ethidium bromide-stained agarose gels displaying AChE and S12 ribosomal protein (S12) PCR products from sham-operated (S) and 1-, 2- and 4-day axotomized (A1, A2, and A4, respectively) SCG. The negative control lane is shown with a –. *B*, quantification of AChE mRNA levels in Sham and 1-, 2-, and 4-day axotomized SCG, expressed as a percentage of sham-operated SCG. Symbols indicate significant differences from sham-operated SCG (*, $p < 0.0001$; $n = 3$-4 independent experiments).

This model was chosen for multiple reasons: i) the adrenergic neurons of the SCG receive predominantly cholinergic inputs and express high levels of AChE; ii) the structure of this ganglion consists of a single major site of input and two major sites of output (internal and external carotid nerves), allowing for simple manipulation; iii) its peripheral

location allows for easy accessibility; and iv) the synaptic structure of these sympathetic neurons is similar to those of the central nervous system (Klimaschewski et al., 1994;Taxi and Eugene, 1995;Klimaschewski et al., 1996). Neuronal injury (axotomy) was achieved with this model by cutting the internal and external carotid nerves as they exit from the ganglion body. The success of axotomy was determined by ptosis of the ipsilateral eyelid and by the concomitant decrease in tyrosine hydroxylase transcript levels (data not shown) (Sun and Zigmond, 1996).

Although initial studies have demonstrated that SCG axotomy resulted in an ~60% decrease in AChE protein activity (Klingman and Klingman, 1969;Viana and Kauffman, 1984), the effect of axotomy on the underlying molecular mechanisms remains poorly defined. Thus, we examined the initial response of AChE mRNA to SCG axotomy. Since the AChE T transcript is predominantly expressed in neurons, we focused on the level of expression of this mRNA (Legay et al., 1993;Seidman et al., 1995). As illustrated in Figure 3A and B, AChE mRNA levels decreased dramatically to ~50% of sham-operated levels ($p < 0.0001$) within 1-day of axotomy. Transcript levels continued to decrease gradually, such that the levels observed within 2- and 4-days of axotomy were approximately 30% and 15% ($p < 0.0001$) of those in sham-operated SCG, respectively. The relative amount of S12 ribosomal protein mRNA, used as an internal control for these assays, did not vary significantly during this period.

Axotomy results in decreased AChE mRNA stability.

Given these observations, we subsequently considered whether axotomy of the SCG also affected post-transcriptional regulation of AChE. Therefore, we first performed *in*

vitro mRNA stability assays to determine whether proteins from sham-operated and 2-day axotomized SCG differentially affect AChE mRNA levels.

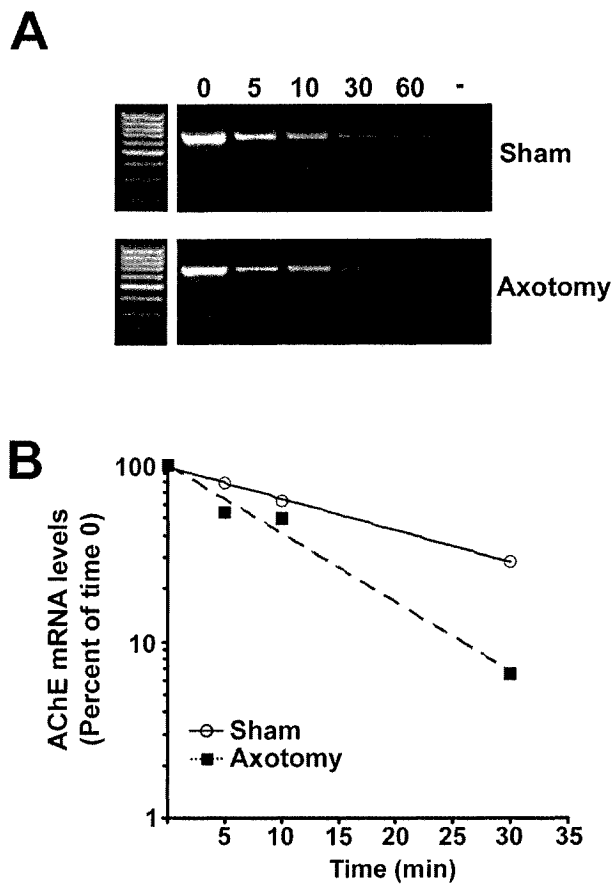

Figure 4. Decline in AChE mRNA levels is accelerated following axotomy of rat SCG. *A*, example of ethidium bromide-stained agarose gels displaying AChE PCR products from *in vitro* mRNA stability assay performed using total RNA from differentiated PC12 cells incubated for 0-, 5-, 10-, 30- or 60- minutes (0, 5, 10, 30 and 60, respectively) with total proteins extracted from sham-operated (Sham) and 2-day axotomized (Axotomy) SCG. *B*, quantification of the remaining AChE mRNA levels after the indicated incubation times expressed as a percent of the mRNA level at time 0 (100%).

These assays are commonly used to assess the stability of specific transcripts in response to particular stimuli (see (Ford and Wilusz, 1999;Kohn et al., 1996;Mobarak et al., 2000)). To this end, exogenous total RNA from NGF-induced differentiated PC12 cells that have a high level of AChE mRNA was incubated with protein extracts from either sham-

operated or 2-day axotomized SCG. Aliquots of the protein-RNA reaction mixture were removed at timed intervals, and mRNA was extracted and used for quantitative RT-PCR.

As shown in Figure 4A and B, AChE transcript levels decreased during the course of the incubation period in the presence of protein extracts from sham-operated and 2-day axotomized SCG, albeit more rapidly in the presence of axotomized SCG proteins. Figure 4A demonstrates that mRNA extracted from the sham-operated protein-RNA reaction mixture could be amplified at all time points examined. Remaining AChE mRNA levels were expressed as a percent of the levels determined at Time 0 (100%). Accordingly, under these conditions, AChE mRNA half-life corresponded to ~20 minutes when RNA was incubated with sham-operated SCG proteins. In comparison, AChE mRNA levels were significantly diminished within 30 minutes of incubation of the RNA with 2-day axotomized SCG protein extracts and undetectable by 60 minutes. The calculated half-life of AChE mRNA when incubated with 2-day axotomized SCG protein extract was ~10 minutes. These results, therefore, suggest that following SCG axotomy there is an alteration in the levels or functions of certain RNA-binding proteins that are important to AChE transcript stability and abundance.

Axotomy alters AChE mRNA and protein interactions.

We performed REMSAs using an *in vitro* transcribed 64 nucleotide transcript encompassing the AU-rich element (ARE; see Figure 5A) to visualize the differential interactions between this domain and RNA-binding proteins.

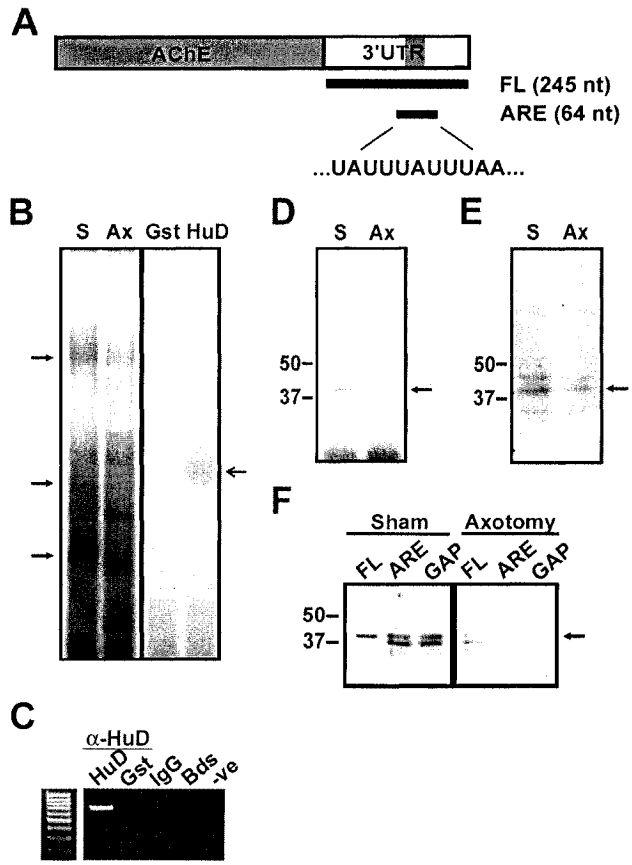

Figure 5. The pattern of protein-RNA interactions with the AChE 3'-UTR, changes following axotomy of rat SCG. *A*, depicts a schematic of the ~2.4 kb AChE transcript including the ARE and its sequence. The full-length 3'UTR probe (FL, 245 nt) and ARE probe (64 nucleotides) consisting of the ARE and flanking domains are also shown. *B*, REMSAs were performed using protein extracts from sham-operated (S) and 2-day axotomized (Ax) SCG incubated with *in vitro* transcribed radiolabeled AChE 3'-UTR ARE probe in the presence of yeast tRNA and heparin. REMSAs were also performed with the GST-HuD fusion protein (HuD) and the GST domain alone, and the ARE probe (open arrow). Representative autoradiograms demonstrate the interaction between RNA and protein complexes. Closed arrows indicate specific RNA-protein complexes that decrease in the axotomy sample. *C*, Specific interaction between the GST-HuD fusion protein (HuD) and AChE mRNA found in a sham-operated SCG total mRNA extract is demonstrated by immunoprecipitation of HuD with a HuD-specific antibody and RT-PCR for AChE performed on the immunoprecipitate. The GST moiety (GST), normal rabbit IgG (IgG) and protein-G beads (Bds) were used as controls for non-specific binding. The AChE PCR product obtained is viewed on an ethidium bromide-stained agarose gel. *D* and *E*, direct binding of specific proteins from sham-operated (S) and 2-day axotomized SCG (Ax) to the ARE was examined using UV-crosslinking assays (*D*) and Northwestern analyses (*E*). Note the decreased binding of a ~ 40 kD protein indicated with the closed arrows. *F*, mRNA-binding protein pull-down assay was performed using biotin-labeled full-length (FL) AChE 3'-UTR, ARE and GAP-43 3'-

UTR (GAP) RNAs incubated with protein extracts from sham-operated (Sham) and 2-day axotomized (Axotomy) SCG. Immunoblots for neuronal Hu proteins were performed with proteins pulled-down by labeled RNA. Bands corresponding to HuD are indicated with a closed arrow. Note the presence of Hu proteins binding more specifically to AChE and GAP-43 mRNAs in the sham-operated protein extract than in the axotomized protein extract.

As illustrated in Figure 5B, REMSAs performed using proteins from sham-operated SCG, resulted in three different complexes (see arrows) that associated with the AChE ARE. In order to decrease non-specific interactions both yeast tRNA and heparin were added to the binding buffer. REMSAs performed with excess cold labeled ARE (100-fold) competed out the complexes (data not shown). When protein extracts from 2-day axotomized SCG were used, the binding intensities for all three complexes were decreased. Notably, the decreased interactions of some of the complexes were more dramatic than others (see second arrow in Figure 5B) in the REMSAs performed with 2-day axotomized SCG protein extracts. The observed banding pattern and decrease in binding intensity were highly reproducible (n = 3 different REMSAs).

To demonstrate that HuD protein specifically associated with the ARE, we performed REMSAs with a GST-HuD fusion protein and excess GST domain alone. As shown in Figure 5B, we found that the complex formed between GST-HuD and the ARE corresponded with one of the complexes formed between the ARE and SCG proteins (compare arrow on right-hand side with arrowhead on left-hand side). In addition, this complex appeared to be absent in the REMSA performed with protein extracts from axotomized SCG. Accordingly, this complex, which possibly corresponds to HuD, exhibits either reduced binding or does not bind to the AChE 3'-UTR following axotomy.

To further demonstrate that HuD associates with AChE mRNA, we incubated the GST-HuD fusion protein with a total RNA extract isolated from sham-operated SCG and

immunoprecipitated HuD protein with a HuD specific antibody (see Methods and Materials), extracted total RNA and used it for RT-PCR. As shown in Figure 5C, AChE mRNA could be amplified from the GST-HuD immunoprecipitate. In addition, GAP-43 mRNA, another known target of HuD (Mobarak et al., 2000), was also amplified from the GST-HuD immunoprecipitate (data not shown). However, neither AChE nor GAP-43 transcripts were amplified from the controls, namely excess GST alone, control IgG or the streptavidin-coated beads, suggesting that the binding was specific. Together, these results strongly support the specificity of the interaction between HuD and AChE.

Interactions between the ARE and proteins from sham-operated and 2-day axotomized SCG were also assessed using UV-crosslinking (UV-XL) assays and Northwestern analyses. UV-XL assays were performed in a similar fashion to the REMSAs, except that the protein-RNA complexes were crosslinked by UV light and proteins making up the complexes were separated by SDS-PAGE, following digestion of the bound and unbound RNA. With the UV-XL assay, we found a protein from sham-operated SCG of approximately 40-42 kDa that could bind the ARE (see Figure 5D). In comparison, we could not detect any proteins from the 2-day axotomized SCG extract that were directly binding the AChE ARE. Likewise, in the Northwestern analyses, two proteins of that approximate mass from sham-operated and 2-day axotomized SCG protein extracts bound the ARE. However, binding intensities of the proteins from 2-day axotomized SCG were considerably decreased (see Figure 5E).

Finally, direct binding of SCG HuD with the AChE ARE was demonstrated by mRNA-binding protein pull-down assays. In this assay, biotin-labeled RNA corresponding to the full-length (FL) AChE 3'-UTR and the ARE were incubated with

protein extracts from sham-operated and 2-day axotomized SCG. The resulting complexes were pulled out of solution and used for Western blotting using an antibody directed to all neuronal Hu proteins, HuD (~40-42 kDa), HuB and HuC (~39 kDa) (Marusich et al., 1994;Okano and Darnell, 1997). Based on the amino acid sequence, HuD has a greater mass than HuB and HuC. As shown in Figure 5F, neuronal Hu proteins from sham-operated SCG associated with AChE FL and ARE transcripts specifically. The specificity of this interaction was confirmed using the GAP-43 3'-UTR, which, as expected, also pulled-down neuronal Hu proteins (see Figure 5F) (Mobarak et al., 2000). Note that proteins pulled out of solution by AChE and GAP-43 transcripts had a similar molecular mass to those binding to the ARE as revealed in UV-XL and Northwestern analyses (see arrow and compare to western shown in Figure 2B). This suggests that one of the proteins binding the AChE ARE is indeed HuD. When the pull-down assay was performed with protein extracts from 2-day axotomized SCG, transcripts for AChE and GAP-43 showed greatly decreased or no interactions with neuronal Hu proteins. Taken together, the results obtained with several different yet complementary approaches indicate that the decrease in AChE mRNA levels following SCG axotomy results from decreased binding of the neuronal Hu proteins, specifically HuD.

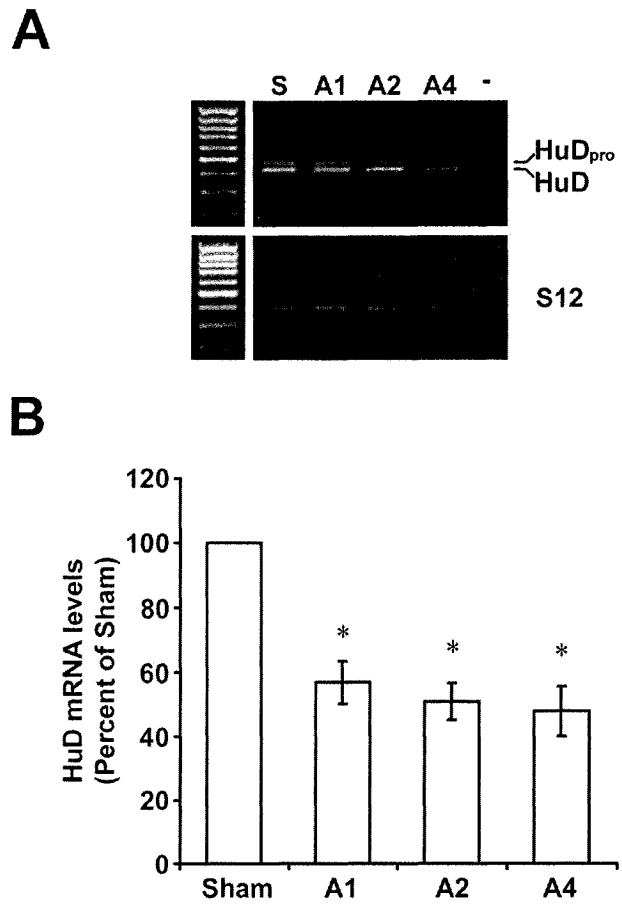

Figure 6. HuD transcript levels decrease following SCG axotomy. *A*, example of ethidium bromide-stained agarose gels displaying HuD (top band is HuD_{pro} and bottom band is HuD) and S12 ribosomal protein (S12) PCR products from sham-operated (S) and 1-, 2- and 4-day axotomized SCG (A1, A2 and A4, respectively). The negative control lane is indicated by –. *B*, quantification of HuD mRNA levels (HuD_{pro} and HuD quantified together) in sham-operated and 1-, 2- and 4-day axotomized SCG expressed as a percent of the mRNA levels present in sham-operated SCG. (*, $p < 0.0001$; $n = 3$ independent experiments).

HuD expression is altered following SCG axotomy.

Since we observed decreased binding of proteins of the approximate molecular mass of HuD, we next specifically examined the effect of SCG axotomy on expression of HuD transcripts and protein. The HuD gene is alternatively spliced at the 3'-end resulting in three possible transcripts termed HuD_{pro}, which is unspliced, HuD, which lacks exon 7

and HuD_{mex}, which lacks exons 6 and 7 (see for review (Deschenes-Furry et al., 2006)). As shown in Figure 6A, sham-operated SCG expressed HuD_{pro} and HuD transcripts; but to different extents, such that the level of expression of HuD was greater than that of HuD_{pro}. As shown in Figure 6A and B, the alternatively spliced HuD_{pro} and HuD transcripts (quantified together by RT-PCR) decrease by ~50% within 1-day of axotomy and remained at this low level of expression for up to four-days. Using antibodies directed to all neuronal Hu proteins, we observed that the protein levels of the band corresponding to HuD (Marusich et al., 1994) were decreased to a similar extent as the transcripts in 2-day axotomized SCG (data not shown). In addition, the reduction in Hu protein levels corresponds well with the diminished protein-RNA interactions observed in the UV-XL, Northwestern and pull-down analyses (compare with Figures 5D, E and F and 2B). These findings, therefore, indicate that reduced HuD expression levels as a result of SCG axotomy lead to parallel changes in HuD binding activity and, consequently, on the overall expression levels of AChE mRNA.

Exogenous expression of HuD maintains AChE mRNA levels following axotomy.

In order to explicitly demonstrate HuD's role in regulating AChE expression following SCG axotomy, we expressed exogenous human HuD in SCG neurons using a replication-deficient Herpes Simplex virus (HSV) containing the complete human HuD cDNA (Anderson et al., 2001;Szabo et al., 1991). For this experiment, the virus was injected into the SCG 4-days prior to axotomy and SCG were removed 2-days following axotomy for analysis. As shown in Figure 7, we observed that AChE mRNA levels decreased dramatically by ~80% following axotomy, similarly to what we observed in

Figure 3. However, when human HuD was expressed in the SCG, AChE mRNA levels remained unchanged. In fact, they were expressed to the same level as in the sham-operated SCG. When comparing the staining intensity seen in ethidium bromide-stained agarose gels (see Figure 7A), injection of HuD-HSV appeared to increase expression levels of AChE transcript in sham-operated SCG as well. In order to control for the effects of viral infection, we injected a LacZ expressing HSV. Injection of the LacZ expressing HSV did not appear to affect the expression level of AChE transcripts in sham-operated SCG (see middle panels of Figure 7A) or following axotomy, suggesting that the response to HSV-HuD was specific to HuD.

To confirm the compensatory effect of HuD exogenous expression on AChE mRNA levels, we also examined expression of another known HuD target, GAP-43 mRNA. We observed that GAP-43 mRNA levels decreased in 2-day axotomized SCG. Expression of exogenous human HuD also prevented the decrease in GAP-43 transcript levels. As with AChE, the levels of GAP-43 mRNA appeared greater in sham-operated, HuD injected SCG (see Figure 7A), upon comparison of the gels. Taken together, these results clearly demonstrate that expression of human HuD in the SCG prior to axotomy can compensate for decreased expression of endogenous HuD, and accordingly prevent the reduction in AChE transcript levels in response to axotomy.

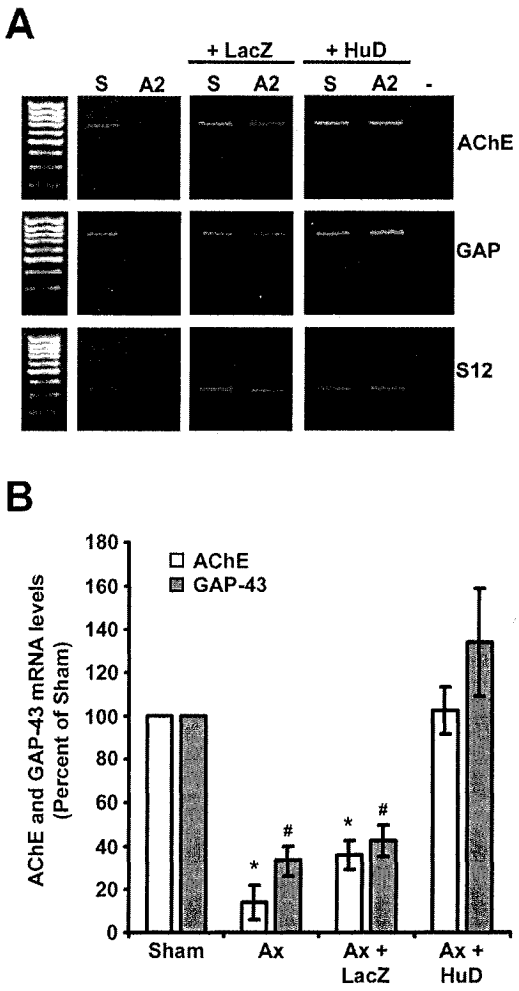

Figure 7. Overexpression of HuD in the SCG rescues the expression of AChE mRNA following axotomy. HSV-HuD and HSV-LacZ were used to infect both rat SCG and axotomy was performed 4-days later. The SCG were removed 2-days following axotomy. *A*, examples of ethidium bromide-stained agarose gels displaying AChE, GAP-43 (GAP) and S12 ribosomal protein (S12) PCR products from sham-operated (S) and 2-day axotomized (A2) control and HSV-LacZ (+ LacZ) or HSV-HuD (+ HuD) infected SCG. The negative control lane is shown with a –. *B*, quantification of AChE and GAP-43 mRNA levels in sham and 2-day axotomized (Ax) control, HSV-LacZ or HSV-HuD infected SCG expressed as a percentage of the control or infected sham-operated SCG. (*, $p < 0.01$; #, $p < 0.008$; $n = 3$ independent experiments).

4.6 Discussion

Numerous studies have previously characterized the expression pattern of AChE molecular forms, activity and transcripts in neurons of the CNS and PNS (Karpel et al., 1994;Bernard et al., 1995;Hammond et al., 1994;Mesulam and Geula, 1991;Koelle, 1954;Hosli and Hosli, 1970). Although the individual roles of transcriptional and post-transcriptional events are being addressed using various cell culture systems, there is by comparison, very little known about the molecular mechanisms controlling AChE expression in neurons *in vivo*. Given the essential role of AChE in the CNS and PNS, its involvement in various disease states and its known non-cholinergic functions (see Introduction), it appears important to gain insights into the molecular mechanisms regulating AChE expression *in vivo*. Accordingly, we examined here the interaction between HuD and AChE mRNAs *in vivo* in the CNS and PNS and following axotomy of the SCG. Our findings indicate that HuD directly binds AChE mRNA and that there is a correspondence between the levels of expression of HuD and AChE mRNA, such that decreased levels of HuD correlate with decreased AChE mRNA stability and levels following SCG axotomy. To our knowledge, this is the first *in vivo* study that has clearly demonstrated the occurrence of a specific molecular mechanism involved in regulating AChE expression in neurons.

In vivo studies characterizing the molecular events regulating AChE expression have mostly been performed with skeletal muscle. For example, the dramatic decrease in muscle AChE mRNA levels observed following denervation was attributed to diminished transcript stability as determined by *in vitro* mRNA stability assays (Boudreau-Lariviere et al., 2000;Grubic et al., 1999). To date, however, the elements and factors mediating

this effect have yet to be identified. By comparison, the specific roles of transcriptional and post-transcriptional regulation of AChE, as well as specific elements and factors mediating these effects, have been identified using cultured muscle, neuronal and hematopoietic cell lines. During differentiation of these cells, post-transcriptional mechanisms corresponding mainly to transcript stability, were shown to be a dominant process regulating AChE expression (Angus et al., 2001;Chan et al., 1998;Deschenes-Furry et al., 2003;Deschenes-Furry et al., 2005;Coleman and Taylor, 1996;Fuentes and Taylor, 1993;Luo et al., 1994;Luo et al., 1999). Specifically, an ARE in the AChE 3'-UTR was identified as a target for Hu family RNA-binding proteins during myogenic and neuronal differentiation (Cuadrado et al., 2003;Deschenes-Furry et al., 2003;Deschenes-Furry et al., 2005). Here, we show that post-transcriptional mechanisms, in particular transcript stability, are also important to AChE mRNA expression *in vivo*. Specifically, we demonstrated using human HuD overexpressing transgenic mice and a series of complementary *in vitro* approaches that HuD associates with the ARE in the AChE 3'UTR.

AREs are found in 1 in 20 human genes and represent the best-characterized determinants of transcript stability (Chen and Shyu, 1995;Bakheet et al., 2006;Xu et al., 1997). However, whether a transcript will be degraded or stabilized largely depends on the presence and nature of RNA-binding proteins that recognize and bind the ARE. One of the prevailing theories surrounding transcript turnover mediated via AREs proposes that the relative abundance of stabilizing versus destabilizing RNA-binding proteins governs the stability pf target mRNAs (Barreau et al., 2005;Deschenes-Furry et al., 2006). Of the increasing number of identified RNA-binding proteins that bind AREs, HuD and

the Hu-family are some of the few that have transcript stabilizing activities. In this regard, following SCG axotomy we observed using an *in vitro* mRNA stability assay, a marked decline in AChE mRNA stability, which most likely results from changes in the levels or activity of key RNA-binding protein and a concomitant decrease in HuD transcript and protein levels. These results therefore, indicate that the reduction in AChE transcripts is mostly associated with a decline in HuD expression since, exogenous expression of HuD can rescue AChE mRNA levels.

Alteration in transcript stability also occurs due to changes in the binding activity of RNA-binding proteins. Accordingly, we observed reduced binding of neuronal Hu proteins to the ARE using various binding assays. In addition to decreased HuD expression, the observed decrease in HuD binding may also result from post-translational modification of HuD, or from interactions with other RNA-binding proteins or structural proteins (Kasashima et al., 1999;Kasashima et al., 2002;Pascale et al., 2004). For instance, during neuronal differentiation HuD binding activity can be controlled by the protein kinase C (PKC) signaling pathway (Mobarak et al., 2000;Pascale et al., 2005) or through methylation by the methyltransferase CARM1 (co-activator associated methyltransferase 1) (Fujiwara et al., 2006).

The Hu family of RNA-binding proteins are known to interact with several other proteins and they are found in ribonucleoprotein complexes often associated with the cytoskeleton (Keene and Tenenbaum, 2002;Pascale et al., 2004). Accordingly, we immunoprecipitated HuD and AChE mRNA from hippocampal cytoskeletal protein extracts of HuD overexpressing transgenic mice. We also observed several RNA-protein complexes that formed with the AChE ARE and SCG protein extracts suggesting that

HuD may be a member of these complexes or that other RNA-binding proteins are interacting with this domain.

HuD is one of the neuronal members of the Embryonic Lethal Abnormal Vision (ELAV)-like Hu family of RNA-binding proteins (see for review (Guhaniyogi and Brewer, 2001;Perrone-Bizzozero and Bolognani, 2002;Deschenes-Furry et al., 2006). This family of mRNA stabilizing proteins includes the other neuronally expressed members HuB and HuC, and the ubiquitously expressed HuR. In addition to binding AREs, the RNA-recognition motifs (RRM) of these proteins bind to long poly(A)-tails and the overall binding efficacy of the RRM is modulated by the length of the poly(A)-tail (Park-Lee et al., 2003;Lopez de Silanes et al., 2004;Beckel-Mitchener et al., 2002;Ma et al., 1997). Given the number and variety of transcripts with AREs, HuD is implicated in multiple neuronal and cellular functions (see for review (Deschenes-Furry et al., 2006)). For instance, cell culture studies, and more recently studies performed with HuD knockout mice, have demonstrated that HuD has a significant role in promoting cell cycle exit (Akamatsu et al., 2005;Marusich et al., 1994;Okano and Darnell, 1997), differentiation and neurite elongation (Anderson et al., 2001;Aranda-Abreu et al., 1999;Mobarak et al., 2000). Within the adult CNS, HuD has also been implicated in plasticity of hippocampal neurons during various learning paradigms (Bolognani et al., 2004;Pascale et al., 2004;Quattrone et al., 2001).

In this context, expression of several proteins involved in neurite outgrowth and extension, including GAP-43, tau, neurofilament M and AChE, are regulated by Hu proteins (Aranda-Abreu et al., 1999;Antic et al., 1999;Deschenes-Furry et al., 2003;Mobarak et al., 2000;Bolognani et al., 2006). Appropriately, we observed that

GAP-43 appears co-regulated with AChE following axotomy such that GAP-43 transcript stability decreased with axotomy and exogenous expression of HuD maintained GAP-43 mRNA levels following axotomy. Given these results, it appears therefore that HuD also plays an essential role *in vivo* by modulating neurite development.

Many of the studies that have characterized AChE expression in neurons have also described changes in AChE protein, activity and mRNA that take place in response to axotomy (Farris et al., 1993;Fernandes et al., 1998;Flumerfelt and Lewis, 1975;Hoover and Hancock, 1985;Klingman and Klingman, 1969;Tetzlaff and Kreutzberg, 1984;Viana and Kauffman, 1984). For example, initial studies performed with SCG showed that axotomy results in decreased AChE activity (Klingman and Klingman, 1969;Viana and Kauffman, 1984). In addition, axotomy of the facial motor nuclei causes a rapid reduction of neuronal AChE activity and transcript levels (Tetzlaff and Kreutzberg, 1984;Fernandes et al., 1998). Importantly, Fernandes *et al.* (1998) also demonstrated that exogenous application of the target-derived trophic factors BDNF and NT4/5, prevented the decline in AChE mRNA levels (Fernandes et al., 1998). To date however, the mechanisms by which this rescue is mediated remain unknown. Given our current findings and the fact that many cellular and molecular effects of SCG axotomy result from loss of NGF (Federoff et al., 1992;Klingman and Klingman, 1969;Schober et al., 1997), it is tempting to speculate that there is a direct link between axotomy, levels of neurotrophic factors, HuD expression and ultimately regulation of key transcripts *in vivo* including AChE and GAP-43.

Recent studies, have also begun to address a putative role for HuD in the regenerative response of motoneurons and sensory neurons following axotomy

(Anderson et al., 2003;Anderson and Steward, 2003). Thus, during the regenerative phase of recovery from axotomy these authors observed elevated levels of HuD and co-localization with ribonucleoprotein granules and GAP-43. In the present study, we observed decreased GAP-43 and HuD transcript levels early in the response to axotomy of SCG neurons (see Figure 7). In addition, exogenous expression of human HuD, which compensated for the decrease in endogenous HuD, rescued the level of expression of GAP-43 transcripts. HuD, therefore, due to its mRNA stabilizing activity of key transcripts involved in neurite outgrowth, is considered to be essential to the regenerative response of peripheral neurons. Most importantly, we have demonstrated by expressing exogenous human HuD in the SCG that HuD is a key factor in maintaining normal neuronal function following injury. Consequently, it is becoming apparent that a better understanding of the molecular mechanisms regulating expression, activity and function of HuD will also increase our basic knowledge of the molecular events taking place during neuronal differentiation, growth and plasticity. In addition, this knowledge will be useful in designing novel therapeutic strategies aimed at modulating HuD levels toward the beneficial treatment of neurodegenerative disorders or promotion of nerve regeneration.

Chapter 5.

General Discussion

In this series of individual studies, we have examined the role of post-transcriptional events in the regulation of AChE expression in neurons and muscle. Taken together, our findings indicate that AChE expression in neurons and muscle is regulated by transcript stability as mediated by the ARE in the 3'-UTR and Hu family proteins, HuD and HuR. Specifically, we demonstrated that the ARE in the AChE 3'-UTR, and HuD and HuR are key regulatory elements that control AChE transcript stability during neuronal and myogenic differentiation, respectively. Furthermore, HuD's role in controlling AChE mRNA levels was confirmed in hippocampal and SCG neurons, inasmuch as SCG axotomy resulted in decreased HuD expression and, correspondingly, in AChE mRNA levels, which was prevented by exogenous expression of human HuD in the SCG. Accordingly, results of these studies have furthered our understanding of the molecular events regulating AChE expression in excitable cells.

5.1 Post-transcriptional regulation of AChE expression

For over a decade, a number of studies have proposed that post-transcriptional mechanisms regulate AChE mRNA levels in various tissues. For instance, separate studies have suggested that increased transcript stability is responsible for the marked accumulation of AChE transcripts observed during myogenic, neuronal and hematopoietic differentiation (Chan et al., 1998;Coleman and Taylor, 1996;Fuentes and Taylor, 1993).

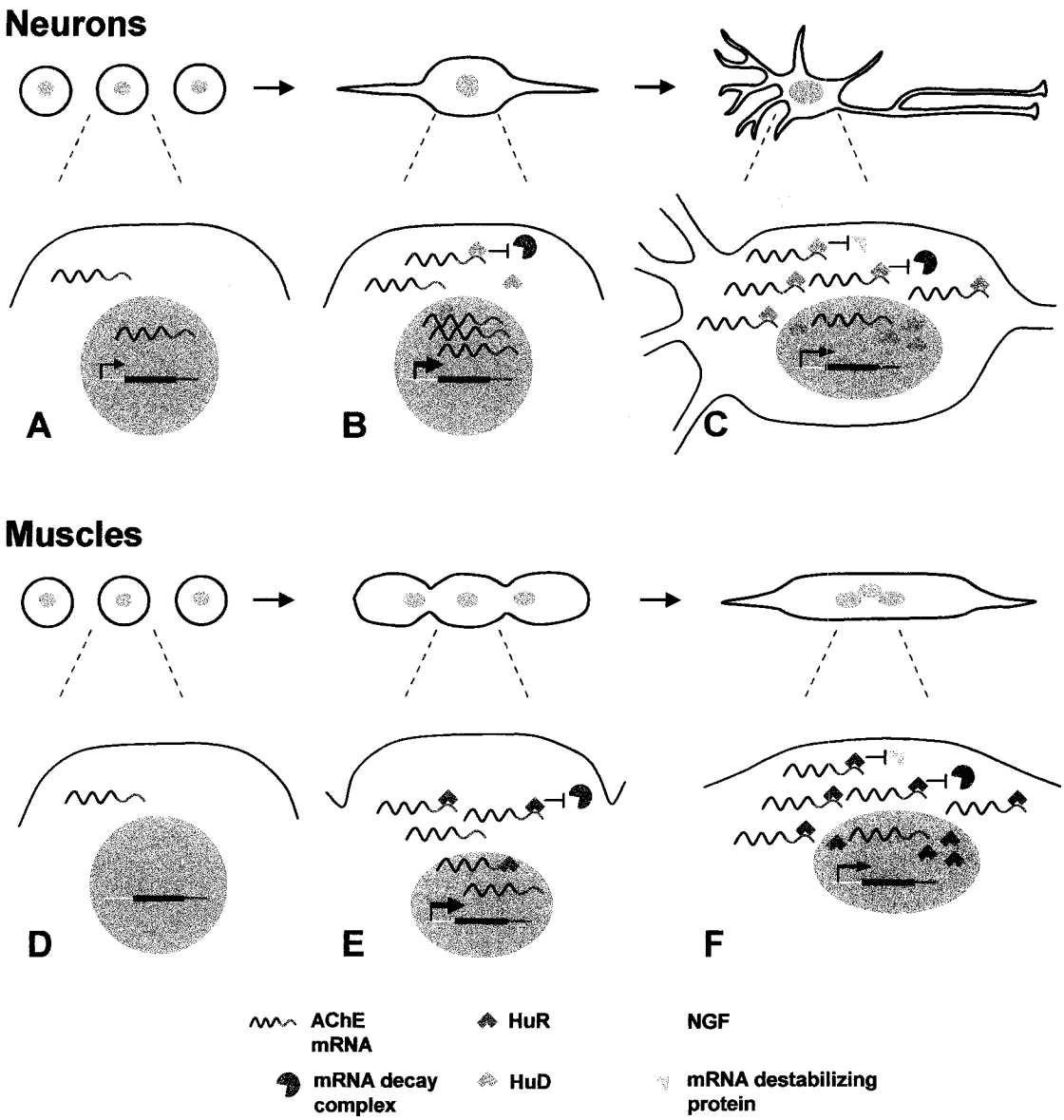

Figure 1. Role of HuD and HuR in regulating AChE expression. *A)* In neurons, low levels of AChE mRNA can be detected prior to differentiation. *B)* NGF-induced differentiation results in a transient increase in transcription and accumulation of AChE mRNA, which is stabilized by HuD. *C)* As differentiation proceeds, transcription returns to basal levels and AChE mRNA is stabilized by HuD. HuD control of AChE mRNA is maintained in adult neurons and is influenced by target-derived trophic factors. *D)* In muscle, prior to myogenic differentiation, myoblasts express very little AChE. *E)* As myogenesis begins, *AChE* gene transcription increases and AChE accumulates in the cytoplasm, where it is stabilized by HuR. *F)* In differentiated myotubes, AChE transcription returns to basal levels and sustained AChE mRNA levels result from HuR stabilization. HuD and HuR binding may also inhibit binding of other destabilizing proteins to the AChE 3'-UTR (*C* and *F*).

As well, diminished AChE mRNA levels observed following muscle denervation were shown to result from decreased transcript stability (Boudreau-Lariviere et al., 2000a). However, the elements and factors mediating AChE mRNA stability had not been identified. In our studies we have confirmed that AChE mRNA levels are regulated by transcript stability in excitable cells; and, importantly, we demonstrated that binding of mRNA stabilizing proteins, HuD and HuR, to the ARE in the 3'-UTR, is responsible for controlling mRNA levels in differentiating neurons and muscle, and *in vivo* in sympathetic neurons. Consequently, these are the first studies that have identified a conserved *cis*-acting element in the 3'-UTR and a conserved family of RBP that can regulate AChE expression in both neurons and muscle (see Figure 1).

AChE, however, is also expressed in non-excitable cells, such as hematopoietic cells, and has a significant role in their differentiation (see *1.5 AChE expression in non-excitable tissues*). Thus, initial studies, examining the molecular mechanisms regulating AChE expression in hematopoietic cells, have demonstrated that accumulation of AChE transcripts during hematopoietic differentiation of a murine erythroleukemia cell line is regulated by increased transcript stability (Chan et al., 1998). Considering that we have established that the ARE and Hu family proteins are important and conserved regulators of AChE expression in excitable cells, we next need to determine whether they are also acting in non-excitable cells. In this context, HuR has recently been associated with differentiation of a myeloid leukemia cell line, such that absence of HuR migration from the nucleus to cytoplasm was linked to the inability of these cells to differentiate (Champelovier et al., 2006). HuR, therefore, appears to have an important role in differentiation of hematopoietic cells, and may also mediate increased AChE transcript

stability. Consequently, future research needs to examine the role of HuR in controlling AChE expression during hematopoietic cell differentiation and, specifically, in leukemias.

AChE and its aberrant expression have also been linked with multiple disease states (see *1.4 AChE expression in disorders of the nervous system*). For instance, elevated AChE mRNA levels have been correlated with aggressive brain cancers, and reduced AChE expression levels or protein activity are observed in leukemias (see for review Soreq et al., 1991;Soreq and Seidman, 2001). In the present studies we have observed that elevated levels of HuD or HuR resulted in greater accumulation of AChE mRNAs in neurons and muscle cells, respectively. As well, reduction in HuD expression following SCG axotomy was paralleled by decreased AChE mRNA levels. These studies, therefore, provide insight into the cause of abnormal AChE expression in cancers and an indication of the regulatory mechanisms that may be influencing AChE expression in cancers.

In this regard, Hu proteins were identified as the ectopically misexpressed proteins in tumors that lead to the production of onconeural antibodies and development of paraneoplastic encephalomyelitis and sensory neuronopathy syndromes (Dalmau et al., 1990;Musunuru and Darnell, 2001;Nabors et al., 1998;Szabo et al., 1991). Atypical HuR and HuD activity, in addition, have increasingly been associated with cancers, including neuroblastomas and breast, ovarian, and colon cancers (Cho et al., 2006;Denkert et al., 2004b;Denkert et al., 2004a;Denkert et al., 2006;Erkinheimo et al., 2003;Heinonen et al., 2005;Lopez de Silanes et al., 2003;Nabors et al., 2001;Lopez de Silanes et al., 2004;Sommer et al., 2005;Behrends et al., 2002;Gultekin et al., 2000). Given the

putative role for AChE in cellular differentiation and the known role of Hu proteins in cell cycle regulation and induction of cellular differentiation (see for review Brennan and Steitz, 2001;Soreq and Seidman, 2001;Keene, 1999;Deschenes-Furry et al., 2006;Perrone-Bizzozero and Bolognani, 2002), it is particularly important to examine the role of Hu proteins in regulating AChE expression in cancers.

5.2 Role of HuR in regulating AChE expression in muscle

AChE expression in muscle is controlled initially by intrinsic properties of the muscle, such that AChE expression increases significantly during myogenesis. As muscle development proceeds, however, AChE expression is predominantly directed by the motor nerve, such that AChE mRNA levels are greater at the junction and decline outside of the endplate (see *1.2.2 Expression and compartmentalization in developing muscle*). Accordingly, AChE expression is regulated differently depending on the subcellular location. Since we have identified HuR as an essential regulator of AChE transcript levels in myotubes, it is conceivable that HuR activity or expression is also controlled by the motor nerve. Thus, HuR would stabilize AChE mRNA at the junction and not in surrounding regions (see Figure 2). Expression of other synaptic protein mRNAs, such as α-dystrobrevin, has also been suggested to depend on distinct post-transcriptional mechanisms acting in different subcellular locations (Newey et al., 2001 and see for review Chakkalakal and Jasmin, 2003). Although previous studies have indicated that HuR is expressed in muscle (Figueroa et al., 2003;Tang et al., 2002), future research needs to address whether HuR is involved in synapse-specific AChE expression

and, particularly, whether HuR is itself expressed synaptically and controlled by the motor nerve.

In this context, recent studies have shown that muscle denervation results in diminished AChE mRNA levels and transcript stability, which is associated with increased binding of suspected destabilizing RBPs (Boudreau-Lariviere et al., 2000a;Grubic et al., 1999). Therefore, assuming HuR controls AChE mRNA in muscle, interaction with AChE mRNA following denervation may be blocked or possibly competed out by other destabilizing ARE RBP, such as AUF1 (see Table 2 and Figure 2 and see for review Barreau et al., 2005;Misquitta et al., 2006). In this regard, many ARE-binding RBPs have antagonistic functions with respect to mRNA metabolism, such as AUF1 that promotes decay and Hu family proteins that block decay. mRNA stability, therefore, is a consequence of functional antagonism between stabilizing and destabilizing proteins (see for review Barreau et al., 2005). For example, the antagonistic effects of HuR and AUF1 have been demonstrated using siRNA to individually decrease the expression of HuR or AUF1, resulting in increased association of the target transcript with the opposite protein and subsequent decreased or increased stability, respectively (Lal et al., 2004). Thus, it appears that competition for target transcripts and the eventual outcome for their longevity are largely determined by the relative abundance of the different RBP (Barreau et al., 2005;Lal et al., 2004). Accordingly, since we know that AChE mRNA is stabilized by HuR in culture, to better understand activity-dependent regulation of AChE transcript stability, future studies need to examine HuR expression and HuR-AChE mRNA interactions in muscle in response to denervation. In addition,

since increased muscle activity has the opposite effect, that of increasing AChE mRNA levels, the putative role of HuR in mediating this effect should also be investigated.

Since nerve-evoked activity is intimately linked to muscle fiber-type, HuR may also be implicated in the fiber-type specific pattern of AChE mRNA expression (see *1.2.3 Fiber-type specific expression*). A distinct role for post-transcriptional mechanisms in modulating fiber-type specific expression of synaptic proteins has recently been described for utrophin, a synaptic protein that has greater levels of expression in slow-contracting muscles, especially outside of the endplate (Gramolini et al., 2001;Chakkalakal and Jasmin, 2003). Notably, fast- and slow-contracting muscles have distinct intracellular calcium transients that are directly implicated in fiber-type specific gene expression (see for review Liu et al., 2005;Olson and Williams, 2000;Schiaffino and Serrano, 2002). Interestingly, HuR has been identified as a downstream effector of calcium signaling in other systems, including cardiac and smooth muscles (Pullmann, Jr. et al., 2005 and see for review Misquitta et al., 2006). Since, previous studies have suggested a specific role for intracellular calcium and its downstream effector, calcineurin, in post-transcriptional regulation of AChE mRNA levels (Luo et al., 1994;Luo et al., 1999), future research needs to examine whether calcium transients and calcineurin can function through HuR to regulate fiber-type specific AChE mRNA expression. In this context, both HuR and calcineurin are important activators of T-cells and the immune response (see for review Im and Rao, 2004;Seko et al., 2006;Stankunas et al., 1999). The role of calcium-dependent signaling in regulation of HuR activity is also significant to the effects of denervation and increased activity on AChE expression and should, therefore, also be examined in these contexts.

In addition to AChE, expression of several other muscle-specific proteins is regulated in a nerve-dependent fashion, including nAChR subunits. For example, following denervation, mRNAs to α-, γ-, and δ-subunits accumulate in the muscle in response to transiently increased gene transcription; suggesting that the sustained increase of these transcripts depends on additional factors, such as transcript stability (see for review Duclert and Changeux, 1995). Since, expression of myogenic factors MyoD and myogenin during myogenesis and regeneration are known to be regulated by HuR; it is plausible that HuR regulates expression levels of these other transcripts as well (Figueroa et al., 2003;Van Der Giessen et al., 2003). In this regard, AREs and Hu proteins appear to have defined roles in regulating transcript levels of proteins with similar functions (see for review Barreau et al., 2005;Deschenes-Furry et al., 2006). HuR, therefore, may regulate expression of synaptic proteins, which would afford the muscle a rapid and efficient approach to precisely regulate expression of this functionally-related group of transcripts. Accordingly, HuR may have a dual regulatory role in muscle, such that it controls expression of synaptic proteins directly through transcript stability and indirectly through regulation of the myogenic transcription factors.

5.3 Role of HuD in regulating AChE expression in neurons

In the present studies, we have established that HuD controls AChE expression during neuronal differentiation and can bind and modulate AChE mRNA expression in the hippocampus of HuD transgenic mice or the rat SCG. Comparison of AChE and HuD expression pattern during development of the nervous system, reveals that both HuD and AChE are expressed in neurons very early following differentiation.

Specifically, AChE is typically expressed before synapse formation and expression of the other cholinergic markers (see *1.3.1 Expression during neuronal differentiation and CNS development*); and HuD expression can be localized to cells exiting the cell cycle, and to those migrating and undergoing terminal differentiation (Wakamatsu and Weston, 1997;Okano and Darnell, 1997;Clayton et al., 1998).

HuD and *AChE* gene expression also appear to co-localize in specific regions of the adult brain. For example, some regions that have relatively high levels of AChE mRNA, such as individual layers of the cortex and cerebellum, the pontine, vestibular and habenular nuclei, and the hippocampus, also express considerable amounts of HuD mRNA (Bernard et al., 1995;Okano and Darnell, 1997). In addition, HuD knockout mice and AChE knockout animals, mice and Zebrafish, show similar developmental defects, such as impaired extension of cranial nerves and motor-sensory defects (Xie et al., 2000;Behra et al., 2002;Akamatsu et al., 2005). Accordingly, HuD appears to be an essential regulator of AChE expression throughout neuronal development and in the adult nervous system.

The marked decrease in AChE protein and mRNA levels in response to axotomy of either motoneurons, sensory or sympathetic neurons has previously been described (see *1.3.3 Expression following neuronal decentralization and axotomy*); however, the molecular mechanisms regulating these effects have never been addressed. In our last study (Chapter 4), we demonstrated that axotomy of the rat SCG results in a substantial decrease in AChE mRNA levels and, specifically, that diminished transcript stability could account for this outcome. This response correlated with decreased levels of expression of HuD and decreased binding to the 3'-UTR. We have, therefore,

demonstrated that post-transcriptional mechanisms, as mediated by HuD, are implicated in target-derived regulation of AChE expression (see Figure 2).

Neuronal plasticity and survival are greatly influenced by target-derived neurotrophic factors. In particular, sympathetic neurons depend on NGF from target tissues for survival as demonstrated by the loss of sympathetic neurons in NGF or tyrosine kinase A (trkA, the NGF receptor) knockout animals (Crowley et al., 1994;Fagan et al., 1996). NGF and TrkA also affect AChE expression in SCG neurons, inasmuch as AChE activity levels are diminished in SCG neurons of TrkA deficient mice and in SCG neurons of mice that have been treated with antibodies directed to NGF (Schober et al., 1997;Klingman and Klingman, 1969). Other neurotrophins, specifically BDNF and NT4/5, were shown to prevent the decrease in AChE transcript and activity levels that result from FMN axotomy (Fernandes et al., 1998). Similarly, in our study, exogenous expression of human HuD in the SCG neurons prior to axotomy inhibited the reduction in AChE mRNA levels. These previous individual observations, combined with our observations during neuronal differentiation and following SCG axotomy, indicate that there is a likely connection between target-derived trophic factors, Hu proteins and *AChE* gene expression. Future research, therefore, is required to address whether neurotrophins, such as BDNF, NT 4/5 and NGF, function through HuD to stabilize AChE transcripts in neurons, and whether neurotrophins modulate HuD levels and activity. In this regard, cell culture studies of neuronal differentiation have demonstrated links between the RNA-binding activity of HuD and NGF-induced intracellular signaling (Mobarak et al., 2000;Pascale et al., 2005).

AChE, in addition to its cholinergic functions, has been demonstrated to stimulate neurite elongation independently of its enzymatic activity (see *1.6.1 Neurite outgrowth*). A role for this enzyme in recovery and regeneration following axonal injury is suggested by the defect in sensory neuron outgrowth observed in AChE mutant Zebrafish (Behra et al., 2002) and increased AChE expression in glial cells during regeneration of injured SCG (Gisiger et al., 1978). HuD, which has a significant role in morphological differentiation of cultured neurons (see for review Deschenes-Furry et al., 2006), has also been suggested to have a role in axonal regeneration. Recent findings have shown that following nerve crush of DRG sensory neurons and facial motoneurons, HuD protein and transcript levels increased 7 days after injury and remained elevated for up to 21 days (Anderson et al., 2003;Anderson and Steward, 2003). This increase in HuD expression was accompanied by a dramatic increase in the mRNA levels of GAP-43, a known target of HuD. In this context, we found that exogenous expression of human HuD in the SCG resulted in maintenance of AChE and GAP-43 mRNA levels following axotomy. Accordingly, future research is required to examine whether there is functional recovery of the SCG, or other neuronal systems, when HuD is overexpressed. In addition, the role of neurotrophins in facilitating recovery should be examined in the context of modulating HuD function. It is particularly important to address these questions given the substantial influence neurotrophins have on nerve damage and recovery (see for review Bregman et al., 2002;Hendriks et al., 2004;Mendell et al., 2001). These additional studies will also further our understanding of the molecular mechanisms involved in the beneficial effects of neurotrophins following injury.

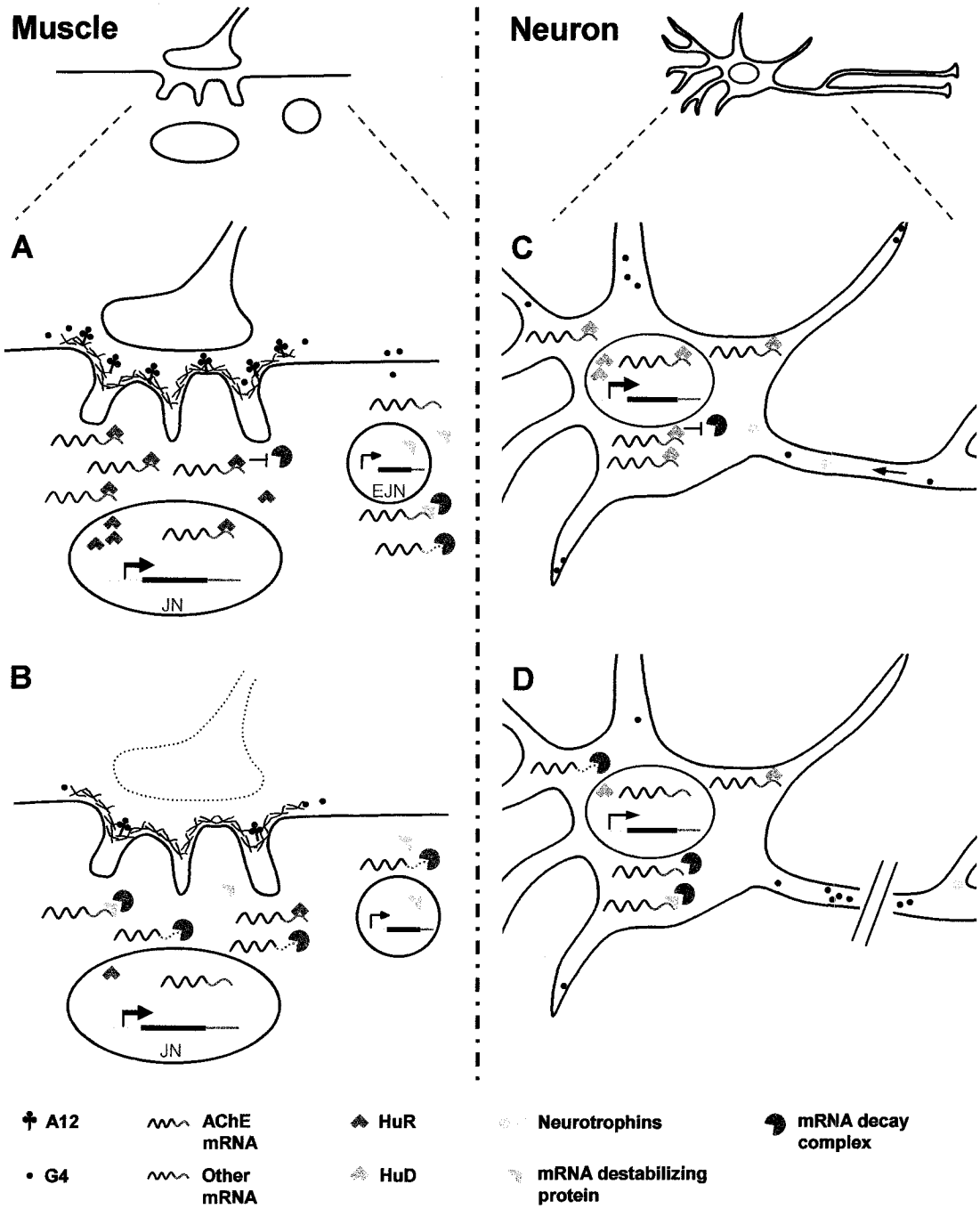

Figure 2. Putative role of Hu family RBPs in muscle and neurons. *A)* Accumulation of AChE transcripts at the NMJ may result from specific stabilization by HuR of mRNAS transcribed in junction nuclei (JN). AChE transcribed by extrajunctional nuclei (EJN) may be targeted by destabilizing proteins. *B)* Denervation may result in decreased HuR binding and increased interactions with destabilizing proteins. *C)* In neurons, AChE mRNA may be stabilized by HuD in response to signaling from neurotrophins. *D)* Following axotomy, loss of target-derived neurotrophins may result in decreased HuD expression and stability of AChE mRNA, and other transcripts.

5.4 Conclusion

AChE is an essential component of cholinergic synapses, whose absence or altered expression is associated with developmental dysfunctions, neuromuscular disorders and several disease states. This enzyme also has various non-cholinergic functions, which are important to development and plasticity of neurons and muscle, as well as of numerous tissues that are not cholinergic or excitable. Given the multifaceted nature of this protein, expression of AChE transcripts and multiple molecular forms is highly regulated in a temporal and tissue-specific manner, and has been the subject of numerous studies. Accordingly, we have demonstrated that in excitable cells AChE expression is controlled by the mRNA-stabilizing proteins HuR and HuD. Importantly, identification of the Hu family of RBPs as essential regulators of AChE expression has culminated in greater insight into the molecular mechanisms that control AChE expression during development, plasticity and disease of excitable and non-excitable tissues (see Figure 2). In addition, the results of our studies have furthered our understanding of the role of Hu proteins in regulating expression of functionally related proteins, and their implication in activity-dependent expression of synaptic proteins and axonal regeneration.

Reference List

1. Adler M, Manley HA, Purcell AL, Deshpande SS, Hamilton TA, Kan RK, Oyler G, Lockridge O, Duysen EG, Sheridan RE (2004) Reduced acetylcholine receptor density, morphological remodeling, and butyrylcholinesterase activity can sustain muscle function in acetylcholinesterase knockout mice. Muscle Nerve 30: 317-327.

2. Akamatsu W, Fujihara H, Mitsuhashi T, Yano M, Shibata S, Hayakawa Y, Okano HJ, Sakakibara S, Takano H, Takano T, Takahashi T, Noda T, Okano H (2005) The RNA-binding protein HuD regulates neuronal cell identity and maturation. Proc Natl Acad Sci U S A 102: 4625-4630.

3. Akamatsu W, Okano HJ, Osumi N, Inoue T, Nakamura S, Sakakibara S, Miura M, Matsuo N, Darnell RB, Okano H (1999) Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems. Proc Natl Acad Sci U S A 96: 9885-9890.

4. Aldunate R, Casar JC, Brandan E, Inestrosa NC (2004) Structural and functional organization of synaptic acetylcholinesterase. Brain Res Brain Res Rev 47: 96-104.

5. Alvarez A, Alarcon R, Opazo C, Campos EO, Munoz FJ, Calderon FH, Dajas F, Gentry MK, Doctor BP, De Mello FG, Inestrosa NC (1998) Stable complexes involving acetylcholinesterase and amyloid-beta peptide change the biochemical properties of the enzyme and increase the neurotoxicity of Alzheimer's fibrils. J Neurosci 18: 3213-3223.

6. Alvarez A, Bronfman F, Perez CA, Vicente M, Garrido J, Inestrosa NC (1995) Acetylcholinesterase, a senile plaque component, affects the fibrillogenesis of amyloid-beta-peptides. Neurosci Lett 201: 49-52.

7. Alvarez A, Opazo C, Alarcon R, Garrido J, Inestrosa NC (1997) Acetylcholinesterase promotes the aggregation of amyloid-beta-peptide fragments by forming a complex with the growing fibrils. J Mol Biol 272: 348-361.

8. Anderson KD, Merhege MA, Morin M, Bolognani F, Perrone-Bizzozero NI (2003) Increased expression and localization of the RNA-binding protein HuD and GAP-43 mRNA to cytoplasmic granules in DRG neurons during nerve regeneration. Exp Neurol 183: 100-108.

9. Anderson KD and Steward O (2003) *In vivo* Expression of the Neuronal-specific RNA-binding Protein HuD in Reference to a Potential Target mRNA (GAP-43) in the Mouse Facial Nucleus During Regeneration. Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience Program No. 78.17.

10. Andres C, Beeri R, Friedman A, Lev-Lehman E, Henis S, Timberg R, Shani M, Soreq H (1997) Acetylcholinesterase-transgenic mice display embryonic modulations in spinal cord choline acetyltransferase and neurexin Ibeta gene expression followed by late-onset neuromotor deterioration. Proc Natl Acad Sci U S A 94: 8173-8178.

11. Andres C, Seidman S, Beeri R, Timberg R, Soreq H (1998) Transgenic acetylcholinesterase induces enlargement of murine neuromuscular junctions but leaves spinal cord synapses intact. Neurochem Int 32: 449-456.

12. Anglister L (1991) Acetylcholinesterase from the motor nerve terminal accumulates on the synaptic basal lamina of the myofiber. J Cell Biol 115: 755-764.

13. Angus LM, Chan RY, Jasmin BJ (2001) Role of intronic E- and N-box motifs in the transcriptional induction of the acetylcholinesterase gene during myogenic differentiation. J Biol Chem 276: 17603-17609.

14. Antar LN, Bassell GJ (2003) Sunrise at the synapse: the FMRP mRNP shaping the synaptic interface. Neuron 37: 555-558.

15. Antic D, Keene JD (1997) Embryonic lethal abnormal visual RNA-binding proteins involved in growth, differentiation, and posttranscriptional gene expression. Am J Hum Genet 61: 273-278.

16. Apel ED, Merlie JP (1995) Assembly of the postsynaptic apparatus. Curr Opin Neurobiol 5: 62-67.

17. Appleyard ME (1992) Secreted acetylcholinesterase: non-classical aspects of a classical enzyme. Trends Neurosci 15: 485-490.

18. Arikawa-Hirasawa E, Rossi SG, Rotundo RL, Yamada Y (2002) Absence of acetylcholinesterase at the neuromuscular junctions of perlecan-null mice. Nat Neurosci 5: 119-123.

19. Atack JR, Perry EK, Bonham JR, Perry RH (1987) Molecular forms of acetylcholinesterase and butyrylcholinesterase in human plasma and cerebrospinal fluid. J Neurochem 48: 1845-1850.

20. Atanasova E, Chiappa S, Wieben E, Brimijoin S (1999) Novel messenger RNA and alternative promoter for murine acetylcholinesterase. J Biol Chem 274: 21078-21084.

21. Auld VJ, Fetter RD, Broadie K, Goodman CS (1995) Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in Drosophila. Cell 81: 757-767.

22. Aziz-Aloya R, Seidman S, Timberg R, Sternfeld M, Zakut H, Soreq H (1993) Expression of a human acetylcholinesterase promoter-reporter construct in developing neuromuscular junctions of Xenopus embryos. Proc Natl Acad Sci U S A 90: 2471-2475.

23. Bacou F, Vigneron P, Massoulie J (1982) Acetylcholinesterase forms in fast and slow rabbit muscle. Nature 296: 661-664.

24. Bakheet T, Williams BR, Khabar KS (2003) ARED 2.0: an update of AU-rich element mRNA database. Nucleic Acids Res 31: 421-423.

25. Bakheet T, Williams BR, Khabar KS (2006) ARED 3.0: the large and diverse AU-rich transcriptome. Nucleic Acids Res 34: D111-D114.

26. Barbosa M, Rios O, Velasquez M, Villalobos J, Ehrmanns J (2001) Acetylcholinesterase and butyrylcholinesterase histochemical activities and tumor cell growth in several brain tumors. Surg Neurol 55: 106-112.

27. Bardoni B, Mandel JL (2002) Advances in understanding of fragile X pathogenesis and FMRP function, and in identification of X linked mental retardation genes. Curr Opin Genet Dev 12: 284-293.

28. Barjot C, Jbilo O, Chatonnet A, Bacou F (1993) Expression of acetylcholinesterase gene during in vitro differentiation of rabbit muscle satellite cells. Neuromuscul Disord 3: 443-446.

29. Barmack NH, Baughman RW, Eckenstein FP (1992) Cholinergic innervation of the cerebellum of rat, rabbit, cat, and monkey as revealed by choline acetyltransferase activity and immunohistochemistry. J Comp Neurol 317: 233-249.

30. Barreau C, Paillard L, Osborne HB (2005) AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res 33: 7138-7150.

31. Bassell GJ, Oleynikov Y, Singer RH (1999) The travels of mRNAs through all cells large and small. FASEB J 13: 447-454.

32. Bazelyansky M, Robey E, Kirsch JF (1986) Fractional diffusion-limited component of reactions catalyzed by acetylcholinesterase. Biochemistry 25: 125-130.

33. Beelman CA, Parker R (1995) Degradation of mRNA in eukaryotes. Cell 81: 179-183.

34. Behra M, Cousin X, Bertrand C, Vonesch JL, Biellmann D, Chatonnet A, Strahle U (2002) Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. Nat Neurosci 5: 111-118.

35. Behrends U, Jandl T, Golbeck A, Lechner B, Muller-Weihrich S, Schmid I, Till H, Berthold F, Voltz R, Mautner JM (2002) Novel products of the HUD, HUC, NNP-1 and alpha-internexin genes identified by autologous antibody screening of a pediatric neuroblastoma library. Int J Cancer 100: 669-677.

36. Belliveau DJ, Krivko I, Kohn J, Lachance C, Pozniak C, Rusakov D, Kaplan D, Miller FD (1997) NGF and neurotrophin-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. J Cell Biol 136: 375-388.

37. Bernard V, Legay C, Massoulie J, Bloch B (1995) Anatomical analysis of the neurons expressing the acetylcholinesterase gene in the rat brain, with special reference to the striatum. Neuroscience 64: 995-1005.

38. Bernstein P, Ross J (1989) Poly(A), poly(A) binding protein and the regulation of mRNA stability. Trends Biochem Sci 14: 373-377.

39. Bertrand C, Chatonnet A, Takke C, Yan YL, Postlethwait J, Toutant JP, Cousin X (2001) Zebrafish acetylcholinesterase is encoded by a single gene localized on linkage group 7. Gene structure and polymorphism; molecular forms and expression pattern during development. J Biol Chem 276: 464-474.

40. Biagioni S, Odorisio T, Poiana G, Scarsella G, Augusti-Tocco G (1989) Acetylcholinesterase in the development of chick dorsal root ganglia. Int J Dev Neurosci 7: 267-273.

41. Biagioni S, Tata AM, De Jaco A, Augusti-Tocco G (2000) Acetylcholine synthesis and neuron differentiation. Int J Dev Biol 44: 689-697.

42. Bigbee JW, Sharma KV, Chan EL, Bogler O (2000) Evidence for the direct role of acetylcholinesterase in neurite outgrowth in primary dorsal root ganglion neurons. Brain Res 861: 354-362.

43. Birney E, Kumar S, Krainer AR (1993) Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. Nucleic Acids Res 21: 5803-5816.

44. Blasina MF, Faria AC, Gardino PF, Hokoc JN, Almeida OM, De Mello FG, Arruti C, Dajas F (2000) Evidence for a noncholinergic function of acetylcholinesterase during development of chicken retina as shown by fasciculin. Cell Tissue Res 299: 173-184.

45. Bon S, Coussen F, Massoulie J (1997) Quaternary associations of acetylcholinesterase. II. The polyproline attachment domain of the collagen tail. J Biol Chem 272: 3016-3021.

46. Bon S, Huet M, Lemonnier M, Rieger F, Massoulie J (1976) Molecular forms of Electrophorus acetylcholinesterase. Molecular weight and composition. Eur J Biochem 68: 523-530.

47. Bon S, Massoulie J (1997) Quaternary associations of acetylcholinesterase. I. Oligomeric associations of T subunits with and without the amino-terminal domain of the collagen tail. J Biol Chem 272: 3007-3015.

48. Bon S, Meflah K, Musset F, Grassi J, Massoulie J (1987) An immunoglobulin M monoclonal antibody, recognizing a subset of acetylcholinesterase molecules from electric organs of Electrophorus and Torpedo, belongs to the HNK-1 anti-carbohydrate family. J Neurochem 49: 1720-1731.

49. Bon S, Vigny M, Massoulie J (1979) Asymmetric and globular forms of acetylcholinesterase in mammals and birds. Proc Natl Acad Sci U S A 76: 2546-2550.

50. Botti SA, Felder CE, Sussman JL, Silman I (1998) Electrotactins: a class of adhesion proteins with conserved electrostatic and structural motifs. Protein Eng 11: 415-420.

51. Boudreau-Lariviere C, Chan RY, Wu J, Jasmin BJ (2000a) Molecular mechanisms underlying the activity-linked alterations in acetylcholinesterase mRNAs in developing versus adult rat skeletal muscles. J Neurochem 74: 2250-2258.

52. Boudreau-Lariviere C, Gisiger V, Michel RN, Hubatsch DA, Jasmin BJ (1997) Fast and slow skeletal muscles express a common basic profile of acetylcholinesterase molecular forms. Am J Physiol 272: C68-C76.

53. Boudreau-Lariviere C, Jasmin BJ (1999) Calcitonin gene-related peptide decreases expression of acetylcholinesterase in mammalian myotubes. FEBS Lett 444: 22-26.

54. Boudreau-Lariviere C, Parry DJ, Jasmin BJ (2000b) Myotubes originating from single fast and slow satellite cells display similar patterns of AChE expression. Am J Physiol Regul Integr Comp Physiol 278: R140-R148.

55. Bourne Y, Taylor P, Marchot P (1995) Acetylcholinesterase inhibition by fasciculin: crystal structure of the complex. Cell 83: 503-512.

56. Bregman BS, Coumans JV, Dai HN, Kuhn PL, Lynskey J, McAtee M, Sandhu F (2002) Transplants and neurotrophic factors increase regeneration and recovery of function after spinal cord injury. Prog Brain Res 137: 257-273.

57. Brennan CM, Steitz JA (2001) HuR and mRNA stabiity. Cell Mol Life Sci 58: 266-277.

58. Brimijoin S (1983) Molecular forms of acetylcholinesterase in brain, nerve and muscle: nature, localization and dynamics. Prog Neurobiol 21: 291-322.

59. Brimijoin S, Hammond P (1996) Transient expression of acetylcholinesterase messenger RNA and enzyme activity in developing rat thalamus studied by quantitative histochemistry and in situ hybridization. Neuroscience 71: 555-565.

60. Brimijoin S, Skau K, Wiermaa MJ (1978) On the origin and fate of external acetylcholinesterase in peripheral nerve. J Physiol 285: 143-158.

61. Brimijoin S, Wiermaa MJ (1978) Rapid orthograde and retrograde axonal transport of acetylcholinesterase as characterized by the stop-flow technique. J Physiol 285: 129-142.

62. Brockman SK, Usiak MF, Younkin SG (1986) Assembly of monomeric acetylcholinesterase into tetrameric and asymmetric forms. J Biol Chem 261: 1201-1207.

63. Brockman SK, Younkin LH, Younkin SG (1984) The effect of spontaneous electromechanical activity on the metabolism of acetylcholinesterase in cultured embryonic rat myotubes. J Neurosci 4: 131-140.

64. Brzin M, Sketelj J, Tennyson VM, Kiauta T, Budininkas-Schoenebeck M (1981) Activity, molecular forms, and cytochemistry of cholinesterases in developing rat diaphragm. Muscle Nerve 4: 505-513.

65. Burd CG, Dreyfuss G (1994) Conserved structures and diversity of functions of RNA-binding proteins. Science 265: 615-621.

66. Burstein SA, Adamson JW, Harker LA (1980) Megakaryocytopoiesis in culture: modulation by cholinergic mechanisms. J Cell Physiol 103: 201-208.

67. Burstein SA, Boyd CN, Dale GL (1985) Quantitation of megakaryocytopoiesis in liquid culture by enzymatic determination of acetylcholinesterase. J Cell Physiol 122: 159-165.

68. Burstein SA, Harker LA (1983) Control of platelet production. Clin Haematol 12: 3-22.

69. Camp S, Bon S, Li Y, Getman DK, Engel AG, Massoulie J, Taylor P (1995) Patients with congenital myasthenia associated with end-plate acetylcholinesterase deficiency show normal sequence, mRNA splicing, and assembly of catalytic subunits. J Clin Invest 95: 333-340.

70. Camp S, Zhang L, Marquez M, de la TB, Long JM, Bucht G, Taylor P (2005) Acetylcholinesterase (AChE) gene modification in transgenic animals: functional consequences of selected exon and regulatory region deletion. Chem Biol Interact 157-158: 79-86.

71. Campos EO, Alvarez A, Inestrosa NC (1998) Brain acetylcholinesterase promotes amyloid-beta-peptide aggregation but does not hydrolyze amyloid precursor protein peptides. Neurochem Res 23: 135-140.

72. Cartaud A, Strochlic L, Guerra M, Blanchard B, Lambergeon M, Krejci E, Cartaud J, Legay C (2004) MuSK is required for anchoring acetylcholinesterase at the neuromuscular junction. J Cell Biol 165: 505-515.

73. Cartaud J, Rieger F, Bon S, Massoulie J (1975) Fine structure of electric ell acetylcholinesterase. Brain Res 88: 127-130.

74. Chakkalakal JV, Jasmin BJ (2003) Localizing synaptic mRNAs at the neuromuscular junction: it takes more than transcription. Bioessays 25: 25-31.

75. Champelovier P, Pautre V, Elatifi M, Dupre I, Rostaing B, Michoud A, Berger F, Seigneurin D (2006) Resistance to phorbol ester-induced differentiation in human myeloid leukemia cells: A hypothetic role for the mRNA stabilization process. Leuk Res.

76. Chan RY, Adatia FA, Krupa AM, Jasmin BJ (1998) Increased expression of acetylcholinesterase T and R transcripts during hematopoietic differentiation is accompanied by parallel elevations in the levels of their respective molecular forms. J Biol Chem 273: 9727-9733.

77. Chan RY, Boudreau-Lariviere C, Angus LM, Mankal FA, Jasmin BJ (1999) An intronic enhancer containing an N-box motif is required for synapse- and tissue-specific expression of the acetylcholinesterase gene in skeletal muscle fibers. Proc Natl Acad Sci U S A 96: 4627-4632.

78. Chan RY, Jasmin BJ (1999) What does acetylcholinesterase do in hematopoietic cells? Gene Ther Mol Biol 3: 347-354.

79. Chang PL (1977) Effect of preganglionic sympathectomy on the cholinesterase activity in the superior cervical ganglia of rats and hamsters. Cell Tissue Res 179: 111-120.

80. Chapron J, De La PS, Delepine L, Koenig J (1997) Schwann cells modify expression of acetylcholinesterase and butyrylcholinesterase at rat neuromuscular junctions. Eur J Neurosci 9: 260-270.

81. Chatel JM, Grassi J, Frobert Y, Massoulie J, Vallette FM (1993) Existence of an inactive pool of acetylcholinesterase in chicken brain. Proc Natl Acad Sci U S A 90: 2476-2480.

82. Chatonnet A, Lockridge O (1989) Comparison of butyrylcholinesterase and acetylcholinesterase. Biochem J 260: 625-634.

83. Chen CY, Shyu AB (1995) AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci 20: 465-470.

84. Chen CY, Xu N, Shyu AB (1995) mRNA decay mediated by two distinct AU-rich elements from c-fos and granulocyte-macrophage colony-stimulating factor transcripts: different deadenylation kinetics and uncoupling from translation. Mol Cell Biol 15: 5777-5788.

85. Cho NH, Kang S, Hong S, An HJ, Choi YH, Jeong GB, Choi HK (2006) Elevation of cyclin B1, active cdc2, and HuR in cervical neoplasia with human papillomavirus type 18 infection. Cancer Lett 232: 170-178.

86. Choi RC, Leung PW, Dong TT, Wan DC, Tsim KW (1996) Calcitonin gene-related peptide increases the expression of acetylcholinesterase in cultured chick myotubes. Neurosci Lett 217: 165-168.

87. Choi RC, Man ML, Ling KK, Ip NY, Simon J, Barnard EA, Tsim KW (2001a) Expression of the P2Y1 nucleotide receptor in chick muscle: its functional role in the regulation of acetylcholinesterase and acetylcholine receptor. J Neurosci 21: 9224-9234.

88. Choi RC, Siow NL, Cheng AW, Ling KK, Tung EK, Simon J, Barnard EA, Tsim KW (2003) ATP acts via P2Y1 receptors to stimulate acetylcholinesterase and acetylcholine receptor expression: transduction and transcription control. J Neurosci 23: 4445-4456.

89. Choi RC, Siow NL, Zhu SQ, Wan DC, Wong YH, Tsim KW (2001b) The cyclic AMP-mediated expression of acetylcholinesterase in myotubes shows contrasting activation and repression between avian and mammalian enzymes. Mol Cell Neurosci 17: 732-745.

90. Choi RC, Yung LY, Dong TT, Wan DC, Wong YH, Tsim KW (1998) The calcitonin gene-related peptide-induced acetylcholinesterase synthesis in cultured chick myotubes is mediated by cyclic AMP. J Neurochem 71: 152-160.

91. Chubb IW, Smith AD (1975a) Isoenzymes of soluble and membrane-bound acetylcholinesterase in boine splanchnic nerve and adrenal medulla. Proc R Soc Lond B Biol Sci 191: 245-261.

92. Chubb IW, Smith AD (1975b) Release of acetylcholinesterase into the perfusate from the ox adrenal gland. Proc R Soc Lond B Biol Sci 191: 263-269.

93. Chung S, Jiang L, Cheng S, Furneaux H (1996) Purification and properties of HuD, a neuronal RNA-binding protein. J Biol Chem 271: 11518-11524.

94. Clayton GH, Perez GM, Smith RL, Owens GC (1998) Expression of mRNA for the elav-like neural-specific RNA binding protein, HuD, during nervous system development. Brain Res Dev Brain Res 109: 271-280.

95. Cohen TV, Randall WR (2006) The regulation of acetylcholinesterase by cis-elements within intron I in cultured contracting myotubes. J Neurochem.

96. Coleman BA, Taylor P (1996) Regulation of acetylcholinesterase expression during neuronal differentiation. J Biol Chem 271: 4410-4416.

97. Combes D, Fedon Y, Grauso M, Toutant JP, Arpagaus M (2000) Four genes encode acetylcholinesterases in the nematodes Caenorhabditis elegans and Caenorhabditis briggsae. cDNA sequences, genomic structures, mutations and in vivo expression. J Mol Biol 300: 727-742.

98. Combes D, Fedon Y, Toutant JP, Arpagaus M (2003) Multiple ace genes encoding acetylcholinesterases of Caenorhabditis elegans have distinct tissue expression. Eur J Neurosci 18: 497-512.

99. Couraud JY, Di Giamberardino L (1980) Axonal transport of the molecular forms of acetylcholinesterase in chick sciatic nerve. J Neurochem 35: 1053-1066.

100. Cousin X, Strahle U, Chatonnet A (2005) Are there non-catalytic functions of acetylcholinesterases? Lessons from mutant animal models. Bioessays 27: 189-200.

101. Coutts M, Brawerman G (1993) A 5' exoribonuclease from cytoplasmic extracts of mouse sarcoma 180 ascites cells. Biochim Biophys Acta 1173: 57-62.

102. Cresnar B, Crne-Finderle N, Breskvar K, Sketelj J (1994) Neural regulation of muscle acetylcholinesterase is exerted on the level of its mRNA. J Neurosci Res 38: 294-299.

103. Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD, . (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76: 1001-1011.

104. Dale HH (1914) The action of certain esters of choline and their relation to muscarine. J Pharmac exp Ther 6: 147-190.

105. Dalmau J, Furneaux HM, Gralla RJ, Kris MG, Posner JB (1990) Detection of the anti-Hu antibody in the serum of patients with small cell lung cancer--a quantitative western blot analysis. Ann Neurol 27: 544-552.

106. Darboux I, Barthalay Y, Piovant M, Hipeau-Jacquotte R (1996) The structure-function relationships in Drosophila neurotactin show that cholinesterasic domains may have adhesive properties. EMBO J 15: 4835-4843.

107. Darvesh S, Hopkins DA (2003) Differential distribution of butyrylcholinesterase and acetylcholinesterase in the human thalamus. J Comp Neurol 463: 25-43.

108. Darvesh S, Hopkins DA, Geula C (2003) Neurobiology of butyrylcholinesterase. Nat Rev Neurosci 4: 131-138.

109. De Jaco A, Augusti-Tocco G, Biagioni S (2002) Alternative acetylcholinesterase molecular forms exhibit similar ability to induce neurite outgrowth. J Neurosci Res 70: 756-765.

110. de Kerchove d'Exaerde A, Cartaud J, Ravel-Chapuis A, Seroz T, Pasteau F, Angus LM, Jasmin BJ, Changeux JP, Schaeffer L (2002) Expression of mutant Ets protein at the neuromuscular synapse causes alterations in morphology and gene expression. EMBO Rep 3: 1075-1081.

111. De La Escalera S., Bockamp EO, Moya F, Piovant M, Jimenez F (1990) Characterization and gene cloning of neurotactin, a Drosophila transmembrane protein related to cholinesterases. EMBO J 9: 3593-3601.

112. De La Porte S, Vigny M, Massoulie J, Koenig J (1984) Action of veratridine on acetylcholinesterase in cultures of rat muscle cells. Dev Biol 106: 450-456.

113. De Repentigny Y, Deschenes-Furry J, Jasmin BJ, Kothary R (2003) Impaired fast axonal transport in neurons of the sciatic nerves from dystonia musculorum mice. J Neurochem 86: 564-571.

114. Dean C, Scholl FG, Choih J, DeMaria S, Berger J, Isacoff E, Scheiffele P (2003) Neurexin mediates the assembly of presynaptic terminals. Nat Neurosci 6: 708-716.

115. Decker CJ, Parker R (1994) Mechanisms of mRNA degradation in eukaryotes. Trends Biochem Sci 19: 336-340.

116. Denkert C, Koch I, von Keyserlingk N, Noske A, Niesporek S, Dietel M, Weichert W (2006) Expression of the ELAV-like protein HuR in human colon cancer: association with tumor stage and cyclooxygenase-2. Mod Pathol.

117. Denkert C, Weichert W, Pest S, Koch I, Licht D, Kobel M, Reles A, Sehouli J, Dietel M, Hauptmann S (2004a) Overexpression of the embryonic-lethal abnormal vision-like protein HuR in ovarian carcinoma is a prognostic factor and is associated with increased cyclooxygenase 2 expression. Cancer Res 64: 189-195.

118. Denkert C, Weichert W, Winzer KJ, Muller BM, Noske A, Niesporek S, Kristiansen G, Guski H, Dietel M, Hauptmann S (2004b) Expression of the ELAV-like protein HuR is associated with higher tumor grade and increased cyclooxygenase-2 expression in human breast carcinoma. Clin Cancer Res 10: 5580-5586.

119. Deschenes-Furry J, Perrone-Bizzozero N, Jasmin BJ (2006) The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity. Bioessays 28: 822-833.

120. Di Giamberardino L, Couraud JY (1978) Rapid accumulation of high molecular weight acetylcholinesterase in transected sciatic nerve. Nature 271: 170-172.

121. Doctor BP, Chapman TC, Christner CE, Deal CD, De La Hoz DM, Gentry MK, Ogert RA, Rush RS, Smyth KK, Wolfe AD (1990) Complete amino acid sequence of fetal bovine serum acetylcholinesterase and its comparison in various regions with other cholinesterases. FEBS Lett 266: 123-127.

122. Dong H, Xiang YY, Farchi N, Ju W, Wu Y, Chen L, Wang Y, Hochner B, Yang B, Soreq H, Lu WY (2004) Excessive expression of acetylcholinesterase impairs glutamatergic synaptogenesis in hippocampal neurons. J Neurosci 24: 8950-8960.

123. Donger C, Krejci E, Serradell AP, Eymard B, Bon S, Nicole S, Chateau D, Gary F, Fardeau M, Massoulie J, Guicheney P (1998) Mutation in the human acetylcholinesterase-associated collagen gene, COLQ, is responsible for congenital myasthenic syndrome with end-plate acetylcholinesterase deficiency (Type Ic). Am J Hum Genet 63: 967-975.

124. Donoso JA, Fernandez HL (1985) Cellular localization of cytochemically stained acetylcholinesterase activity in adult rat skeletal muscle. J Neurocytol 14: 795-808.

125. Drews U (1975) Cholinesterase in embryonic development. Prog Histochem Cytochem 7: 1-52.

126. Dreyfuss G, Matunis MJ, Pinol-Roma S, Burd CG (1993) hnRNP proteins and the biogenesis of mRNA. Annu Rev Biochem 62: 289-321.

127. Drummond DR, Armstrong J, Colman A (1985) The effect of capping and polyadenylation on the stability, movement and translation of synthetic messenger RNAs in Xenopus oocytes. Nucleic Acids Res 13: 7375-7394.

128. Duclert A, Changeux JP (1995) Acetylcholine receptor gene expression at the developing neuromuscular junction. Physiol Rev 75: 339-368.

129. Duclert A, Savatier N, Schaeffer L, Changeux JP (1996) Identification of an element crucial for the sub-synaptic expression of the acetylcholine receptor epsilon-subunit gene. J Biol Chem 271: 17433-17438.

130. Dupree JL, Bigbee JW (1994) Retardation of neuritic outgrowth and cytoskeletal changes accompany acetylcholinesterase inhibitor treatment in cultured rat dorsal root ganglion neurons. J Neurosci Res 39: 567-575.

131. Dutta-Choudhury TA, Rosenberry TL (1984) Human erythrocyte acetylcholinesterase is an amphipathic protein whose short membrane-binding domain is removed by papain digestion. J Biol Chem 259: 5653-5660.

132. Duysen EG, Stribley JA, Fry DL, Hinrichs SH, Lockridge O (2002) Rescue of the acetylcholinesterase knockout mouse by feeding a liquid diet; phenotype of the adult acetylcholinesterase deficient mouse. Brain Res Dev Brain Res 137: 43-54.

133. Ehrlich G, Viegas-Pequignot E, Ginzberg D, Sindel L, Soreq H, Zakut H (1992) Mapping the human acetylcholinesterase gene to chromosome 7q22 by fluorescent in situ hybridization coupled with selective PCR amplification from a somatic hybrid cell panel and chromosome-sorted DNA libraries. Genomics 13: 1192-1197.

134. Eichler J, Silman I (1995) The activity of an endoplasmic reticulum-localized pool of acetylcholinesterase is modulated by heat shock. J Biol Chem 270: 4466-4472.

135. Ekstrom TJ, Klump WM, Getman D, Karin M, Taylor P (1993) Promoter elements and transcriptional regulation of the acetylcholinesterase gene. DNA Cell Biol 12: 63-72.

136. Engel AG, Lambert EH, Gomez MR (1977) A new myasthenic syndrome with end-plate acetylcholinesterase deficiency, small nerve terminals, and reduced acetylcholine release. Ann Neurol 1: 315-330.

137. Engel AG, Ohno K, Shen XM, Sine SM (2003a) Congenital myasthenic syndromes: multiple molecular targets at the neuromuscular junction. Ann N Y Acad Sci 998: 138-160.

138. Engel AG, Ohno K, Sine SM (1999) Congenital myasthenic syndromes: recent advances. Arch Neurol 56: 163-167.

139. Engel AG, Ohno K, Sine SM (2003b) Congenital myasthenic syndromes: progress over the past decade. Muscle Nerve 27: 4-25.

140. Engel AK, Kreutzberg GW (1986) Changes of acetylcholinesterase molecular forms in regenerating motor neurons. Neuroscience 18: 467-473.

141. Eranko L (1972) Biochemical and histochemical observations on the postnatal development of cholinesterases in the sympathetic ganglion of the rat. Histochem J 4: 545-559.

142. Erkinheimo TL, Lassus H, Sivula A, Sengupta S, Furneaux H, Hla T, Haglund C, Butzow R, Ristimaki A (2003) Cytoplasmic HuR expression correlates with poor outcome and with cyclooxygenase 2 expression in serous ovarian carcinoma. Cancer Res 63: 7591-7594.

143. Fagan AM, Zhang H, Landis S, Smeyne RJ, Silos-Santiago I, Barbacid M (1996) TrkA, but not TrkC, receptors are essential for survival of sympathetic neurons in vivo. J Neurosci 16: 6208-6218.

144. Fairman K, Giacobini E, Chiappinelli V (1976) Developmental variations of tyrosine hydroxylase and acetylcholinesterase in embryonic and post-hatching chicken sympathetic ganglia. Brain Res 102: 301-312.

145. Falugi C, Raineri M (1985) Acetylcholinesterase (AChE) and pseudocholinesterase (BuChE) activity distribution pattern in early developing chick limbs. J Embryol Exp Morphol 86: 89-108.

146. Fan J, Yang X, Wang W, Wood WH, III, Becker KG, Gorospe M (2002) Global analysis of stress-regulated mRNA turnover by using cDNA arrays. Proc Natl Acad Sci U S A 99: 10611-10616.

147. Farina KL, Singer RH (2002) The nuclear connection in RNA transport and localization. Trends Cell Biol 12: 466-472.

148. Farris TW, Woolf NJ, Oh JD, Butcher LL (1993) Reestablishment of laminar patterns of cortical acetylcholinesterase activity following axotomy of the medial cholinergic pathway in the adult rat. Exp Neurol 121: 77-92.

149. Federoff HJ, Geschwind MD, Geller AI, Kessler JA (1992) Expression of nerve growth factor in vivo from a defective herpes simplex virus 1 vector prevents effects of axotomy on sympathetic ganglia. Proc Natl Acad Sci U S A 89: 1636-1640.

150. Feng G, Krejci E, Molgo J, Cunningham JM, Massoulie J, Sanes JR (1999) Genetic analysis of collagen Q: roles in acetylcholinesterase and butyrylcholinesterase assembly and in synaptic structure and function. J Cell Biol 144: 1349-1360.

151. Fernandes KJ, Kobayashi NR, Jasmin BJ, Tetzlaff W (1998) Acetylcholinesterase gene expression in axotomized rat facial motoneurons is differentially regulated by neurotrophins: correlation with trkB and trkC mRNA levels and isoforms. J Neurosci 18: 9936-9947.

152. Fernandez HL, Donoso JA (1988) Exercise selectively increases G4 AChe activity in fast-twitch muscle. J Appl Physiol 65: 2245-2252.

153. Fernandez HL, Duell MJ, Festoff BW (1979) Neurotrophic control of 16S acetylcholinesterase at the vertebrate neuromuscular junction. J Neurobiol 10: 441-454.

154. Fernandez HL, Moreno RD, Inestrosa NC (1996) Tetrameric (G4) acetylcholinesterase: structure, localization, and physiological regulation. J Neurochem 66: 1335-1346.

155. Fernandez HL, Seiter TC (1984) Subcellular distribution of acetylcholinesterase asymmetric forms during postnatal development of mammalian skeletal muscle. FEBS Lett 170: 147-151.

156. Fernandez-Valle C, Rotundo RL (1989) Regulation of acetylcholinesterase synthesis and assembly by muscle activity. Effects of tetrodotoxin. J Biol Chem 264: 14043-14049.

157. Figueroa A, Cuadrado A, Fan J, Atasoy U, Muscat GE, Munoz-Canoves P, Gorospe M, Munoz A (2003) Role of HuR in skeletal myogenesis through coordinate regulation of muscle differentiation genes. Mol Cell Biol 23: 4991-5004.

158. Fitzpatrick-McElligott S, Stent GS (1981) Appearance and localization of acetylcholinesterase in embryos of the leech Helobdella triserialis. J Neurosci 1: 901-907.

159. Fuentes ME, Inestrosa NC (1988) Characterization of a tetrameric G4 form of acetylcholinesterase from bovine brain: a comparison with the dimeric G2 form of the electric organ. Mol Cell Biochem 81: 53-64.

160. Fuentes ME, Rosenberry TL, Inestrosa NC (1988) A 13 kDa fragment is responsible for the hydrophobic aggregation of brain G4 acetylcholinesterase. Biochem J 256: 1047-1050.

161. Fuentes ME, Taylor P (1993) Control of acetylcholinesterase gene expression during myogenesis. Neuron 10: 679-687.

162. Gaspersic R, Koritnik B, Crne-Finderle N, Sketelj J (1999) Acetylcholinesterase in the neuromuscular junction. Chem Biol Interact 119-120: 301-308.

163. Gennari K, Brunner J, Brodbeck U (1987) Tetrameric detergent-soluble acetylcholinesterase from human caudate nucleus: subunit composition and number of active sites. J Neurochem 49: 12-18.

164. Getman DK, Eubanks JH, Camp S, Evans GA, Taylor P (1992) The human gene encoding acetylcholinesterase is located on the long arm of chromosome 7. Am J Hum Genet 51: 170-177.

165. Getman DK, Mutero A, Inoue K, Taylor P (1995) Transcription factor repression and activation of the human acetylcholinesterase gene. J Biol Chem 270: 23511-23519.

166. Geula C, Mesulam MM (1989) Cortical cholinergic fibers in aging and Alzheimer's disease: a morphometric study. Neuroscience 33: 469-481.

167. Geula C, Mesulam MM, Kuo CC, Tokuno H (1995) Postnatal development of cortical acetylcholinesterase-rich neurons in the rat brain: permanent and transient patterns. Exp Neurol 134: 157-178.

168. Geula C, Mesulam MM, Tokuno H, Kuo CC (1993) Developmentally transient expression of acetylcholinesterase within cortical pyramidal neurons of the rat brain. Brain Res Dev Brain Res 76: 23-31.

169. Giacobini E (2003) Cholinesterases: new roles in brain function and in Alzheimer's disease. Neurochem Res 28: 515-522.

170. Gibney G, MacPhee-Quigley K, Thompson B, Vedvick T, Low MG, Taylor SS, Taylor P (1988) Divergence in primary structure between the molecular forms of acetylcholinesterase. J Biol Chem 263: 1140-1145.

171. Gilad GM, Gilad VH (1988) Early polyamine treatment enhances survival of sympathetic neurons after postnatal axonal injury or immunosympathectomy. Brain Res 466: 175-181.

172. Gisiger V, Sherker S, Gardiner PF (1991) Swimming training increases the G4 acetylcholinesterase content of both fast ankle extensors and flexors. FEBS Lett 278: 271-273.

173. Gisiger V, Stephens H (1982) Correlation between the acetylcholinesterase content in motor nerves and their muscles. J Physiol (Paris) 78: 720-728.

174. Gisiger V, Stephens HR (1983) Asymmetric and globular forms of AChE in slow and fast muscles of 129/ReJ normal and dystrophic mice. J Neurochem 41: 919-929.

175. Gisiger V, Vigny M, Gautron J, Rieger F (1978) Acetylcholinesterase of rat sympathetic ganglion: molecular forms, localization and effects of denervation. J Neurochem 30: 501-516.

176. Gomez JL, Nieto-Ceron S, Campoy FJ, Munoz-Delgado E, Vidal CJ (2003) Purification and properties of hydrophilic dimers of acetylcholinesterase from mouse erythrocytes. Int J Biochem Cell Biol 35: 1109-1118.

177. Gramolini AO, Angus LM, Schaeffer L, Burton EA, Tinsley JM, Davies KE, Changeux JP, Jasmin BJ (1999) Induction of utrophin gene expression by heregulin in skeletal muscle cells: role of the N-box motif and GA binding protein. Proc Natl Acad Sci U S A 96: 3223-3227.

178. Gramolini AO, Belanger G, Thompson JM, Chakkalakal JV, Jasmin BJ (2001) Increased expression of utrophin in a slow vs. a fast muscle involves posttranscriptional events. Am J Physiol Cell Physiol 281: C1300-C1309.

179. Greene LA, Rukenstein A (1981) Regulation of acetylcholinesterase activity by nerve growth factor. Role of transcription and dissociation from effects on proliferation and neurite outgrowth. J Biol Chem 256: 6363-6367.

180. Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A 73: 2424-2428.

181. Greenfield SA (1984) Acetylcholinesterase may have novel functions in the brain. Trends Neurosci 7: 364-368.

182. Greenfield SA (1991) A noncholinergic action of acetylcholinesterase (AChE) in the brain: from neuronal secretion to the generation of movement. Cell Mol Neurobiol 11: 55-77.

183. Greenspan RJ, Finn JA, Jr., Hall JC (1980) Acetylcholinesterase mutants in Drosophila and their effects on the structure and function of the central nervous system. J Comp Neurol 189: 741-774.

184. Grifman M, Galyam N, Seidman S, Soreq H (1998) Functional redundancy of acetylcholinesterase and neuroligin in mammalian neuritogenesis. Proc Natl Acad Sci U S A 95: 13935-13940.

185. Grisaru D, Deutsch V, Shapira M, Pick M, Sternfeld M, Melamed-Book N, Kaufer D, Galyam N, Gait MJ, Owen D, Lessing JB, Eldor A, Soreq H (2001) ARP, a peptide derived from the stress-associated acetylcholinesterase variant, has hematopoietic growth promoting activities. Mol Med 7: 93-105.

186. Grisaru D, Lev-Lehman E, Shapira M, Chaikin E, Lessing JB, Eldor A, Eckstein F, Soreq H (1999a) Human osteogenesis involves differentiation-dependent increases in the morphogenically active 3' alternative splicing variant of acetylcholinesterase. Mol Cell Biol 19: 788-795.

187. Grisaru D, Sternfeld M, Eldor A, Glick D, Soreq H (1999b) Structural roles of acetylcholinesterase variants in biology and pathology. Eur J Biochem 264: 672-686.

188. Gruber H, Zenker W (1973) Acetylcholinesterase: histochemical differentiation between motor and sensory nerve fibres. Brain Res 51: 207-214.

189. Grubic Z, Komel R, Walker WF, Miranda AF (1995) Myoblast fusion and innervation with rat motor nerve alter distribution of acetylcholinesterase and its mRNA in cultures of human muscle. Neuron 14: 317-327.

190. Grubic Z, Zajc-Kreft K, Brank M, Mars T, Komel R, Miranda AF (1999) Control levels of acetylcholinesterase expression in the mammalian skeletal muscle. Chem Biol Interact 119-120: 309-319.

191. Guhaniyogi J, Brewer G (2001) Regulation of mRNA stability in mammalian cells. Gene 265: 11-23.

192. Gultekin SH, Rosai J, Demopoulos A, Graus YF, Posner JB, Dalmau J, Rosenblum MK (2000) Hu Immunolabeling as a Marker of Neural and Neuroendocrine Differentiation in Normal and Neoplastic Human Tissues: Assessment Using a Recombinant Anti-Hu Fab Fragment. Int J Surg Pathol 8: 109-117.

193. Hall JC, Kankel DR (1976) Genetics of acetylcholinesterase in Drosophila melanogaster. Genetics 83: 517-535.

194. Hall LM, Spierer P (1986) The Ace locus of Drosophila melanogaster: structural gene for acetylcholinesterase with an unusual 5' leader. EMBO J 5: 2949-2954.

195. Hall ZW (1973) Multiple forms of acetylcholinesterase and their distribution in endplate and non-endplate regions of rat diaphragm muscle. J Neurobiol 4: 343-361.

196. Hall ZW, Sanes JR (1993) Synaptic structure and development: the neuromuscular junction. Cell 72 Suppl: 99-121.

197. Hammond P, Rao R, Koenigsberger C, Brimijoin S (1994) Regional variation in expression of acetylcholinesterase mRNA in adult rat brain analyzed by in situ hybridization. Proc Natl Acad Sci U S A 91: 10933-10937.

198. Heinonen M, Bono P, Narko K, Chang SH, Lundin J, Joensuu H, Furneaux H, Hla T, Haglund C, Ristimaki A (2005) Cytoplasmic HuR expression is a prognostic factor in invasive ductal breast carcinoma. Cancer Res 65: 2157-2161.

199. Hendriks WT, Ruitenberg MJ, Blits B, Boer GJ, Verhaagen J (2004) Viral vector-mediated gene transfer of neurotrophins to promote regeneration of the injured spinal cord. Prog Brain Res 146: 451-476.

200. Hendry IA (1975a) The effects of axotomy on the development of the rat superior cervical ganglion. Brain Res 90: 235-244.

201. Hendry IA (1975b) The response of adrenergic neurones to axotomy and nerve growth factor. Brain Res 94: 87-97.

202. Hesketh JE (1996) mRNA targeting: signals in the 3'-untranslated sequences for sorting of some mRNAs. Biochem Soc Trans 24: 521-527.

203. Hilleren P and Parker R (1999a) Mechanisms of mRNA Surveillance in Eukaryotes. Annu Rev Genet 33: 229-260.

204. Hilleren P, Parker R (1999b) mRNA surveillance in eukaryotes: kinetic proofreading of proper translation termination as assessed by mRNP domain organization? RNA 5: 711-719.

205. Holmes C, Jones SA, Budd TC, Greenfield SA (1997) Non-cholinergic, trophic action of recombinant acetylcholinesterase on mid-brain dopaminergic neurons. J Neurosci Res 49: 207-218.

206. Hosli E, Hosli L (1970) The presence of acetylcholinesterase in cultures of cerebellum and brain stem. Brain Res 19: 494-496.

207. Hotelier T, Renault L, Cousin X, Negre V, Marchot P, Chatonnet A (2004) ESTHER, the database of the alpha/beta-hydrolase fold superfamily of proteins. Nucleic Acids Res 32: D145-D147.

208. Ichtchenko K, Hata Y, Nguyen T, Ullrich B, Missler M, Moomaw C, Sudhof TC (1995) Neuroligin 1: a splice site-specific ligand for beta-neurexins. Cell 81: 435-443.

209. Im SH, Rao A (2004) Activation and deactivation of gene expression by Ca2+/calcineurin-NFAT-mediated signaling. Mol Cells 18: 1-9.

210. Inestrosa NC, Alvarez A, Calderon F (1996a) Acetylcholinesterase is a senile plaque component that promotes assembly of amyloid beta-peptide into Alzheimer's filaments. Mol Psychiatry 1: 359-361.

211. Inestrosa NC, Alvarez A, Perez CA, Moreno RD, Vicente M, Linker C, Casanueva OI, Soto C, Garrido J (1996b) Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. Neuron 16: 881-891.

212. Inestrosa NC, Alvarez J (1988) Axons grow in the aging rat but fast transport and acetylcholinesterase content remain unchanged. Brain Res 441: 331-338.

213. Inestrosa NC, Fuentes ME, Anglister L, Futerman AH, Silman I (1988) A membrane-associated dimer of acetylcholinesterase from Xenopus skeletal muscle is solubilized by phosphatidylinositol-specific phospholipase C. Neurosci Lett 90: 186-190.

214. Inestrosa NC, Miller JB, Silberstein L, Ziskind-Conhaim L, Hall ZW (1983) Developmental regulation of 16S acetylcholinesterase and acetylcholine receptors in a mouse muscle cell line. Exp Cell Res 147: 393-405.

215. Inestrosa NC, Moreno RD, Fuentes ME (1994) Monomeric amphiphilic forms of acetylcholinesterase appear early during brain development and may correspond to biosynthetic precursors of the amphiphilic G4 forms. Neurosci Lett 173: 155-158.

216. Inestrosa NC, Reiness CG, Reichardt LF, Hall ZW (1981) Cellular localization of the molecular forms of acetylcholinesterase in rat pheochromocytoma PC12 cells treated with nerve growth factor. J Neurosci 1: 1260-1267.

217. Inestrosa NC, Roberts WL, Marshall TL, Rosenberry TL (1987) Acetylcholinesterase from bovine caudate nucleus is attached to membranes by a novel subunit distinct from those of acetylcholinesterases in other tissues. J Biol Chem 262: 4441-4444.

218. Jacobs Anderson JS, Parker R (2000) Computational identification of cis-acting elements affecting post-transcriptional control of gene expression in Saccharomyces cerevisiae. Nucleic Acids Res 28: 1604-1617.

219. Jasmin BJ, Gardiner PF, Gisiger V (1991) Muscle acetylcholinesterase adapts to compensatory overload by a general increase in its molecular forms. J Appl Physiol 70: 2485-2489.

220. Jasmin BJ, Gisiger V (1990) Regulation by exercise of the pool of G4 acetylcholinesterase characterizing fast muscles: opposite effect of running training in antagonist muscles. J Neurosci 10: 1444-1454.

221. Jasmin BJ, Lee RK, Rotundo RL (1993) Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction. Neuron 11: 467-477.

222. Jiang JX, Choi RC, Siow NL, Lee HH, Wan DC, Tsim KW (2003) Muscle induces neuronal expression of acetylcholinesterase in neuron-muscle co-culture: transcriptional regulation mediated by cAMP-dependent signaling. J Biol Chem 278: 45435-45444.

223. Johnson CD, Rand JB, Herman RK, Stern BD, Russell RL (1988) The acetylcholinesterase genes of C. elegans: identification of a third gene (ace-3) and mosaic mapping of a synthetic lethal phenotype. Neuron 1: 165-173.

224. Johnson G, Moore SW (2000) Cholinesterases modulate cell adhesion in human neuroblastoma cells in vitro. Int J Devl Neurosci 18: 781-790.

225. Johnson G, Moore SW (2001) Association of the HNK-1 epitope with the detergent-soluble G4 isoform of acetylcholinesterase from human neuroblastoma cells. Int J Dev Neurosci 19: 439-445.

226. Johnson G, Moore SW (2003) Human acetylcholinesterase binds to mouse laminin-1 and human collagen IV by an electrostatic mechanism at the peripheral anionic site. Neurosci Lett 337: 37-40.

227. Johnson G, Moore SW (2004) Identification of a structural site on acetylcholinesterase that promotes neurite outgrowth and binds laminin-1 and collagen IV. Biochem Biophys Res Commun 319: 448-455.

228. Jones SA, Holmes C, Budd TC, Greenfield SA (1995) The effect of acetylcholinesterase on outgrowth of dopaminergic neurons in organotypic slice culture of rat mid-brain. Cell Tissue Res 279: 323-330.

229. Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI (1982) Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J Cell Biol 94: 253-262.

230. Kamel F, Hoppin JA (2004) Association of pesticide exposure with neurologic dysfunction and disease. Environ Health Perspect 112: 950-958.

231. Karlsson E, Mbugua PM, Rodriguez-Ithurralde D (1984) Fasciculins, anticholinesterase toxins from the venom of the green mamba Dendroaspis angusticeps. J Physiol (Paris) 79: 232-240.

232. Karpel R, Aziz-Aloya R, Sternfeld M, Ehrlich G, Ginzberg D, Tarroni P, Clementi F, Zakut H, Soreq H (1994) Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. Exp Cell Res 210: 268-277.

233. Karpel R, Sternfeld M, Ginzberg D, Guhl E, Graessmann A, Soreq H (1996) Overexpression of alternative human acetylcholinesterase forms modulates process extensions in cultured glioma cells. J Neurochem 66: 114-123.

234. Kasa P, Mann SP, Karcsu S, Toth L, Jordan S (1973) Transport of choline acetyltransferase and acetylcholinesterase in the rat sciatic nerve: a biochemical and electron histochemical study. J Neurochem 21: 431-436.

235. Kaufer D, Friedman A, Seidman S, Soreq H (1998) Acute stress facilitates long-lasting changes in cholinergic gene expression. Nature 393: 373-377.

236. Keene JD (1999) Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. Proc Natl Acad Sci U S A 96: 5-7.

237. Keene JD (2001) Ribonucleoprotein infrastructure regulating the flow of genetic information between the genome and the proteome. Proc Natl Acad Sci U S A 98: 7018-7024.

238. Kenan DJ, Query CC, Keene JD (1991) RNA recognition: towards identifying determinants of specificity. Trends Biochem Sci 16: 214-220.

239. Kimbell LM, Ohno K, Engel AG, Rotundo RL (2004) C-terminal and heparin-binding domains of collagenic tail subunit are both essential for anchoring acetylcholinesterase at the synapse. J Biol Chem 279: 10997-11005.

240. Klinar B, Brzin M (1978) Cytochemical demonstration of the postnatal development of cholinesterases in the sympathetic ganglion of the rat. Neuroscience 3: 1129-1134.

241. Klinar B, Brzin M (1979) Histochemical and cytochemical observations on the acetylcholinesterase (AChE) localization in the superior cervical ganglion (SCG) after preganglionic sympathectomy of the neonatal rat. Experientia 35: 754-756.

242. Klingman GI, Klingman JD (1969) Cholinesterases of rat sympathetic ganglia after immunosympathectomy, decentralization and axotomy. J Neurochem 16: 261-268.

243. Koelle GB (1955) The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons. J Pharmacol Exp Ther 114: 167-184.

244. Koenig E, Koelle GB (1961) Mode of regeneration of acetylcholinesterase in cholinergic neurons following irreversible inactivation. J Neurochem 8: 169-188.

245. Koenig J, Oren M, Melone MA (1982) Establishment of neuromuscular contacts in cultures of rat embryonic cells: effect of tetrodotoxin on maturation of muscle fibers and on formation and maintenance of acetylcholinesterase and acetylcholine receptor clusters. Dev Neurosci 5: 314-325.

246. Koenig J, Vigny M (1978) Neural induction of the 16S acetylcholinesterase in muscle cell cultures. Nature 271: 75-77.

247. Koenigsberger C, Chiappa S, Brimijoin S (1997) Neurite differentiation is modulated in neuroblastoma cells engineered for altered acetylcholinesterase expression. J Neurochem 69: 1389-1397.

248. Koenigsberger C, Hammond P, Brimijoin S (1998) Developmental expression of acetyl- and butyrylcholinesterase in the rat: enzyme and mRNA levels in embryonic dorsal root ganglia. Brain Res 787: 248-258.

249. Koike S, Schaeffer L, Changeux JP (1995) Identification of a DNA element determining synaptic expression of the mouse acetylcholine receptor delta-subunit gene. Proc Natl Acad Sci U S A 92: 10624-10628.

250. Korner CG, Wahle E (1997) Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. J Biol Chem 272: 10448-10456.

251. Kostovic I, Rakic P (1980) Cytology and time of origin of interstitial neurons in the white matter in infant and adult human and monkey telencephalon. J Neurocytol 9: 219-242.

252. Kostovic I, Skavic J, Strinovic D (1988) Acetylcholinesterase in the human frontal associative cortex during the period of cognitive development: early laminar shifts and late innervation of pyramidal neurons. Neurosci Lett 90: 107-112.

253. Krejci E, Coussen F, Duval N, Chatel JM, Legay C, Puype M, Vandekerckhove J, Cartaud J, Bon S, Massoulie J (1991) Primary structure of a collagenic tail

peptide of Torpedo acetylcholinesterase: co-expression with catalytic subunit induces the production of collagen-tailed forms in transfected cells. EMBO J 10: 1285-1293.

254. Kreutzberg GW (1969) Neuronal dynamics and axonal flow. IV. Blockage of intra-axonal enzyme transport by colchicine. Proc Natl Acad Sci U S A 62: 722-728.

255. Kristt DA (1989) Acetylcholinesterase in immature thalamic neurons: relation to afferentation, development, regulation and cellular distribution. Neuroscience 29: 27-43.

256. Lagnado CA, Brown CY, Goodall GJ (1994) AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: the functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). Mol Cell Biol 14: 7984-7995.

257. Lal A, Mazan-Mamczarz K, Kawai T, Yang X, Martindale JL, Gorospe M (2004) Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs. EMBO J 23: 3092-3102.

258. Landwehrmeyer B, Probst A, Palacios JM, Mengod G (1993) Expression of acetylcholinesterase messenger RNA in human brain: an in situ hybridization study. Neuroscience 57: 615-634.

259. Layer PG (1983) Comparative localization of acetylcholinesterase and pseudocholinesterase during morphogenesis of the chicken brain. Proc Natl Acad Sci U S A 80: 6413-6417.

260. Layer PG, Alber R, Rathjen FG (1988) Sequential activation of butyrylcholinesterase in rostral half somites and acetylcholinesterase in motoneurones and myotomes preceding growth of motor axons. Development 102: 387-396.

261. Layer PG, Kaulich S (1991) Cranial nerve growth in birds is preceded by cholinesterase expression during neural crest cell migration and the formation of an HNK-1 scaffold. Cell Tissue Res 265: 393-407.

262. Layer PG, Sporns O (1987) Spatiotemporal relationship of embryonic cholinesterases with cell proliferation in chicken brain and eye. Proc Natl Acad Sci U S A 84: 284-288.

263. Layer PG, Weikert T, Alber R (1993) Cholinesterases regulate neurite growth of chick nerve cells in vitro by means of a non-enzymatic mechanism. Cell Tissue Res 273: 219-226.

264. Layer PG, Willbold E (1995) Novel functions of cholinesterases in development, physiology and disease. Prog Histochem Cytochem 29: 1-94.

265. Lazar M, Salmeron E, Vigny M, Massoulie J (1984) Heavy isotope-labeling study of the metabolism of monomeric and tetrameric acetylcholinesterase forms in the murine neuronal-like T 28 hybrid cell line. J Biol Chem 259: 3703-3713.

266. Lazar M, Vigny M (1980) Modulation of the distribution of acetylcholinesterase molecular forms in a murine neuroblastoma x sympathetic ganglion cell hybrid cell line. J Neurochem 35: 1067-1079.

267. Lebel JM, Dussault JH, Puymirat J (1994) Overexpression of the beta 1 thyroid receptor induces differentiation in neuro-2a cells. Proc Natl Acad Sci U S A 91: 2644-2648.

268. Legay C (2000) Why so many forms of acetylcholinesterase? Microsc Res Tech 49: 56-72.

269. Legay C, Bon S, Massoulie J (1993a) Expression of a cDNA encoding the glycolipid-anchored form of rat acetylcholinesterase. FEBS Lett 315: 163-166.

270. Legay C, Bon S, Vernier P, Coussen F, Massoulie J (1993b) Cloning and expression of a rat acetylcholinesterase subunit: generation of multiple molecular forms and complementarity with a Torpedo collagenic subunit. J Neurochem 60: 337-346.

271. Legay C, Huchet M, Massoulie J, Changeux JP (1995) Developmental regulation of acetylcholinesterase transcripts in the mouse diaphragm: alternative splicing and focalization. Eur J Neurosci 7: 1803-1809.

272. Legay C, Mankal FA, Massoulie J, Jasmin BJ (1999) Stability and secretion of acetylcholinesterase forms in skeletal muscle cells. J Neurosci 19: 8252-8259.

273. Lev-Lehman E, Deutsch V, Eldor A, Soreq H (1997) Immature human megakaryocytes produce nuclear-associated acetylcholinesterase. Blood 89: 3644-3653.

274. Lewis PR, Shute CC (1966) The distribution of cholinesterase in cholinergic neurons demonstrated with the electron microscope. J Cell Sci 1: 381-390.

275. Li B, Stribley JA, Ticu A, Xie W, Schopfer LM, Hammond P, Brimijoin S, Hinrichs SH, Lockridge O (2000) Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. J Neurochem 75: 1320-1331.

276. Li Y, Camp S, Rachinsky TL, Getman D, Taylor P (1991) Gene Structure of Mammalian Achetylcholinesterase Alternative Exons Dictate Tissue-Specific Expression. J Biol Chem 266: 23083-23090.

277. Li Y, Camp S, Rachinsky TL, Bongiorno C, Taylor P (1993) Promoter elements and transcriptional control of the mouse acetylcholinesterase gene. J Biol Chem 268: 3563-3572.

278. Linkhart TA, Wilson BW (1975) Role of muscle contraction in trophic regulation of chick muscle acetylcholinesterase activity. Exp Neurol 48: 557-568.

279. Liu J, Dalmau J, Szabo A, Rosenfeld M, Huber J, Furneaux H (1995) Paraneoplastic encephalomyelitis antigens bind to the AU-rich elements of mRNA. Neurology 45: 544-550.

280. Liu Y, Shen T, Randall WR, Schneider MF (2005) Signaling pathways in activity-dependent fiber type plasticity in adult skeletal muscle. J Muscle Res Cell Motil 26: 13-21.

281. Lopez de Silanes I, Fan J, Yang X, Zonderman AB, Potapova O, Pizer ES, Gorospe M (2003) Role of the RNA-binding protein HuR in colon carcinogenesis. Oncogene 22: 7146-7154.

282. Lopez de Silanes I, Zhan M, Lal A, Yang X, Gorospe M (2004) Identification of a target RNA motif for RNA-binding protein HuR. Proc Natl Acad Sci U S A 101: 2987-2992.

283. Lubinska L, Niemierko S (1971) Velocity and intensity of bidirectional migration of acetylcholinesterase in transected nerves. Brain Res 27: 329-342.

284. Lucas CA, Kreutzberg GW (1985) Regulation of acetylcholinesterase secretion from neuronal cell cultures.--1. Actions of nerve growth factor, cytoskeletal inhibitors and tunicamycin. Neuroscience 14: 349-360.

285. Luo Z, Fuentes ME, Taylor P (1994) Regulation of acetylcholinesterase mRNA stability by calcium during differentiation from myoblasts to myotubes. J Biol Chem 269: 27216-27223.

286. Luo ZD, Camp S, Mutero A, Taylor P (1998) Splicing of 5' introns dictates alternative splice selection of acetylcholinesterase pre-mRNA and specific expression during myogenesis. J Biol Chem 273: 28486-28495.

287. Luo ZD, Wang Y, Werlen G, Camp S, Chien KR, Taylor P (1999) Calcineurin enhances acetylcholinesterase mRNA stability during C2-C12 muscle cell differentiation. Mol Pharmacol 56: 886-894.

288. Lwebuga-Mukasa JS, Lappi S, Taylor P (1976) Molecular forms of acetylcholinesterase from Torpedo californica: their relationship to synaptic membranes. Biochemistry 15: 1425-1434.

289. Mack A, Robitzki A (2000) The key role of butyrylcholinesterase during neurogenesis and neural disorders: an antisense-5'butyrylcholinesterase-DNA study. Prog Neurobiol 60: 607-628.

290. Malinger G, Zakut H, Soreq H (1989) Cholinoceptive properties of human primordial, preantral, and antral oocytes: in situ hybridization and biochemical evidence for expression of cholinesterase genes. J Mol Neurosci 1: 77-84.

291. Mangus DA, Evans MC, Jacobson A (2003) Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. Genome Biol 4: 223.

292. Marchand A, Chapouthier G, Massoulie J (1977) Developmental aspects of acetylcholinesterase activity in chick brain. FEBS Lett 78: 233-236.

293. Marnay A (1937) Cholinestérase dans l'organe électrique de la Torpille. Compt Rend Soc Biol 126: 573-574.

294. Marnay A, Nachmansohn D (1937) Cholinestérase dans le muscle strié. Compt Rend Soc Biol 124: 942-944.

295. Marnay A, Nachmansohn D (1938) Cholinesterase in voluntary muscle. J Physiol 92: 37-47.

296. Massoulie J, Bon S (1982) The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. Annu Rev Neurosci 5: 57-106.

297. Massoulie J, Pezzementi L, Bon S, Krejci E, Vallette FM (1993) Molecular and cellular biology of cholinesterases. Prog Neurobiol 41: 31-91.

298. Maulet Y, Camp S, Gibney G, Rachinsky TL, Ekstrom TJ, Taylor P (1990) Single gene encodes glycophospholipid-anchored and asymmetric acetylcholinesterase forms: alternative coding exons contain inverted repeat sequences. Neuron 4: 289-301.

299. McMahan UJ, Sanes JR, Marshall LM (1978) Cholinesterase is associated with the basal lamina at the neuromuscular junction. Nature 271: 172-174.

300. Mendell LM, Munson JB, Arvanian VL (2001) Neurotrophins and synaptic plasticity in the mammalian spinal cord. J Physiol 533: 91-97.

301. Mendez R, Richter JD (2001) Translational control by CPEB: a means to the end. Nat Rev Mol Cell Biol 2: 521-529.

302. Merlie JP (1984) Biogenesis of the acetylcholine receptor, a multisubunit integral membrane protein. Cell 36: 573-575.

303. Meshorer E, Erb C, Gazit R, Pavlovsky L, Kaufer D, Friedman A, Glick D, Ben Arie N, Soreq H (2002) Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. Science 295: 508-512.

304. Meshorer E, Toiber D, Zurel D, Sahly I, Dori A, Cagnano E, Schreiber L, Grisaru D, Tronche F, Soreq H (2004) Combinatorial complexity of 5' alternative acetylcholinesterase transcripts and protein products. J Biol Chem 279: 29740-29751.

305. Mesulam MM, Geula C (1988) Acetylcholinesterase-rich pyramidal neurons in the human neocortex and hippocampus: absence at birth, development during the life span, and dissolution in Alzheimer's disease. Ann Neurol 24: 765-773.

306. Mesulam MM, Geula C (1991) Acetylcholinesterase-rich neurons of the human cerebral cortex: cytoarchitectonic and ontogenetic patterns of distribution. J Comp Neurol 306: 193-220.

307. Mesulam MM, Geula C, Cosgrove R, Mash D, Brimijoin S (1991) Immunocytochemical demonstration of axonal and perikaryal acetylcholinesterase in human cerebral cortex. Brain Res 539: 233-238.

308. Mesulam MM, Geula C, Moran MA (1987) Anatomy of cholinesterase inhibition in Alzheimer's disease: effect of physostigmine and tetrahydroaminoacridine on plaques and tangles. Ann Neurol 22: 683-691.

309. Michel RN, Vu CQ, Tetzlaff W, Jasmin BJ (1994) Neural regulation of acetylcholinesterase mRNAs at mammalian neuromuscular synapses. J Cell Biol 127: 1061-1069.

310. Miki A, Fujimoto E, Mizoguti H (1983) Acetylcholinesterase activity in neural crest cells of the early chick embryo. Histochemistry 78: 81-93.

311. Milde-Langosch K (2005) The Fos family of transcription factors and their role in tumourigenesis. Eur J Cancer 41: 2449-2461.

312. Mis K, Mars T, Jevsek M, Brank M, Zajc-Kreft K, Grubic Z (2003) Localization of mRNAs encoding acetylcholinesterase and butyrylcholinesterase in the rat spinal cord by nonradioactive in situ hybridization. J Histochem Cytochem 51: 1633-1644.

313. Misquitta CM, Chen T, Grover AK (2006) Control of protein expression through mRNA stability in calcium signalling. Cell Calcium.

314. Mitchell P, Tollervey D (2000) mRNA stability in eukaryotes. Curr Opin Genet Dev 10: 193-198.

315. Mobarak CD, Anderson KD, Morin M, Beckel-Mitchener A, Rogers SL, Furneaux H, King P, Perrone-Bizzozero NI (2000) The RNA-binding protein

HuD is required for GAP-43 mRNA stability, GAP-43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells. Mol Biol Cell 11: 3191-3203.

316. Mor I, Grisaru D, Titelbaum L, Evron T, Richler C, Wahrman J, Sternfeld M, Yogev L, Meiri N, Seidman S, Soreq H (2001) Modified testicular expression of stress-associated "readthrough" acetylcholinesterase predicts male infertility. FASEB J 15: 2039-2041.

317. Morel N, Leroy J, Ayon A, Massoulie J, Bon S (2001) Acetylcholinesterase H and T dimers are associated through the same contact. Mutations at this interface interfere with the C-terminal T peptide, inducing degradation rather than secretion. J Biol Chem 276: 37379-37389.

318. Morel N, Massoulie J (2000) Comparative expression of homologous proteins. A novel mode of transcriptional regulation by the coding sequence folding compatibility of chimeras. J Biol Chem 275: 7304-7312.

319. Musunuru K, Darnell RB (2001) Paraneoplastic neurologic disease antigens: RNA-binding proteins and signaling proteins in neuronal degeneration. Annu Rev Neurosci 24: 239-262.

320. Nabors LB, Furneaux HM, King PH (1998) HuR, a novel target of anti-Hu antibodies, is expressed in non-neural tissues. J Neuroimmunol 92: 152-159.

321. Nabors LB, Gillespie GY, Harkins L, King PH (2001) HuR, a RNA stability factor, is expressed in malignant brain tumors and binds to adenine- and uridine-rich elements within the 3' untranslated regions of cytokine and angiogenic factor mRNAs. Cancer Res 61: 2154-2161.

322. Navaratnam V, Lewis PR (1970) Cholinesterase-containing neurones in the spinal cord of the rat. Brain Res 18: 411-425.

323. Newey SE, Gramolini AO, Wu J, Holzfeind P, Jasmin BJ, Davies KE, Blake DJ (2001) A novel mechanism for modulating synaptic gene expression: differential localization of alpha-dystrobrevin transcripts in skeletal muscle. Mol Cell Neurosci 17: 127-140.

324. Nielsen FC, Christiansen J (1992) Endonucleolysis in the turnover of insulin-like growth factor II mRNA. J Biol Chem 267: 19404-19411.

325. Nieto-Ceron S, Moral-Naranjo MT, Munoz-Delgado E, Vidal CJ, Campoy FJ (2004) Molecular properties of acetylcholinesterase in mouse spleen. Neurochem Int 45: 129-139.

326. Nijholt I, Farchi N, Kye M, Sklan EH, Shoham S, Verbeure B, Owen D, Hochner B, Spiess J, Soreq H, Blank T (2004) Stress-induced alternative splicing of acetylcholinesterase results in enhanced fear memory and long-term potentiation. Mol Psychiatry 9: 174-183.

327. Ochs S (1972) Rate of fast axoplasmic transport in mammalian nerve fibres. J Physiol 227: 627-645.

328. Okano HJ, Akamatsu W, Yano M, Mitsuhashi T, Fujihara H, Shibata S, Takahashi T, Noda T, Okano H (2005) RNA-binding protein Hu family regulates neural stem cell proliferation and differentiation. Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience Program No. 598.13

329. Okano HJ, Darnell RB (1997) A hierarchy of Hu RNA binding proteins in developing and adult neurons. J Neurosci 17: 3024-3037.

330. Olson EN, Williams RS (2000) Remodeling muscles with calcineurin. Bioessays 22: 510-519.

331. Olson PF, Fessler LI, Nelson RE, Sterne RE, Campbell AG, Fessler JH (1990) Glutactin, a novel Drosophila basement membrane-related glycoprotein with sequence similarity to serine esterases. EMBO J 9: 1219-1227.

332. Ott P, Brodbeck U (1984) Amphiphile dependency of the monomeric and dimeric forms of acetylcholinesterase from human erythrocyte membrane. Biochim Biophys Acta 775: 71-76.

333. Pannese E, Luciano L, Iurato S, Reale E (1971) Cholinesterase activity in spinal ganglia neuroblasts: a histochemical study at the electron microscope. J Ultrastruct Res 36: 46-67.

334. Paoletti F, Mocali A, Vannucchi AM (1992) Acetylcholinesterase in murine erythroleukemia (Friend) cells: evidence for megakaryocyte-like expression and potential growth-regulatory role of enzyme activity. Blood 79: 2873-2879.

335. Paraoanu LE, Layer PG (2004) Mouse acetylcholinesterase interacts in yeast with the extracellular matrix component laminin-1beta. FEBS Lett 576: 161-164.

336. Pascale A, Amadio M, Scapagnini G, Lanni C, Racchi M, Provenzani A, Govoni S, Alkon DL, Quattrone A (2005) Neuronal ELAV proteins enhance mRNA stability by a PKCalpha-dependent pathway. Proc Natl Acad Sci U S A 102: 12065-12070.

337. Paulus JM, Maigne J, Keyhani E (1981) Mouse megakaryocytes secrete acetylcholinesterase. Blood 58: 1100-1106.

338. Peng HB, Xie H, Rossi SG, Rotundo RL (1999) Acetylcholinesterase clustering at the neuromuscular junction involves perlecan and dystroglycan. J Cell Biol 145: 911-921.

339. Perrier AL, Massoulie J, Krejci E (2002) PRiMA: the membrane anchor of acetylcholinesterase in the brain. Neuron 33: 275-285.

340. Perrier NA, Kherif S, Perrier AL, Dumas S, Mallet J, Massoulie J (2003) Expression of PRiMA in the mouse brain: membrane anchoring and accumulation of 'tailed' acetylcholinesterase. Eur J Neurosci 18: 1837-1847.

341. Perrier NA, Salani M, Falasca C, Bon S, Augusti-Tocco G, Massoulie J (2005) The readthrough variant of acetylcholinesterase remains very minor after heat shock, organophosphate inhibition and stress, in cell culture and in vivo. J Neurochem 94: 629-638.

342. Perrone-Bizzozero N, Bolognani F (2002) Role of HuD and other RNA-binding proteins in neural development and plasticity. J Neurosci Res 68: 121-126.

343. Perry C, Sklan EH, Birikh K, Shapira M, Trejo L, Eldor A, Soreq H (2002) Complex regulation of acetylcholinesterase gene expression in human brain tumors. Oncogene 21: 8428-8441.

344. Postuma RB, Saez-Valero J, Small DH (1999) Inhibition of neurite outgrowth from chick sympathetic neurons by cholinesterase inhibitors is not mediated by binding to cholinesterases. Neurosci Lett 266: 77-80.

345. Pregelj P, Sketelj J (2002) Role of load bearing in acetylcholinesterase regulation in rat skeletal muscles. J Neurosci Res 67: 114-121.

346. Pullmann R, Jr., Juhaszova M, Lopez dS, I, Kawai T, Mazan-Mamczarz K, Halushka MK, Gorospe M (2005) Enhanced proliferation of cultured human vascular smooth muscle cells linked to increased function of RNA-binding protein HuR. J Biol Chem 280: 22819-22826.

347. Puymirat J, Etongue-Mayer P, Dussault JH (1995) Thyroid hormones stabilize acetylcholinesterase mRNA in neuro-2A cells that overexpress the beta 1 thyroid receptor. J Biol Chem 270: 30651-30656.

348. Quinn DM (1987) Acetylcholinesterase: Enzyme structure, reaction dynamics, and virtual transition states. Chem Rev 87: 955-979.

349. Rachinsky TL, Camp S, Li Y, Ekstrom TJ, Newton M, Taylor P (1990) Molecular cloning of mouse acetylcholinesterase: tissue distribution of alternatively spliced mRNA species. Neuron 5: 317-327.

350. Rachinsky TL, Crenshaw EB, III, Taylor P (1992) Assignment of the gene for acetylcholinesterase to distal mouse chromosome 5. Genomics 14: 511-514.

351. Radic Z, Duran R, Vellom DC, Li Y, Cervenansky C, Taylor P (1994) Site of fasciculin interaction with acetylcholinesterase. J Biol Chem 269: 11233-11239.

352. Rajagopalan LE, Malter JS (1997) Regulation of eukaryotic messenger RNA turnover. Prog Nucleic Acid Res Mol Biol 56: 257-286.

353. Rakonczay Z, Vincendon G, Zanetta JP (1981) Heterogeneity of rat brain acetylcholinesterase: a study by gel filtration and gradient centrifugation. J Neurochem 37: 662-669.

354. Ravin HA, Zacks SI, Seligman AM (1952) Histochemical localization of acetyl cholinesterase in the central nervous system and myoneural junction. J Natl Cancer Inst 13: 257-259.

355. Razon N, Soreq H, Roth E, Bartal A, Silman I (1984) Characterization of activities and forms of cholinesterases in human primary brain tumors. Exp Neurol 84: 681-695.

356. Rees T, Hammond PI, Soreq H, Younkin S, Brimijoin S (2003) Acetylcholinesterase promotes beta-amyloid plaques in cerebral cortex. Neurobiol Aging 24: 777-787.

357. Reyes AE, Perez DR, Alvarez A, Garrido J, Gentry MK, Doctor BP, Inestrosa NC (1997) A monoclonal antibody against acetylcholinesterase inhibits the formation of amyloid fibrils induced by the enzyme. Biochem Biophys Res Commun 232: 652-655.

358. Rieger F, Vigny M (1976) Solubilization and physiochemical characterization of rat brain acetylcholinesterase: development and maturation of its molecular forms. J Neurochem 27: 121-129.

359. Rieger F, Bon S, Massoulie J (1973) [Electron microscopic studies on stretched and globular acetylcholinesterase molecules of the electric eel (Electrophorus electricus)]. Eur J Biochem 34: 539-547.

360. Rieger F, Chetelat R, Nicolet M, Kamal L, Poullet M (1980a) Presence of tailed, asymmetric forms of acetylcholinesterase in the central nervous system of vertebrates. FEBS Lett 121: 169-174.

361. Rieger F, Goudou D, Tran LH (1984) Increase of junctional and background 16S (tailed, asymmetric) acetylcholinesterase during postnatal maturation of rat and mouse sternocleidomastoid muscle. J Neurochem 42: 601-606.

362. Rieger F, Shelanski ML, Greene LA (1980b) The effects of nerve growth factor on acetylcholinesterase and its multiple forms in cultures of rat PC12 pheochromocytoma cells: increased total specific activity and appearance of the 16 S molecular form. Dev Biol 76: 238-243.

363. Rimer M, Randall WR (1999) Denervation of chicken skeletal muscle causes an increase in acetylcholinesterase mRNA synthesis. Biochem Biophys Res Commun 260: 251-255.

364. Robertson RT (1987) A morphogenic role for transiently expressed acetylcholinesterase in developing thalamocortical systems? Neurosci Lett 75: 259-264.

365. Robertson RT, Poon HK, Mirrafati SJ, Yu J (1989) Transient patterns of acetylcholinesterase activity in developing thalamus: a comparative study in rodents. Brain Res Dev Brain Res 48: 309-315.

366. Robertson RT, Yu BP, Liu HH, Liu NH, Kageyama GH (1991) Development of cholinesterase histochemical staining in cerebellar cortex: transient expression of "nonspecific" cholinesterase in Purkinje cells of the nodulus and uvula. Exp Neurol 114: 330-342.

367. Roegiers F, Jan YN (2000) Staufen: a common component of mRNA transport in oocytes and neurons? Trends Cell Biol 10: 220-224.

368. Rosenberry TL (1979) Quantitative simulation of endplate currents at neuromuscular junctions based on the reaction of acetylcholine with acetylcholine receptor and acetylcholinesterase. Biophys J 26: 263-289.

369. Rosenberry TL, Richardson JM (1977) Structure of 18S and 14S acetylcholinesterase. Identification of collagen-like subunits that are linked by disulfide bonds to catalytic subunits. Biochemistry 16: 3550-3558.

370. Ross J (1995) mRNA stability in mammalian cells. Microbiol Rev 59: 423-450.

371. Rossi SG, Dickerson IM, Rotundo RL (2003) Localization of the calcitonin gene-related peptide receptor complex at the vertebrate neuromuscular junction and its role in regulating acetylcholinesterase expression. J Biol Chem 278: 24994-25000.

372. Rossi SG, Rotundo RL (1992) Cell surface acetylcholinesterase molecules on multinucleated myotubes are clustered over the nucleus of origin. J Cell Biol 119: 1657-1667.

373. Rossi SG, Vazquez AE, Rotundo RL (2000) Local control of acetylcholinesterase gene expression in multinucleated skeletal muscle fibers: individual nuclei respond to signals from the overlying plasma membrane. J Neurosci 20: 919-928.

374. Rotundo RL (1984) Asymmetric acetylcholinesterase is assembled in the Golgi apparatus. Proc Natl Acad Sci U S A 81: 479-483.

375. Rotundo RL (1988) Biogenesis of acetylcholinesterase molecular forms in muscle. Evidence for a rapidly turning over, catalytically inactive precursor pool. J Biol Chem 263: 19398-19406.

376. Rotundo RL (1990) Nucleus-specific translation and assembly of acetylcholinesterase in multinucleated muscle cells. J Cell Biol 110: 715-719.

377. Rotundo RL (2003) Expression and localization of acetylcholinesterase at the neuromuscular junction. J Neurocytol 32: 743-766.

378. Rotundo RL, Fambrough DM (1979) Molecular forms of chicken embryo acetylcholinesterase in vitro and in vivo. Isolation and characterization. J Biol Chem 254: 4790-4799.

379. Rotundo RL, Gomez AM, Fernandez-Valle C, Randall WR (1988) Allelic variants of acetylcholinesterase: genetic evidence that all acetylcholinesterase forms in avian nerves and muscles are encoded by a single gene. Proc Natl Acad Sci U S A 85: 7805-7809.

380. Rotundo RL, Rossi SG, Kimbell LM, Ruiz C, Marrero E (2005) Targeting acetylcholinesterase to the neuromuscular synapse. Chem Biol Interact 157-158: 15-21.

381. Sachs A (1990) The role of poly(A) in the translation and stability of mRNA. Curr Opin Cell Biol 2: 1092-1098.

382. Sachs AB (1993) Messenger RNA degradation in eukaryotes. Cell 74: 413-421.

383. Sastry BV, Janson VE, Chaturvedi AK (1981) Inhibition of human sperm motility by inhibitors of choline acetyltransferase. J Pharmacol Exp Ther 216: 378-384.

384. Schiaffino S, Serrano A (2002) Calcineurin signaling and neural control of skeletal muscle fiber type and size. Trends Pharmacol Sci 23: 569-575.

385. Schlaepfer WW, Torack RM (1967) The ultrastructural localization of cholinesterase activity in the peripheral nervous system of rats. J Neuropathol Exp Neurol 26: 137.

386. Schlaggar BL, De Carlos JA, O'Leary DD (1993) Acetylcholinesterase as an early marker of the differentiation of dorsal thalamus in embryonic rats. Brain Res Dev Brain Res 75: 19-30.

387. Schober A, Minichiello L, Keller M, Huber K, Layer PG, Roig-Lopez JL, Garcia-Arraras JE, Klein R, Unsicker K (1997) Reduced Acetylcholinesterase (AChE) Activity in Adrenal Medulla and Loss of Sympathetic Preganglionic neurons in TrkA-Deficient but Not TrkB-Deficient, Mice. J Neurosci 17: 891-903.

388. Scholl FG, Scheiffele P (2003) Making connections: cholinesterase-domain proteins in the CNS. Trends Neurosci 26: 618-624.

389. Schumacher M, Camp S, Maulet Y, Newton M, MacPhee-Quigley K, Taylor SS, Friedmann T, Taylor P (1986) Primary structure of Torpedo californica acetylcholinesterase deduced from its cDNA sequence. Nature 319: 407-409.

390. Schumacher M, Maulet Y, Camp S, Taylor P (1988) Multiple messenger RNA species give rise to the structural diversity in acetylcholinesterase. J Biol Chem 263: 18979-18987.

391. Seidman S, Aziz-Aloya RB, Timberg R, Loewenstein Y, Velan B, Shafferman A, Liao J, Norgaard-Pedersen B, Brodbeck U, Soreq H (1994) Overexpressed monomeric human acetylcholinesterase induces subtle ultrastructural modifications in developing neuromuscular junctions of Xenopus laevis embryos. J Neurochem 62: 1670-1681.

392. Seidman S, Sternfeld M, Aziz-Aloya R, Timberg R, Kaufer-Nachum D, Soreq H (1995) Synaptic and epidermal accumulations of human acetylcholinesterase are encoded by alternative 3'-terminal exons. Mol Cell Biol 15: 2993-3002.

393. Seko Y, Cole S, Kasprzak W, Shapiro BA, Ragheb JA (2006) The role of cytokine mRNA stability in the pathogenesis of autoimmune disease. Autoimmun Rev 5: 299-305.

394. Sharma KV, Bigbee JW (1998) Acetylcholinesterase antibody treatment results in neurite detachment and reduced outgrowth from cultured neurons: further evidence for a cell adhesive role for neuronal acetylcholinesterase. J Neurosci Res 53: 454-464.

395. Sharma KV, Koenigsberger C, Brimijoin S, Bigbee JW (2001) Direct evidence for an adhesive function in the noncholinergic role of acetylcholinesterase in neurite outgrowth. J Neurosci Res 63: 165-175.

396. Shatkin AJ (1985) mRNA cap binding proteins: essential factors for initiating translation. Cell 40: 223-224.

397. Shyu AB, Belasco JG, Greenberg ME (1991) Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. Genes Dev 5: 221-231.

398. Shyu AB, Greenberg ME, Belasco JG (1989) The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes Dev 3: 60-72.

399. Sikorav JL, Krejci E, Massoulie J (1987) cDNA sequences of Torpedo marmorata acetylcholinesterase: primary structure of the precursor of a catalytic subunit; existence of multiple 5'-untranslated regions. EMBO J 6: 1865-1873.

400. Silman I, Futerman AH (1987) Modes of attachment of acetylcholinesterase to the surface membrane. Eur J Biochem 170: 11-22.

401. Siow NL, Choi RC, Cheng AW, Jiang JX, Wan DC, Zhu SQ, Tsim KW (2002) A cyclic AMP-dependent pathway regulates the expression of acetylcholinesterase during myogenic differentiation of C2C12 cells. J Biol Chem 277: 36129-36136.

402. Siow NL, Xie HQ, Choi RC, Tsim KW (2005) ATP induces the post-synaptic gene expression in neuron-neuron synapses: Transcriptional regulation of AChE catalytic subunit. Chem Biol Interact 157-158: 423-426.

403. Sjostrand J, Frizell M, Hasselgren PO (1970) Effects of colchicine on axonal transport in peripheral nerves. J Neurochem 17: 1563-1570.

404. Skau KA, Brimijoin S (1978) Release of acetylcholinesterase from rat hemidiaphragm preparations stimulated through the phrenic nerve. Nature 275: 224-226.

405. Sketelj J, Crne-Finderle N, Ribaric S, Brzin M (1991) Interactions between intrinsic regulation and neural modulation of acetylcholinesterase in fast and slow skeletal muscles. Cell Mol Neurobiol 11: 35-54.

406. Sketelj J, Crne-Finderle N, Strukelj B, Trontelj JV, Pette D (1998) Acetylcholinesterase mRNA level and synaptic activity in rat muscles depend on nerve-induced pattern of muscle activation. J Neurosci 18: 1944-1952.

407. Small DH, Reed G, Whitefield B, Nurcombe V (1995) Cholinergic regulation of neurite outgrowth from isolated chick sympathetic neurons in culture. J Neurosci 15: 144-151.

408. Sofroniew MV, Howe CL, Mobley WC (2001) Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci 24: 1217-1281.

409. Sommer S, Cui Y, Brewer G, Fuqua SA (2005) The c-Yes 3'-UTR contains adenine/uridine-rich elements that bind AUF1 and HuR involved in mRNA decay in breast cancer cells. J Steroid Biochem Mol Biol 97: 219-229.

410. Soreq H, Ben Aziz R, Prody CA, Seidman S, Gnatt A, Neville L, Lieman-Hurwitz J, Lev-Lehman E, Ginzberg D, Lipidot-Lifson Y, . (1990) Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G + C-rich attenuating structure. Proc Natl Acad Sci U S A 87: 9688-9692.

411. Soreq H, Lapidot-Lifson Y, Zakut H (1991) A role for cholinesterases in tumorigenesis? Cancer Cells 3: 511-516.

412. Soreq H, Patinkin D, Lev-Lehman E, Grifman M, Ginzberg D, Eckstein F, Zakut H (1994) Antisense oligonucleotide inhibition of acetylcholinesterase gene expression induces progenitor cell expansion and suppresses hematopoietic apoptosis ex vivo. Proc Natl Acad Sci U S A 91: 7907-7911.

413. Soreq H, Seidman S (2001) Acetylcholinesterase--new roles for an old actor. Nat Rev Neurosci 2: 294-302.

414. Stankunas K, Graef IA, Neilson JR, Park SH, Crabtree GR (1999) Signaling through calcium, calcineurin, and NF-AT in lymphocyte activation and development. Cold Spring Harb Symp Quant Biol 64: 505-516.

415. Stephenson J, Czepulkowski B, Hirst W, Mufti GJ (1996) Deletion of the acetylcholinesterase locus at 7q22 associated with myelodysplastic syndromes (MDS) and acute myeloid leukaemia (AML). Leuk Res 20: 235-241.

416. Sternfeld M, Ming G, Song H, Sela K, Timberg R, Poo M, Soreq H (1998) Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. J Neurosci 18: 1240-1249.

417. Sternfeld M, Shoham S, Klein O, Flores-Flores C, Evron T, Idelson GH, Kitsberg D, Patrick JW, Soreq H (2000) Excess "read-through" acetylcholinesterase attenuates but the "synaptic" variant intensifies neurodeterioration correlates. Proc Natl Acad Sci U S A 97: 8647-8652.

418. Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L, Silman I (1991) Atomic structure of acetylcholinesterase from Torpedo californica: a prototypic acetylcholine-binding protein. Science 253: 872-879.

419. Sveistrup H, Chan RY, Jasmin BJ (1995) Chronic enhancement of neuromuscular activity increases acetylcholinesterase gene expression in skeletal muscle. Am J Physiol 269: C856-C862.

420. Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, Henson J, Posner JB, Furneaux HM (1991) HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. Cell 67: 325-333.

421. Tang K, Breen EC, Wagner PD (2002) Hu protein R-mediated posttranscriptional regulation of VEGF expression in rat gastrocnemius muscle. Am J Physiol Heart Circ Physiol 283: H1497-H1504.

422. Taylor P, Radic Z (1994) The Cholinesterases: from Genes to Proteins. Annu Rev Pharmocol Toxicol 34: 281-320.

423. Tennyson VM, Brzin M, Slotwiner P (1971) The appearance of acetylcholinesterase in the myotome of the embryonic rabbit. An electron microscope cytochemical and biochemical study. J Cell Biol 51: 703-721.

424. Tetzlaff W, Kreutzberg GW (1984) Enzyme changes in the rat facial nucleus following a conditioning lesion. Exp Neurol 85: 547-564.

425. Thullbery MD, Cox HD, Schule T, Thompson CM, George KM (2005) Differential localization of acetylcholinesterase in neuronal and non-neuronal cells. J Cell Biochem 96: 599-610.

426. Torres JC, Behrens MI, Inestrosa NC (1983) Neural 16S acetylcholinesterase is solubilized by heparin. Biochem J 215: 201-204.

427. Torres JC, Inestrosa NC (1983) Heparin solubilizes asymmetric acetylcholinesterase from rat neuromuscular junction. FEBS Lett 154: 265-268.

428. Tsim KW, Choi RC, Dong TT, Wan DC (1997) A globular, not asymmetric, form of acetylcholinesterase is expressed in chick motor neurons: down-regulation toward maturity and after denervation. J Neurochem 68: 479-487.

429. Tsim KW, Greenberg I, Rimer M, Randall WR, Salpeter MM (1992) Transcripts for the acetylcholine receptor and acetylcholine esterase show distribution differences in cultured chick muscle cells. J Cell Biol 118: 1201-1212.

430. Tung EK, Choi RC, Siow NL, Jiang JX, Ling KK, Simon J, Barnard EA, Tsim KW (2004) P2Y2 receptor activation regulates the expression of acetylcholinesterase and acetylcholine receptor genes at vertebrate neuromuscular junctions. Mol Pharmacol 66: 794-806.

431. Uchida N, Hoshino S, Katada T (2004) Identification of a human cytoplasmic poly(A) nuclease complex stimulated by poly(A)-binding protein. J Biol Chem 279: 1383-1391.

432. Ueda H, Ohno S, Kobayashi T (2000) Myotonic dystrophy and myotonic dystrophy protein kinase. Prog Histochem Cytochem 35: 187-251.

433. Ulrich J, Meier-Ruge W, Probst A, Meier E, Ipsen S (1990) Senile plaques: staining for acetylcholinesterase and A4 protein: a comparative study in the hippocampus and entorhinal cortex. Acta Neuropathol (Berl) 80: 624-628.

434. Van Der Giessen K, Di Marco S, Clair E, Gallouzi IE (2003) RNAi-mediated HuR depletion leads to the inhibition of muscle cell differentiation. J Biol Chem 278: 47119-47128.

435. van Kesteren RE, Spencer GE (2003) The role of neurotransmitters in neurite outgrowth and synapse formation. Rev Neurosci 14: 217-231.

436. Verdiere M, Derer M, Rieger F (1982) Multiple molecular forms of rat superior cervical ganglion acetylcholinesterase: developmental aspects in primary cell culture and during postnatal maturation in vivo. Dev Biol 89: 509-515.

437. Viana GB, Kauffman FC (1984) Cholinesterase activity in the rat superior cervical ganglion: effect of denervation and axotomy. Brain Res 304: 37-45.

438. Vieweg WV, Julius DA, Fernandez A, Beatty-Brooks M, Hettema JM, Pandurangi AK (2006) Posttraumatic stress disorder: clinical features, pathophysiology, and treatment. Am J Med 119: 383-390.

439. Vigny M, Di Giamberardino L, Couraud JY, Rieger F, Koenig J (1976a) Molecular forms of chicken acetylcholinesterase: effect of denervation. FEBS Lett 69: 277-280.

440. Vigny M, Koenig J, Rieger F (1976b) The motor end-plate specific form of acetylcholinesterase: appearance during embryogenesis and re-innervation of rat muscle. J Neurochem 27: 1347-1353.

441. Vigny M, Martin GR, Grotendorst GR (1983) Interactions of asymmetric forms of acetylcholinesterase with basement membrane components. J Biol Chem 258: 8794-8798.

442. Vijayan VK (1979) Distribution of cholinergic neurotransmitter enzymes in the hippocampus and the dentate gyrus of the adult and the developing mouse. Neuroscience 4: 121-137.

443. Voshol H, van Zuylen CW, Orberger G, Vliegenthart JF, Schachner M (1996) Structure of the HNK-1 carbohydrate epitope on bovine peripheral myelin glycoprotein P0. J Biol Chem 271: 22957-22960.

444. Wakamatsu Y, Weston JA (1997) Sequential expression and role of Hu RNA-binding proteins during neurogenesis. Development 124: 3449-3460.

445. Wan DC, Choi RC, Siow NL, Tsim KW (2000) The promoter of human acetylcholinesterase is activated by a cyclic adenosine 3',5'-monophosphate-dependent pathway in cultured NG108-15 neuroblastoma cells. Neurosci Lett 288: 81-85.

446. Wang Z, Kiledjian M (2001) Functional link between the mammalian exosome and mRNA decapping. Cell 107: 751-762.

447. Weikert T, Layer PG (1994) The carbohydrate epitope HNK-1 is present on all inactive, but not on all active forms of chicken butyrylcholinesterase. Neurosci Lett 176: 9-12.

448. Weill CO, Vorlova S, Berna N, Ayon A, Massoulie J (2002) Transcriptional regulation of gene expression by the coding sequence: An attempt to enhance expression of human AChE. Biotechnol Bioeng 80: 490-497.

449. Weinberg CB, Hall ZW (1979) Junctional form of acetylcholinesterase restored at nerve-free endplates. Dev Biol 68: 631-635.

450. Whyte KA, Greenfield SA (2003) Effects of acetylcholinesterase and butyrylcholinesterase on cell survival, neurite outgrowth, and voltage-dependent calcium currents of embryonic ventral mesencephalic neurons. Exp Neurol 184: 496-509.

451. Wilson GM, Brewer G (1999) The search for trans-acting factors controlling messenger RNA decay. Prog Nucleic Acid Res Mol Biol 62: 257-291.

452. Wollemann M, Zoltan L (1962) Cholinesterase activity of cerebral tumors and tumorous cysts. Arch Neurol 6: 161-167.

453. Wright CI, Geula C, Mesulam MM (1993) Neurological cholinesterases in the normal brain and in Alzheimer's disease: relationship to plaques, tangles, and patterns of selective vulnerability. Ann Neurol 34: 373-384.

454. Xie W, Stribley JA, Chatonnet A, Wilder PJ, Rizzino A, McComb RD, Taylor P, Hinrichs SH, Lockridge O (2000) Postnatal developmental delay and supersensitivity to organophosphate in gene-targeted mice lacking acetylcholinesterase. J Pharmacol Exp Ther 293: 896-902.

455. Xu N, Chen CY, Shyu AB (1997) Modulation of the fate of cytoplasmic mRNA by AU-rich elements: key sequence features controlling mRNA deadenylation and decay. Mol Cell Biol 17: 4611-4621.

456. Yano M, Okano HJ, Okano H (2005) Involvement of Hu and heterogeneous nuclear ribonucleoprotein K in neuronal differentiation through p21 mRNA post-transcriptional regulation. J Biol Chem 280: 12690-12699.

457. Yew DT, Chan WY (1999) Early appearance of acetylcholinergic, serotoninergic, and peptidergic neurons and fibers in the developing human central nervous system. Microsc Res Tech 45: 389-400.

458. Younkin SG, Rosenstein C, Collins PL, Rosenberry TL (1982) Cellular localization of the molecular forms of acetylcholinesterase in rat diaphragm. J Biol Chem 257: 13630-13637.

459. Zajicek J (1954) Studies on the histogenesis of blood platelets. Acta Haematol 12: 238-244.

460. Zhao Z, Chang FC, Furneaux HM (2000) The identification of an endonuclease that cleaves within an HuR binding site in mRNA. Nucleic Acids Res 28: 2695-2701.

461. Zubiaga AM, Belasco JG, Greenberg ME (1995) The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. Mol Cell Biol 15: 2219-2230.

Reference list: Chapter 2

1. Massoulie, J., Pezzementi, L., Bon, S., Krejci, E., and Vallette, F. M. (1993) *Prog. Neurobiol.* 41, 31–91

2. Legay, C. (2000) *Microsc. Res. Tech.* 49, 56–72

3. Taylor, P., and Radic, Z. (1994) *Annu. Rev. Pharmacol. Toxicol.* 34, 281–320

4. Soreq, H., and Seidman, S. (2001) *Nat. Rev. Neurosci.* 2, 294–302

5. Legay, C., Huchet, M., Massoulie, J., and Changeux, J. P. (1995) *Eur. J. Neurosci.* 7, 1803–1809

6. Li, Y., Camp, S., and Taylor, P. (1993) *J. Biol. Chem.* 268, 5790–5797

7. Rachinsky, T. L., Camp, S., Li, Y., Ekstrom, T. J., Newton, M., and Taylor, P. (1990) *Neuron* 5, 317–327

8. Layer, P. G., and Willbold, E. (1995) *Prog. Histochem. Cytochem.* 29, 1–94

9. Bernard, V., Legay, C., Massoulie, J., and Bloch, B. (1995) *Neuroscience* 64, 995–1005

10. Hammond, P., Rao, R., Koenigsberger, C., and Brimijoin, S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 10933–10937

11. Landwehrmeyer, B., Probst, A., Palacios, J. M., and Mengod, G. (1993) *Neuroscience* 57, 615–634

12. Legay, C., Bon, S., Vernier, P., Coussen, F., and Massoulie, J. (1993) *J. Neurochem.* 60, 337–346

13. Brimijoin, S., and Hammond, P. (1996) *Neuroscience* 71, 555–565

14. Robertson, R. T., and Yu, J. (1993) *News Physiol. Sci.* 8, 266–272

15. Blasina, M. F., Faria, A. C., Gardino, P. F., Hokoc, J. N., Almeida, O. M., De Mello, F. G., Arruti, C., and Dajas, F. (2000) *Cell Tissue Res.* 299, 173–184

16. Tetzlaff, W., Alexander, S. W., Miller, F. D., and Bisby, M. A. (1991) *J. Neurosci.* 11, 2528–2544

17. Dupree, J. L., and Bigbee, J. W. (1996) *J. Neurocytol.* 25, 439–454

18. Jones, S. A., Holmes, C., Budd, T. C., and Greenfield, S. A. (1995) *Cell Tissue Res.* 279, 323–330

19. Koenigsberger, C., Chiappa, S., and Brimijoin, S. (1997) *J. Neurochem.* 69, 1389–1397

20. Sharma, K. V., and Bigbee, J. W. (1998) *J. Neurosci. Res.* 53, 454–464

21. Srivatsan, M., and Peretz, B. (1997) *Neuroscience* 77, 921–931

22. Sternfeld, M., Ming, G., Song, H., Sela, K., Timberg, R., Poo, M., and Soreq, H. (1998) *J. Neurosci.* 18, 1240–1249

23. Angus, L. M., Chan, R. Y., and Jasmin, B. J. (2001) *J. Biol. Chem.* 276, 17603–17609

24. Boudreau-Lariviere, C., Chan, R. Y., Wu, J., and Jasmin, B. J. (2000) *J. Neurochem.* 74, 2250–2258

25. Chan, R. Y., Boudreau-Lariviere, C., Angus, L. M., Mankal, F. A., and Jasmin, B. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 4627–4632

26. Krejci, E., Legay, C., Thomine, S., Sketelj, J., and Massoulie, J. (1999) *J. Neurosci.* 19, 10672–10679

27. Luo, Z. D., Wang, Y., Werlen, G., Camp, S., Chien, K. R., and Taylor, P. (1999) *Mol. Pharmacol.* 56, 886–894

28. Pregelj, P., and Sketelj, J. (2002) *J. Neurosci. Res.* 67, 114–121

29. Rimer, M., and Randall, W. R. (1999) *Biochem. Biophys. Res. Commun.* 260, 251–255

30. Siow, N. L., Choi, R. C., Cheng, A. W., Jiang, J. X., Wan, D. C., Zhu, S. Q., and Tsim, K. W. (2002) *J. Biol. Chem.* 277, 36129–36136

31. Greene, L. A., and Rukenstein, A. (1981) *J. Biol. Chem.* 256, 6363–6367

32. Coleman, B. A., and Taylor, P. (1996) *J. Biol. Chem.* 271, 4410–4416

33. Greene, L. A., Aletta, J. M., Ruckenstein, A., and Greene, S. H. (1986) *Methods Enzymol.* 147, 207–216

34. Mobarak, C. D., Anderson, K. D., Morin, M., Beckel-Mitchener, A., Rogers, S. L., Furneaux, H., King, P., and Perrone-Bizzozero, N. I. (2000) *Mol. Biol. Cell* 11, 3191–3203

35. Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. (1961) *Biochem. Pharmacol.* 7, 88–95

36. Jasmin, B. J., and Gisiger, V. (1990) *J. Neurosci.* 10, 1444–1454

37. Boudreau-Lariviere, C., Sveistrup, H., Parry, D. J., and Jasmin, B. J. (1996) *Neuroscience* 73, 613–622

38. Michel, R. N., Vu, C. Q., Tetzlaff, W., and Jasmin, B. J. (1994) *J. Cell Biol.* 127, 1061–1069

39. Jasmin, B. J., Lee, R. K., and Rotundo, R. L. (1993) *Neuron* 11, 467–477

40. Forster, E., Otten, U., and Frotscher, M. (1993) *Neurosci. Lett.* 155, 216–219

41. Boudreau-Lariviere, C., and Jasmin, B. J. (1999) *FEBS Lett.* 444, 22–26

42. Chan, R. Y., Adatia, F. A., Krupa, A. M., and Jasmin, B. J. (1998) *J. Biol. Chem.* 273, 9727–9733

43. Fuentes, M. E., and Taylor, P. (1993) *Neuron* 10, 679–687

44. Sketelj, J., Crne-Finderle, N., Strukelj, B., Trontelj, J. V., and Pette, D. (1998) *J. Neurosci.* 18, 1944–1952

45. Li, Y., Liu, L., Kang, J., Sheng, J. G., Barger, S. W., Mrak, R. E., and Griffin, W. S. (2000) *J. Neurosci.* 20, 149–155

46. Meshorer, E., Erb, C., Gazit, R., Pavlovsky, L., Kaufer, D., Friedman, A., Glick, D., Ben Arie, N., and Soreq, H. (2002) *Science* 295, 508–512

47. Alterio, J., Mallet, J., and Biguet, N. F. (2001) *Mol. Cell Neurosci.* 17, 179–189

48. Hew, Y., Lau, C., Grzelczak, Z., and Keeley, F. W. (2000) *J. Biol. Chem.* 275, 24857–24864

49. Wilson, G. M., and Brewer, G. (1999) *Methods* 17, 74–83

50. Erondu, N. E., Nwankwo, J., Zhong, Y., Boes, M., Dake, B., and Bar, R. S. (1999) *Mol. Endocrinol.* 13, 495–504

51. Sagesser, R., Martinez, E., Tsagris, M., and Tabler, M. (1997) *Nucleic Acids Res.* 25, 3816–3822

52. Chung, S., Eckrich, M., Perrone-Bizzozero, N., Kohn, D. T., and Furneaux, H. (1997) *J. Biol. Chem.* 272, 6593–6598

53. Inestrosa, N. C., Reiness, C. G., Reichardt, L. F., and Hall, Z. W. (1981) *J. Neurosci.* 1, 1260–1267

54. Lucas, C. A., Czlonkowska, A., and Kreutzberg, G. W. (1980) *Neurosci. Lett.* 18, 333–337

55. Tsai, K. C., Cansino, V. V., Kohn, D. T., Neve, R. L., and Perrone-Bizzozero, N. I. (1997) *J. Neurosci.* 17, 1950–1958

56. Rodriguez-Pascual, F., Hausding, M., Ihrig-Biedert, I., Furneaux, H., Levy, A. P., Forstermann, U., and Kleinert, H. (2000) *J. Biol. Chem.* 275, 26040–26049

57. Yeap, B. B., Voon, D. C., Vivian, J. P., McCulloch, R. K., Thomson, A. M., Giles, K. M., Czyzyk-Krzeska, M. F., Furneaux, H., Wilce, M. C., Wilce, J. A., and Leedman, P. J. (2002) *J. Biol. Chem.* 277, 27183–27192

58. Antic, D., Lu, N., and Keene, J. D. (1999) *Genes Dev.* 13, 449–461

59. Getman, D. K., Mutero, A., Inoue, K., and Taylor, P. (1995) *J. Biol. Chem.* 270, 23511–23519

60. Luo, Z., Fuentes, M. E., and Taylor, P. (1994) *J. Biol. Chem.* 269, 27216–27223

61. Kaufer, D., Friedman, A., Seidman, S., and Soreq, H. (1998) *Nature* 393, 373–377

62. Karpel, R., Aziz-Aloya, R., Sternfeld, M., Ehrlich, G., Ginzberg, D., Tarroni, P., Clementi, F., Zakut, H., and Soreq, H. (1994) *Exp. Cell Res.* 210, 268–277

63. Peng, S. S., Chen, C. Y., and Shyu, A. B. (1996) *Mol. Cell. Biol.* 16, 1490–1499

64. Xu, N., Chen, C. Y., and Shyu, A. B. (1997) *Mol. Cell. Biol.* 17, 4611–4621

65. Guhaniyogi, J., and Brewer, G. (2001) *Gene (Amst.)* 265, 11–23

66. Malter, J. A. (2001) *J. Neurosci. Res.* 66, 311–316

67. Perrone-Bizzozero, N., and Bolognani, F. (2002) *J. Neurosci. Res.* 68, 121–126

68. Manohar, C. F., Short, M. L., Nguyen, A., Nguyen, N. N., Chagnovich, D., Yang, Q., and Cohn, S. L. (2002) *J. Biol. Chem.* 277, 1967–1973

69. Cuadrado, A., Navarro-Yubero, C., Furneaux, H., Kinter, J., Sonderegger, P., and Munoz, A. (2002) *Nucleic Acids Res.* 30, 2202–2211

70. Aranda-Abreu, G. E., Behar, L., Chung, S., Furneaux, H., and Ginzburg, I. (1999) *J. Neurosci.* 19, 6907–6917

71. Anderson, K. D., Sengupta, J., Morin, M., Neve, R. L., Valenzuela, C. F., and Perrone-Bizzozero, N. I. (2001) *Exp. Neurol.* 168, 250–258

72. Dobashi, Y., Shoji, M., Wakata, Y., and Kameya, T. (1998) *Biochem. Biophys. Res. Commun.* 244, 226–229

73. Laroia, G., Cuesta, R., Brewer, G., and Schneider, R. J. (1999) *Science* 284, 499–502

74. Wan, D. C., Choi, R. C., Siow, N. L., and Tsim, K. W. (2000) *Neurosci. Lett.* 288, 81–85

75. Perrone-Bizzozero, N. I., Neve, R. L., Irwin, N., Lewis, S. E., Fischer, I., and Benowitz, L. I. (1991) *Mol. Cell Neurosci.* 2, 402–409

76. Federoff, H. J., Grabczyk, E., and Fishman, M. C. (1988) *J. Biol. Chem.* 263, 19290–19295

77. Huang, E. J., and Reichardt, L. F. (2001) *Annu. Rev. Neurosci.* 24, 677–736

78. Sofroniew, M. V., Howe, C. L., and Mobley, W. C. (2001) *Annu. Rev. Neurosci.* 24, 1217–1281

79. Fernandes, K. J., Kobayashi, N. R., Jasmin, B. J., and Tetzlaff, W. (1998) *J. Neurosci.* 18, 9936–9947

80. Palacios, G., Mengod, G., Sarasa, M., Baudier, J., and Palacios, J. M. (1994) *Brain Res. Mol. Brain Res.* 24, 107–117

81. Tetzlaff, W., Zwiers, H., Lederis, K., Cassar, L., and Bisby, M. A. (1989) *J. Neurosci.* 9, 1303–1313

Reference list: Chapter 3

1. Legay, C. (2000) *Microsc. Res. Tech.* **49,** 56–72

2. Rotundo, R. L. (2003) *J. Neurocytol.* **32,** 743–766

3. Massoulie, J., Pezzementi, L., Bon, S., Krejci, E., and Vallette, F. M. (1993) *Prog. Neurobiol. (Oxford)* **41,** 31–91

4. Soreq, H., and Seidman, S. (2001) *Nat. Rev. Neurosci.* **2,** 294–302

5. Alvarez, A., Alarcon, R., Opazo, C., Campos, E. O., Munoz, F. J., Calderon, F. H., Dajas, F., Gentry, M. K., Doctor, B. P., De Mello, F. G., and Inestrosa, N. C. (1998) *J. Neurosci.* **18,** 3213–3223

6. Inestrosa, N. C., Alvarez, A., Perez, C. A., Moreno, R. D., Vicente, M., Linker, C., Casanueva, O. I., Soto, C., and Garrido, J. (1996) *Neuron* **16,** 881–891

7. Ulrich, J., Meier-Ruge, W., Probst, A., Meier, E., and Ipsen, S. (1990) *Acta Neuropathol. (Berl.)* **80,** 624–628

8. Engel, A. G., Ohno, K., and Sine, S. M. (1999) *Arch. Neurol.* **56,** 163–167

9. Ohno, K., Brengman, J., Tsujino, A., and Engel, A. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9654–9659

10. Legay, C., Huchet, M., Massoulie, J., and Changeux, J. P. (1995) *Eur. J. Neurosci.* **7,** 1803–1809

11. Legay, C., Mankal, F. A., Massoulie, J., and Jasmin, B. J. (1999) *J. Neurosci.* **19,** 8252–8259

12. Li, Y., Camp, S., Rachinsky, T. L., Getman, D., and Taylor, P. (1991) *J. Biol. Chem.* **266,** 23083–23090

13. Li, Y., Camp, S., and Taylor, P. (1993) *J. Biol. Chem.* **268,** 5790–5797

14. Hall, Z. W. (1973) *J. Neurobiol.* **4,** 343–361

15. Grubic, Z., Komel, R., Walker, W. F., and Miranda, A. F. (1995) *Neuron* **14,** 317–327

16. Jasmin, B. J., Lee, R. K., and Rotundo, R. L. (1993) *Neuron* **11,** 467–477

17. Michel, R. N., Vu, C. Q., Tetzlaff, W., and Jasmin, B. J. (1994) *J. Cell Biol.* **127,** 1061–1069

18. Fernandez, H. L., and Stiles, J. R. (1984) *Neurosci. Lett.* **44,** 187–192

19. Boudreau-Lariviere, C., Chan, R. Y., Wu, J., and Jasmin, B. J. (2000) *J. Neurochem.* **74,** 2250–2258

20. Cresnar, B., Crne-Finderle, N., Breskvar, K., and Sketelj, J. (1994) *J. Neurosci. Res.* **38,** 294–299

21. Vigny, M., Koenig, J., and Rieger, F. (1976) *J. Neurochem.* **27,** 1347–1353

22. Prives, J. M., and Paterson, B. M. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 3208–3211

23. Fuentes, M. E., and Taylor, P. (1993) *Neuron* **10,** 679–687

24. Vallette, F. M., Fauquet, M., and Teillet, M. A. (1987) *Dev. Biol.* **120,** 77–84

25. Angus, L. M., Chan, R. Y., and Jasmin, B. J. (2001) *J. Biol. Chem.* **276,** 17603–17609

26. Mutero, A., Camp, S., and Taylor, P. (1995) *J. Biol. Chem.* **270,** 1866–1872

27. Siow, N. L., Choi, R. C., Cheng, A. W., Jiang, J. X., Wan, D. C., Zhu, S. Q., and Tsim, K. W. (2002) *J. Biol. Chem.* **277,** 36129–36136

28. Deschenes-Furry, J., Belanger, G., Perrone-Bizzozero, N., and Jasmin, B. J. (2003) *J. Biol. Chem.* **278,** 5710–5717

29. Beckel-Mitchener, A. C., Miera, A., Keller, R., and Perrone-Bizzozero, N. I. (2002) *J. Biol. Chem.* **277,** 27996–28002

30. Chan, R. Y., Boudreau-Lariviere, C., Angus, L. M., Mankal, F. A., and Jasmin, B. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4627–4632

31. Mobarak, C. D., Anderson, K. D., Morin, M., Beckel-Mitchener, A., Rogers, S. L., Furneaux, H., King, P., and Perrone-Bizzozero, N. I. (2000) *Mol. Biol. Cell* **11,** 3191–3203

32. Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A., and Mehtali, M. (1996) *J. Virol.* **70,** 4805–4810

33. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2509–2514

34. Ng, P., and Graham, F. L. (2002) *Methods Mol. Med.* **69,** 389–414

35. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) *J. Gen. Virol.* **36,** 59–74

36. Forster, E., Otten, U., and Frotscher, M. (1993) *Neurosci. Lett.* **155,** 216–219
37. Legay, C., Bon, S., Vernier, P., Coussen, F., and Massoulie, J. (1993) *J. Neurochem.* **60,** 337–346

38. Boudreau-Lariviere, C., Sveistrup, H., Parry, D. J., and Jasmin, B. J. (1996) *Neuroscience* **73,** 613–622

39. Misquitta, C. M., Iyer, V. R., Werstiuk, E. S., and Grover, A. K. (2001) *Mol. Cell. Biochem.* **224,** 53–67

40. Chung, S., Eckrich, M., Perrone-Bizzozero, N., Kohn, D. T., and Furneaux, H. (1997) *J. Biol. Chem.* **272,** 6593–6598

41. Brennan, C. M., and Steitz, J. A. (2001) *Cell. Mol. Life Sci.* **58,** 266–277

42. Guhaniyogi, J., and Brewer, G. (2001) *Gene (Amst.)* **265,** 11–23

43. Rachinsky, T. L., Camp, S., Li, Y., Ekstrom, T. J., Newton, M., and Taylor, P. (1990) *Neuron* **5,** 317–327

44. Cuadrado, A., Navarro-Yubero, C., Furneaux, H., and Munoz, A. (2003) *J. Neurochem.* **86,** 763–773

45. Gramolini, A. O., and Jasmin, B. J. (1999) *Nucleic Acids Res.* **27,** 3603–3609

46. Gramolini, A. O., Belanger, G., and Jasmin, B. J. (2001) *J. Cell Biol.* **154,** 1173–1183

47. Perrone-Bizzozero, N., and Bolognani, F. (2002) *J. Neurosci. Res.* **68,** 121–126

48. Ma, W. J., Cheng, S., Campbell, C., Wright, A., and Furneaux, H. (1996) *J. Biol. Chem.* **271,** 8144–8151

49. Tang, K., Breen, E. C., and Wagner, P. D. (2002) *Am. J. Physiol.* **283,** H1497–H1504

50. Van Der Giessen, K., Di Marco, S., Clair, E., and Gallouzi, I. E. (2003) *J. Biol. Chem.* **278,** 47119–47128

51. Choi, R. C., Siow, N. L., Zhu, S. Q., Wan, D. C., Wong, Y. H., and Tsim, K. W. (2001) *Mol. Cell. Neurosci.* **17,** 732–745

52. Luo, Z., Fuentes, M. E., and Taylor, P. (1994) *J. Biol. Chem.* **269,** 27216–27223

53. Freyssenet, D., Connor, M. K., Takahashi, M., and Hood, D. A. (1999) *Am. J. Physiol.* **277,** E26–E32

54. Figueroa, A., Cuadrado, A., Fan, J., Atasoy, U., Muscat, G. E., Munoz-Canoves, P., Gorospe, M., and Munoz, A. (2003) *Mol. Cell. Biol.* **23,** 4991–5004

55. Park-Lee, S., Kim, S., and Laird-Offringa, I. A. (2003) *J. Biol. Chem.* **278,** 39801–39808

56. Yeap, B. B., Voon, D. C., Vivian, J. P., McCulloch, R. K., Thomson, A. M., Giles, K. M., Czyzyk-Krzeska, M. F., Furneaux, H., Wilce, M. C., Wilce, J. A., and Leedman, P. J. (2002) *J. Biol. Chem.* **277,** 27183–27192

57. Wilson, T., and Treisman, R. (1988) *Nature* **336,** 396–399

58. Chen, C. Y., Gherzi, R., Ong, S. E., Chan, E. L., Raijmakers, R., Pruijn, G. J., Stoecklin, G., Moroni, C., Mann, M., and Karin, M. (2001) *Cell* **107,** 451–464

59. Zhao, Z., Chang, F. C., and Furneaux, H. M. (2000) *Nucleic Acids Res.* **28,** 2695–2701

60. Aranda-Abreu, G. E., Behar, L., Chung, S., Furneaux, H., and Ginzburg, I. (1999) *J. Neurosci.* **19,** 6907–6917

61. Cok, S. J., Acton, S. J., and Morrison, A. R. (2003) *J. Biol. Chem.* **278,** 36157–36162

62. Duclert, A., and Changeux, J. P. (1995) *Physiol. Rev.* **75,** 339–368

63. Parker, M. H., Seale, P., and Rudnicki, M. A. (2003) *Nat. Rev. Genet.* **4,** 497–507

64. Sanes, J. R., and Lichtman, J. W. (1999) *Annu. Rev. Neurosci.* **22,** 389–442

65. Schaeffer, L., de Kerchove, d'Exaerde, A., and Changeux, J. P. (2001) *Neuron* **31,** 15–22

66. Akamatsu, W., Okano, H. J., Osumi, N., Inoue, T., Nakamura, S., Sakakibara, S., Miura, M., Matsuo, N., Darnell, R. B., and Okano, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9885–9890

67. Anderson, K. D., Morin, M. A., Beckel-Mitchener, A., Mobarak, C. D., Neve, R. L., Furneaux, H. M., Burry, R., and Perrone-Bizzozero, N. I. (2000) *J. Neurochem.* **75,** 1103–1114

68. Dobashi, Y., Shoji, M., Wakata, Y., and Kameya, T. (1998) *Biochem. Biophys. Res. Commun.* **244,** 226–229

69. Irwin, N., Chao, S., Goritchenko, L., Horiuchi, A., Greengard, P., Nairn, A. C., and Benowitz, L. I. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 12427–12431

70. Ohashi, S., Koike, K., Omori, A., Ichinose, S., Ohara, S., Kobayashi, S., Sato, T. A., and Anzai, K. (2002) *J. Biol. Chem.* **277,** 37804–37810

71. Sawaoka, H., Dixon, D. A., Oates, J. A., and Boutaud, O. (2003) *J. Biol. Chem.* **278,** 13928–13935

72. Lal, A., Mazan-Mamczarz, K., Kawai, T., Yang, X., Martindale, J. L., and Gorospe, M. (2004) *EMBO J.* **23,** 3092–3102

73. Lasa, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J., and Clark, A. R. (2000) *Mol. Cell. Biol.* **20,** 4265–4274

74. Raineri, I., Wegmueller, D., Gross, B., Certa, U., and Moroni, C. (2004) *Nucleic Acids Res.* **32,** 1279–1288

75. Wang, W., Fan, J., Yang, X., Furer-Galban, S., Lopez de Silanes, I, von Kobbe, C., Guo, J., Georas, S. N., Foufelle, F., Hardie, D. G., Carling, D., and Gorospe, M. (2002) *Mol. Cell. Biol.* **22,** 3425–3436

76. Li, H., Park, S., Kilburn, B., Jelinek, M. A., Henschen-Edman, A., Aswad, D. W., Stallcup, M. R., and Laird-Offringa, I. A. (2002) *J. Biol. Chem.* **277,** 44623–44630

77. Fan, X. C., and Steitz, J. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15293–15298

Reference List: Chapter 4

Akamatsu W, Fujihara H, Mitsuhashi T, Yano M, Shibata S, Hayakawa Y, Okano HJ, Sakakibara S, Takano H, Takano T, Takahashi T, Noda T, Okano H (2005) The RNA-binding protein HuD regulates neuronal cell identity and maturation. Proc Natl Acad Sci U S A 102: 4625-4630.

Alterio J, Mallet J, Biguet NF (2001) Multiple complexes involved in tyrosine hydroxylase mRNA stability in rat adrenal medulla, after reserpine stimulation. Mol Cell Neurosci 17: 179-189.

Anderson KD, Merhege MA, Morin M, Bolognani F, Perrone-Bizzozero NI (2003) Increased expression and localization of the RNA-binding protein HuD and GAP-43 mRNA to cytoplasmic granules in DRG neurons during nerve regeneration. Exp Neurol 183: 100-108.

Anderson KD, Sengupta J, Morin M, Neve RL, Valenzuela CF, Perrone-Bizzozero NI (2001) Overexpression of HuD accelerates neurite outgrowth and increases GAP-43 mRNA expression in cortical neurons and retinoic acid-induced embryonic stem cells in vitro. Exp Neurol 168: 250-258.

Anderson, K. D. and Steward, O. *In vivo* Expression of the Neuronal-specific RNA-binding Protein HuD in Reference to a Potential Target mRNA (GAP-43) in the Mouse Facial Nucleus During Regeneration. Society for Neuroscience Program No. 78.17. 2003. Ref Type: Abstract

Angus LM, Chan RY, Jasmin BJ (2001) Role of intronic E- and N-box motifs in the transcriptional induction of the acetylcholinesterase gene during myogenic differentiation. J Biol Chem 276: 17603-17609.

Antic D, Lu N, Keene JD (1999) ELAV tumor antigen, Hel-N1, increases translation of neurofilament M mRNA and induces formation of neurites in human teratocarcinoma cells. Genes Dev 13: 449-461.

Aranda-Abreu GE, Behar L, Chung S, Furneaux H, Ginzburg I (1999) Embryonic lethal abnormal vision-like RNA-binding proteins regulate neurite outgrowth and tau expression in PC12 cells. J Neurosci 19: 6907-6917.

Bakheet T, Williams BR, Khabar KS (2006) ARED 3.0: the large and diverse AU-rich transcriptome. Nucleic Acids Res 34: D111-D114.

Barreau C, Paillard L, Osborne HB (2005) AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res 33: 7138-7150.

Beckel-Mitchener AC, Miera A, Keller R, Perrone-Bizzozero NI (2002) Poly(A) Tail Length-dependent Stabilization of GAP-43 mRNA by the RNA-binding Protein HuD. J Biol Chem 277: 27996-28002.

Bernard V, Legay C, Massoulie J, Bloch B (1995) Anatomical analysis of the neurons expressing the acetylcholinesterase gene in the rat brain, with special reference to the striatum. Neuroscience 64: 995-1005.

Bolognani F, Merhege MA, Twiss J, Perrone-Bizzozero NI (2004) Dendritic localization of the RNA-binding protein HuD in hippocampal neurons: association with polysomes and upregulation during contextual learning. Neurosci Lett 371: 152-157.

Bolognani F, Tanner DC, Merhege M, Deschenes-Furry J, Jasmin B, Perrone-Bizzozero NI (2006) In vivo post-transcriptional regulation of GAP-43 mRNA by overexpression of the RNA-binding protein HuD. J Neurochem 96: 790-801.

Boudreau-Lariviere C, Chan RY, Wu J, Jasmin BJ (2000) Molecular mechanisms underlying the activity-linked alterations in acetylcholinesterase mRNAs in developing versus adult rat skeletal muscles. J Neurochem 74: 2250-2258.

Boudreau-Lariviere C, Sveistrup H, Parry DJ, Jasmin BJ (1996) Ciliary neurotrophic factor: regulation of acetylcholinesterase in skeletal muscle and distribution of messenger RNA encoding its receptor in synaptic versus extrasynaptic compartments. Neuroscience 73: 613-622.

Brimijoin S, Hammond P (1996) Transient expression of acetylcholinesterase messenger RNA and enzyme activity in developing rat thalamus studied by quantitative histochemistry and in situ hybridization. Neuroscience 71: 555-565.

Chan RY, Adatia FA, Krupa AM, Jasmin BJ (1998) Increased expression of acetylcholinesterase T and R transcripts during hematopoietic differentiation is accompanied by parallel elevations in the levels of their respective molecular forms. J Biol Chem 273: 9727-9733.

Chen CY, Shyu AB (1995) AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci 20: 465-470.

Chung S, Eckrich M, Perrone-Bizzozero N, Kohn DT, Furneaux H (1997) The Elav-like proteins bind to a conserved regulatory element in the 3'-untranslated region of GAP-43 mRNA. J Biol Chem 272: 6593-6598.

Coleman BA, Taylor P (1996) Regulation of acetylcholinesterase expression during neuronal differentiation. J Biol Chem 271: 4410-4416.

Cuadrado A, Navarro-Yubero C, Furneaux H, Munoz A (2003) Neuronal HuD gene encoding a mRNA stability regulator is transcriptionally repressed by thyroid hormone. J Neurochem 86: 763-773.

Deschenes-Furry J, Belanger G, Mwanjewe J, Lunde JA, Parks RJ, Perrone-Bizzozero N, Jasmin BJ (2005) The RNA-binding protein HuR binds to acetylcholinesterase transcripts and regulates their expression in differentiating skeletal muscle cells. J Biol Chem 280: 25361-25368.

Deschenes-Furry J, Belanger G, Perrone-Bizzozero N, Jasmin BJ (2003) Post-transcriptional regulation of Acetylcholinesterase mRNAs in nerve growth factor-treated PC12 cells by the RNA-binding protein HuD. J Biol Chem 278: 5710-5717.

Deschenes-Furry J, Perrone-Bizzozero N, Jasmin BJ (2006) The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity. Bioessays 28: 822-833.

Erondu NE, Nwankwo J, Zhong Y, Boes M, Dake B, Bar RS (1999) Transcriptional and posttranscriptional regulation of insulin-like growth factor binding protein-3 by cyclic adenosine 3',5'-monophosphate: messenger RNA stabilization is accompanied by decreased binding of a 42-kDa protein to a uridine-rich domain in the 3'-untranslated region. Mol Endocrinol 13: 495-504.

Farris TW, Woolf NJ, Oh JD, Butcher LL (1993) Reestablishment of laminar patterns of cortical acetylcholinesterase activity following axotomy of the medial cholinergic pathway in the adult rat. Exp Neurol 121: 77-92.

Federoff HJ, Geschwind MD, Geller AI, Kessler JA (1992) Expression of nerve growth factor in vivo from a defective herpes simplex virus 1 vector prevents effects of axotomy on sympathetic ganglia. Proc Natl Acad Sci U S A 89: 1636-1640.

Fernandes KJ, Kobayashi NR, Jasmin BJ, Tetzlaff W (1998) Acetylcholinesterase gene expression in axotomized rat facial motoneurons is differentially regulated by neurotrophins: correlation with trkB and trkC mRNA levels and isoforms. J Neurosci 18: 9936-9947.

Flumerfelt BA, Lewis PR (1975) Cholinesterase activity in the hypoglossal nucleus of the rat and the changes produced by axotomy: a light and electron microscopic study. J Anat 119: 309-331.

Ford LP, Wilusz J (1999) An in vitro system using HeLa cytoplasmic extracts that reproduces regulated mRNA stability. Methods 17: 21-27.

Forster E, Otten U, Frotscher M (1993) Developmental neurotrophin expression in slice cultures of rat hippocampus. Neurosci Lett 155: 216-219.

Fuentes ME, Taylor P (1993) Control of acetylcholinesterase gene expression during myogenesis. Neuron 10: 679-687.

Fujiwara T, Mori Y, Chu DL, Koyama Y, Miyata S, Tanaka H, Yachi K, Kubo T, Yoshikawa H, Tohyama M (2006) CARM1 Regulates Proliferation of PC12 Cells by Methylating HuD. Mol Cell Biol 26: 2273-2285.

Greene LA, Rukenstein A (1981) Regulation of acetylcholinesterase activity by nerve growth factor. Role of transcription and dissociation from effects on proliferation and neurite outgrowth. J Biol Chem 256: 6363-6367.

Grifman M, Galyam N, Seidman S, Soreq H (1998) Functional redundancy of acetylcholinesterase and neuroligin in mammalian neuritogenesis. Proc Natl Acad Sci U S A 95: 13935-13940.

Grubic Z, Zajc-Kreft K, Brank M, Mars T, Komel R, Miranda AF (1999) Control levels of acetylcholinesterase expression in the mammalian skeletal muscle. Chem Biol Interact 119-120: 309-319.

Guhaniyogi J, Brewer G (2001) Regulation of mRNA stability in mammalian cells. Gene 265: 11-23.

Hammond P, Rao R, Koenigsberger C, Brimijoin S (1994) Regional variation in expression of acetylcholinesterase mRNA in adult rat brain analyzed by in situ hybridization. Proc Natl Acad Sci U S A 91: 10933-10937.

Hew Y, Lau C, Grzelczak Z, Keeley FW (2000) Identification of a GA-rich sequence as a protein-binding site in the 3'-untranslated region of chicken elastin mRNA with a potential role in the developmental regulation of elastin mRNA stability. J Biol Chem 275: 24857-24864.

Hoover DB, Hancock JC (1985) Effect of facial nerve transection on acetylcholinesterase, choline acetyltransferase and [3H]quinuclidinyl benzilate binding in rat facial nuclei. Neuroscience 15: 481-487.

Hosli E, Hosli L (1970) The presence of acetylcholinesterase in cultures of cerebellum and brain stem. Brain Res 19: 494-496.

Jasmin BJ, Lee RK, Rotundo RL (1993) Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction. Neuron 11: 467-477.

Jiang JX, Choi RC, Siow NL, Lee HH, Wan DC, Tsim KW (2003) Muscle induces neuronal expression of acetylcholinesterase in neuron-muscle co-culture: transcriptional regulation mediated by cAMP-dependent signaling. J Biol Chem 278: 45435-45444.

Karpel R, Aziz-Aloya R, Sternfeld M, Ehrlich G, Ginzberg D, Tarroni P, Clementi F, Zakut H, Soreq H (1994) Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. Exp Cell Res 210: 268-277.

Kasashima K, Sakashita E, Saito K, Sakamoto H (2002) Complex formation of the neuron-specific ELAV-like Hu RNA-binding proteins. Nucleic Acids Res 30: 4519-4526.

Kasashima K, Terashima K, Yamamoto K, Sakashita E, Sakamoto H (1999) Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation. Genes Cells 4: 667-683.

Kaufer D, Friedman A, Seidman S, Soreq H (1998) Acute stress facilitates long-lasting changes in cholinergic gene expression. Nature 393: 373-377.

Keene JD, Tenenbaum SA (2002) Eukaryotic mRNPs may represent posttranscriptional operons. Mol Cell 9: 1161-1167.

Klimaschewski L, Kummer W, Heym C (1996) Localization, regulation and functions of neurotransmitters and neuromodulators in cervical sympathetic ganglia. Microsc Res Tech 35: 44-68.

Klimaschewski L, Tran TD, Nobiling R, Heym C (1994) Plasticity of postganglionic sympathetic neurons in the rat superior cervical ganglion after axotomy. Microsc Res Tech 29: 120-130.

Klingman GI, Klingman JD (1969) Cholinesterases of rat sympathetic ganglia after immunosympathectomy, decentralization and axotomy. J Neurochem 16: 261-268.

Koelle GB (1954) The histochemical localization of cholinesterases in the central nervous system of the rat. J Comp Neurol 100: 211-235.

Koenigsberger C, Chiappa S, Brimijoin S (1997) Neurite differentiation is modulated in neuroblastoma cells engineered for altered acetylcholinesterase expression. J Neurochem 69: 1389-1397.

Kohn DT, Tsai KC, Cansino VV, Neve RL, Perrone-Bizzozero NI (1996) Role of highly conserved pyrimidine-rich sequences in the 3' untranslated region of the GAP-43 mRNA in mRNA stability and RNA-protein interactions. Brain Res Mol Brain Res 36: 240-250.

Legay C (2000) Why so many forms of acetylcholinesterase? Microsc Res Tech 49: 56-72.

Legay C, Bon S, Vernier P, Coussen F, Massoulie J (1993) Cloning and expression of a rat acetylcholinesterase subunit: generation of multiple molecular forms and complementarity with a Torpedo collagenic subunit. J Neurochem 60: 337-346.

Lev-Lehman E, Deutsch V, Eldor A, Soreq H (1997) Immature human megakaryocytes produce nuclear-associated acetylcholinesterase. Blood 89: 3644-3653.

Lev-Lehman E, Evron T, Broide RS, Meshorer E, Ariel I, Seidman S, Soreq H (2000) Synaptogenesis and myopathy under acetylcholinesterase overexpression. J Mol Neurosci 14: 93-105.

Lopez de Silanes I, Zhan M, Lal A, Yang X, Gorospe M (2004) Identification of a target RNA motif for RNA-binding protein HuR. Proc Natl Acad Sci U S A 101: 2987-2992.

Luo Z, Fuentes ME, Taylor P (1994) Regulation of acetylcholinesterase mRNA stability by calcium during differentiation from myoblasts to myotubes. J Biol Chem 269: 27216-27223.

Luo ZD, Wang Y, Werlen G, Camp S, Chien KR, Taylor P (1999) Calcineurin enhances acetylcholinesterase mRNA stability during C2-C12 muscle cell differentiation. Mol Pharmacol 56: 886-894.

Ma WJ, Chung S, Furneaux H (1997) The Elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA. Nucleic Acids Res 25: 3564-3569.

Marusich MF, Furneaux HM, Henion PD, Weston JA (1994) Hu neuronal proteins are expressed in proliferating neurogenic cells. J Neurobiol 25: 143-155.

Massoulie J, Pezzementi L, Bon S, Krejci E, Vallette FM (1993) Molecular and cellular biology of cholinesterases. Prog Neurobiol 41: 31-91.

Meshorer E, Erb C, Gazit R, Pavlovsky L, Kaufer D, Friedman A, Glick D, Ben Arie N, Soreq H (2002) Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. Science 295: 508-512.

Meshorer E, Toiber D, Zurel D, Sahly I, Dori A, Cagnano E, Schreiber L, Grisaru D, Tronche F, Soreq H (2004) Combinatorial complexity of 5' alternative acetylcholinesterase transcripts and protein products. J Biol Chem 279: 29740-29751.

Mesulam MM, Geula C (1991) Acetylcholinesterase-rich neurons of the human cerebral cortex: cytoarchitectonic and ontogenetic patterns of distribution. J Comp Neurol 306: 193-220.

Michel RN, Vu CQ, Tetzlaff W, Jasmin BJ (1994) Neural regulation of acetylcholinesterase mRNAs at mammalian neuromuscular synapses. J Cell Biol 127: 1061-1069.

Mobarak CD, Anderson KD, Morin M, Beckel-Mitchener A, Rogers SL, Furneaux H, King P, Perrone-Bizzozero NI (2000) The RNA-binding protein HuD is required for GAP-43 mRNA stability, GAP-43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells. Mol Biol Cell 11: 3191-3203.

Neve RL, Howe JR, Hong S, Kalb RG (1997) Introduction of the glutamate receptor subunit 1 into motor neurons in vitro and in vivo using a recombinant herpes simplex virus. Neuroscience 79: 435-447.

Okano HJ, Darnell RB (1997) A hierarchy of Hu RNA binding proteins in developing and adult neurons. J Neurosci 17: 3024-3037.

Park-Lee S, Kim S, Laird-Offringa IA (2003) Characterization of the interaction between neuronal RNA-binding protein HuD and AU-rich RNA. J Biol Chem 278: 39801-39808.

Pascale A, Amadio M, Scapagnini G, Lanni C, Racchi M, Provenzani A, Govoni S, Alkon DL, Quattrone A (2005) Neuronal ELAV proteins enhance mRNA stability by a PKCalpha-dependent pathway. Proc Natl Acad Sci U S A 102: 12065-12070.

Pascale A, Gusev PA, Amadio M, Dottorini T, Govoni S, Alkon DL, Quattrone A (2004) Increase of the RNA-binding protein HuD and posttranscriptional up-regulation of the GAP-43 gene during spatial memory. Proc Natl Acad Sci U S A 101: 1217-1222.

Perrone-Bizzozero N, Bolognani F (2002) Role of HuD and other RNA-binding proteins in neural development and plasticity. J Neurosci Res 68: 121-126.

Perrone-Bizzozero NI, Neve RL, Irwin N, Lewis SE, Fischer I, Benowitz LI (1991) Post-transcriptional regulation of GAP-43 mRNA levels during neuronal differentiation and nerve regeneration. Mol Cell Neurosci 2: 402-409.

Perry C, Sklan EH, Birikh K, Shapira M, Trejo L, Eldor A, Soreq H (2002) Complex regulation of acetylcholinesterase gene expression in human brain tumors. Oncogene 21: 8428-8441.

Quattrone A, Pascale A, Nogues X, Zhao W, Gusev P, Pacini A, Alkon DL (2001) Posttranscriptional regulation of gene expression in learning by the neuronal ELAV-like mRNA-stabilizing proteins. Proc Natl Acad Sci U S A 98: 11668-11673.

Rotundo RL (2003) Expression and localization of acetylcholinesterase at the neuromuscular junction. J Neurocytol 32: 743-766.

Sagesser R, Martinez E, Tsagris M, Tabler M (1997) Detection and isolation of RNA-binding proteins by RNA-ligand screening of a cDNA expression library. Nucleic Acids Res 25: 3816-3822.

Schober A, Minichiello L, Keller M, Huber K, Layer PG, Roig-Lopez JL, Garcia-Arraras JE, Klein R, Unsicker K (1997) Reduced Acetylcholinesterase (AChE) Activity in Adrenal Medulla and Loss of Sympathetic Preganglionic neurons in TrkA-Deficient but Not TrkB-Deficient, Mice. J Neurosci 17: 891-903.

Seidman S, Sternfeld M, Aziz-Aloya R, Timberg R, Kaufer-Nachum D, Soreq H (1995) Synaptic and epidermal accumulations of human acetylcholinesterase are encoded by alternative 3'-terminal exons. Mol Cell Biol 15: 2993-3002.

Sharma KV, Koenigsberger C, Brimijoin S, Bigbee JW (2001) Direct evidence for an adhesive function in the noncholinergic role of acetylcholinesterase in neurite outgrowth. J Neurosci Res 63: 165-175.

Siow NL, Choi RC, Cheng AW, Jiang JX, Wan DC, Zhu SQ, Tsim KW (2002) A cyclic AMP-dependent pathway regulates the expression of acetylcholinesterase during myogenic differentiation of C2C12 cells. J Biol Chem 277: 36129-36136.

Siow NL, Xie HQ, Choi RC, Tsim KW (2005) ATP induces the post-synaptic gene expression in neuron-neuron synapses: Transcriptional regulation of AChE catalytic subunit. Chem Biol Interact 157-158: 423-426.

Soreq H, Seidman S (2001) Acetylcholinesterase--new roles for an old actor. Nat Rev Neurosci 2: 294-302.

Sternfeld M, Ming G, Song H, Sela K, Timberg R, Poo M, Soreq H (1998) Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. J Neurosci 18: 1240-1249.

Sun Y, Zigmond RE (1996) Involvement of leukemia inhibitory factor in the increases in galanin and vasoactive intestinal peptide mRNA and the decreases in neuropeptide Y and tyrosine hydroxylase mRNA in sympathetic neurons after axotomy. J Neurochem 67: 1751-1760.

Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, Henson J, Posner JB, Furneaux HM (1991) HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. Cell 67: 325-333.

Taxi J, Eugene D (1995) Effects of axotomy, deafferentation, and reinnervation on sympathetic ganglionic synapses: a comparative study. Int Rev Cytol 159: 195-263.

Tenenbaum SA, Lager PJ, Carson CC, Keene JD (2002) Ribonomics: identifying mRNA subsets in mRNP complexes using antibodies to RNA-binding proteins and genomic arrays. Methods 26: 191-198.

Tetzlaff W, Kreutzberg GW (1984) Enzyme changes in the rat facial nucleus following a conditioning lesion. Exp Neurol 85: 547-564.

Viana GB, Kauffman FC (1984) Cholinesterase activity in the rat superior cervical ganglion: effect of denervation and axotomy. Brain Res 304: 37-45.

Wan DC, Choi RC, Siow NL, Tsim KW (2000) The promoter of human acetylcholinesterase is activated by a cyclic adenosine 3',5'-monophosphate-dependent pathway in cultured NG108-15 neuroblastoma cells. Neurosci Lett 288: 81-85.

Wilson GM, Brewer G (1999) Identification and characterization of proteins binding A + U-rich elements. Methods 17: 74-83.

Xu N, Chen CY, Shyu AB (1997) Modulation of the fate of cytoplasmic mRNA by AU-rich elements: key sequence features controlling mRNA deadenylation and decay. Mol Cell Biol 17: 4611-4621.

Young C, Lindsay S, Vater R, Slater CR (1998) An improved method for the simultaneous demonstration of mRNA and esterase activity at the human neuromuscular junction. Histochem J 30: 7-11.

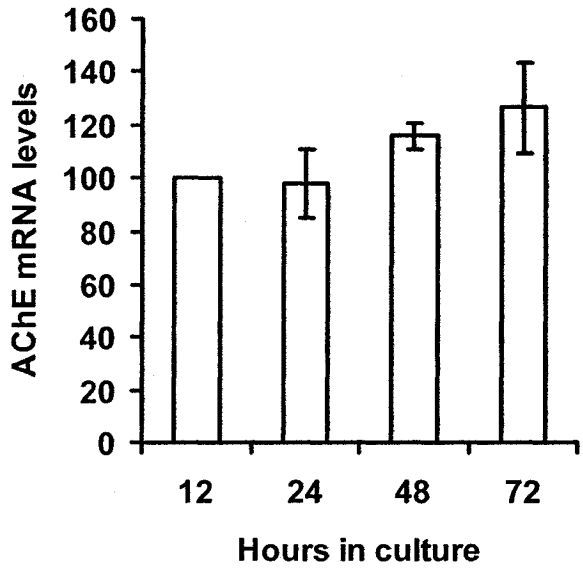

Figure 1. AChE mRNA levels remain unchanged during proliferative growth of PC12 cells. AChE mRNA levels from undifferentiated PC12 cells cultured for 12-, 24-, 48-, and 72-hours were quantified by RT-PCR and expressed as a percentage of the level observed after 12-hours of culture (n = 2 independent experiments).

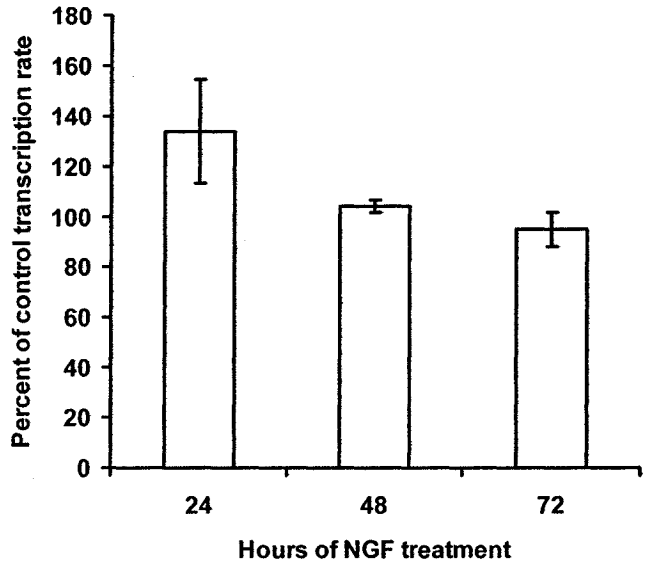

Figure 2. AChE transcription rate increases early during neuronal differentiation of PC12 cells. AChE transcription rate was determined by nuclear run-on assays performed with nuclei extracted from control, 24-, 48- and 72-hour differentiated PC12 cells (n = 2 independent experiments).

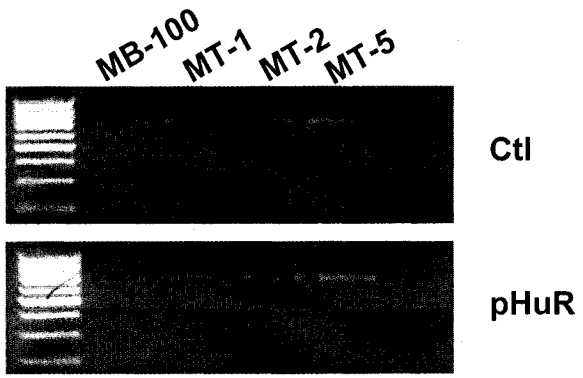

Figure 1. Overexpression of HuR increases AChE mRNA levels in C2C12 myotubes. Representative ethidium bromide-stained agarose gels displaying AChE RT-PCR products from 100% confluent myoblasts (MB-100), 1-, 2-, and 5-day myotubes (MT-1, MT-2 and MT-5, respectively) of control (Ctl) and HuR overexpressing (pHuR) C2C12 cells.

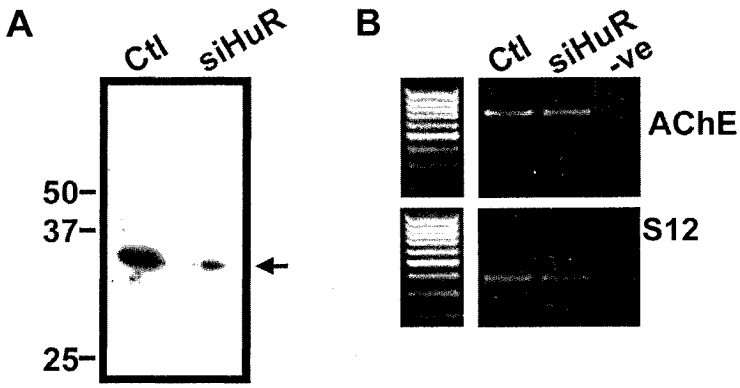

Figure 2. Knockdown of HuR protein levels decreases AChE mRNA levels. *A*, Western blot depicting decreased HuR protein levels in undifferentiated C2C12 cells transiently transfected with siRNAs specifically directed to HuR (siHuR) compared to control (Ctl) cells. Arrow points to the band corresponding to HuR. *B*, representative ethidium bromide-stained agarose gels displaying AChE and S12 ribosomal protein (S12) RT-PCR products in control (Ctl) and C2C12 cells transiently transfected with HuR siRNAs (siHuR).

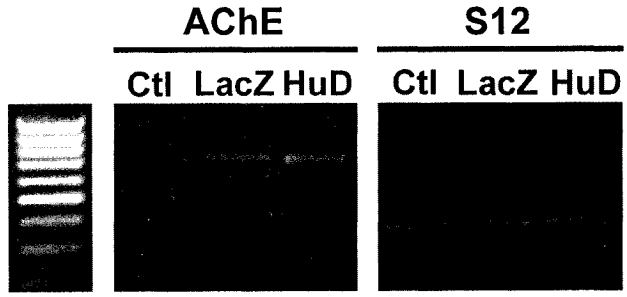

Figure 3. Overexpression of HuD in C2C12 cells increases AChE mRNA levels. Representative ethidium bromide-stained agarose gels displaying AChE and S12 ribosomal protein (S12) RT-PCR products from control (Ctl) C2C12 cells, and cells infected with a LacZ expressing adenovirus or HuD expressing adenovirus.

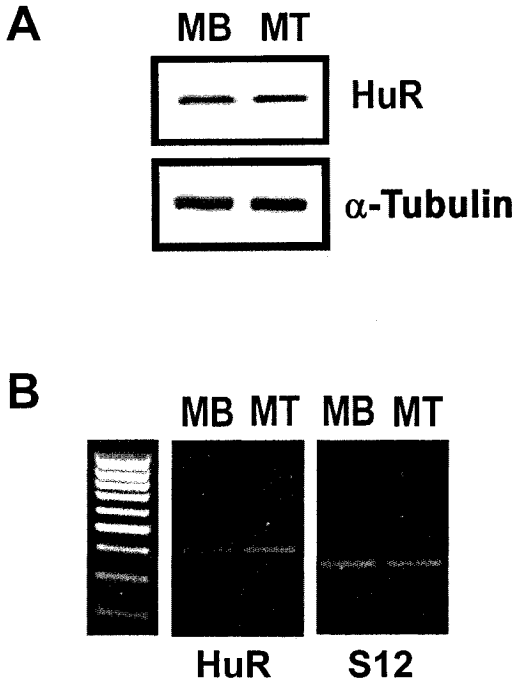

Figure 4. HuR protein and mRNA levels remain unchanged during myogenic differentiation. *A*, representative immunoblot displaying HuR and α-tubulin protein levels in C2C12 myoblasts (MB) and 2-day differentiated myotubes (MT). *B*, representative ethidium bromide-stained agarose gels displaying HuR and S12 ribosomal protein (S12) RT-PCR products from C2C12 myoblasts and 2-day differentiated myotubes.

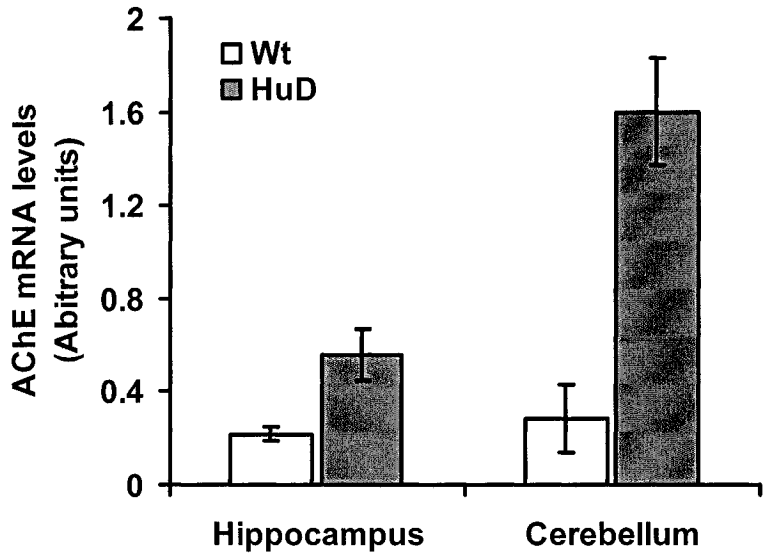

Figure 1. AChE mRNA levels are increased in specific brain regions of HuD transgenic mice. AChE mRNA levels in the hippocampus and cerebellum of wildtype (Wt) and HuD overexpressing transgenic mice were quantified by RT-PCR and expressed as arbitrary units.

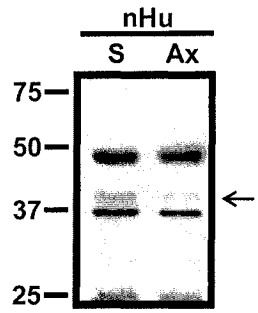

Figure 2. Neuronal Hu proteins decrease following SCG axotomy. Representative immunoblot displaying neuronal Hu proteins from sham-operated (S) and 2-day axotomized (Ax) rat SCG. Arrow points to the band corresponding to HuD.

Appendix D: Julie Deschênes-Furry CV

EDUCATION

1998-present	Doctoral candidate Cellular and Molecular Medicine, Neuroscience University of Ottawa
1994-1998	Bachelors of Science Honors Physiology University of Ottawa

PUBLICATIONS

Journal articles:

1. Deschênes-Furry J, Mousavi K, Bolognani F, Neve RL, Parks RJ, Perrone-Bizzozero N, Jasmin BJ. Role of the RNA-binding protein HuD in post-transcriptional regulation of acetylcholinesterase following axotomy of the superior cervical ganglion. 2006 Accepted J. Neurosci.

2. Bolognani F, Tanner DC, Merhege M, Deschênes-Furry J, Jasmin B, Perrone-Bizzozero NI. In vivo post-transcriptional regulation of GAP-43 mRNA by overexpression of the RNA-binding protein HuD. J Neurochem. 2006; 96: 790-801.

3. Deschênes-Furry J, Angus LM, Bélanger G, Mwanjewe J, Jasmin BJ. Role of ELAV-like RNA-binding proteins HuD and HuR in the post-transcriptional regulation of acetylcholinesterase in neurons and skeletal muscle cells. Chem Biol Interact. 2005; 15: 43-49.

4. Deschênes-Furry J, Belanger G, Mwanjewe J, Lunde JA, Parks RJ, Perrone-Bizzozero N, Jasmin BJ. The RNA-binding protein HuR binds to acetylcholinesterase transcripts and regulates their expression in differentiating skeletal muscle cells. J Biol Chem 2005; 280: 25361-25368.

5. Deschênes-Furry J, Bélanger G, Perrone-Bizzozero N, and Jasmin BJ. Post-transcriptional regulation of acetylcholinesterase mRNAs in nerve growth factor-treated PC12 cells by the RNA-binding protein HuD. J. Biol Chem. 2003; 278:5710-5717.

6. De Repentigny Y*, Deschênes-Furry J*, Jasmin BJ, and Kothary, R. Impaired fast axonal transport in neurons of the sciatic nerves from *dystonia musculorum* mice. J. Neurochem. 2003; 86:564-571 (* Equal first authors)

7. Chakkalakal JV, Stocksley MA, Harrison M, Angus LM, Deschênes-Furry J, St-Pierre S, Megeney LA, Chin ER, Michel RN, and Jasmin BJ. Expression of utrophin A mRNA correlates with the oxidative capacity of skeletal muscle fibers and is regulated by calcineurin/NFAT signaling. Proc. Natl. Acad. Sci. U.S.A. 2003; 100:7791-7796

Review articles and book chapters:

1. Deschênes-Furry J, Perrone-Bizzozero N, Jasmin BJ. The RNA-binding protein HuD: a regulatory of neuronal differentiation, maintenance and plasticity. BioEssays 2006; 28: 822-833.

2. Deschênes-Furry J, Angus LM, Belanger G, Mwanjewe J, Jasmin BJ. Role of ELAV-like RNA-binding proteins HuD and HuR in the post-transcriptional regulation of acetylcholinesterase in neurons and skeletal muscle cells. Chem Biol Interact 2005; 157-158: 43-49.

3. Deschênes-Furry JL, Angus LM, Bélanger G, Mousavi K, Jasmin BJ. Neurotrophins regulate acetylcholinesterase expression in neurons via post-transcriptional mechanisms. In: Inestrosa, N.C. and Campos, E.O. (Eds) Cholinesterases in the Second Millennium: Biomolecular and Pathological Aspects, Diseno e Impresiones, Chili, 2004; pp 25-30.

Meeting abstracts:

1. Deschênes-Furry J, Bélanger G, Angus LM, Lunde JA, and Jasmin BJ. The RNA-binding protein HuR regulates acetylcholinesterase mRNA expression during myogenic differentiation. Presentation No. 2047. American Society for Cell Biology, 2003

2. Deschênes-Furry JL, Mousavi K, Smith LE, Parks RJ, Perrone-Bizzozero N, and Jasmin BJ. Post-transcriptional regulation of acetylcholinesterase mRNAs in axotomized neurons. Program No. 471.15. Society for Neuroscience, 2003

3. Jasmin BJ, Angus LM, Bélanger G, Chakkalakal JV, Deschênes-Furry J, Jones MA, Stocksley MA, Thompson J. Multiple signaling events regulate the expression and localization of utrophin in skeletal muscle fibers. Mol. Biol. Muscle Dev. Regen., 2003

4. Chakkalakal JV, Stocksley MA, Deschênes-Furry J, Chin ER, Harrison M-A., Michel RN, and Jasmin BJ. Expression of Utrophin A mRNA Correlates with the Metabolic Efficiency of Muscle and Involves Calcineurin. Program No. 598.7.

Experimental Biology, 2003

5. Deschênes-Furry JL, Bélanger G, Perrone-Bizzozero NI, and Jasmin BJ. Nerve growth factor regulates acetylcholinesterase expression in PC12 cells via post-transcriptional mechanisms. Program No. 30.17. Society for Neuroscience, 2002.

6. Bélanger G, Deschênes-Furry JL, Angus LM, and Jasmin BJ. Plasticity of acetylcholinesterase expression in skeletal muscle involves cis-acting elements located in the 3'UTR. Program No. 28.13. Society for Neuroscience, 2002.

7. Jasmin BJ, Angus LM, Bélanger G, Deschênes JL, Nasrallah F. Transcriptional and post-transcriptional events controlling expression of acetylcholinesterase in developing and adult muscles. Xith International Symposium on Cholinergic Mechanisms, 2002

8. Deschênes JL, Viau F, Chin E, Michel RN, and Jasmin BJ. Role of calcineurin in the regulation of synaptic proteins in skeletal muscle. Program No. 411.1. Society for Neuroscience, 2000

ADDITIONAL PROFESSIONAL ACTIVITIES

Jan-April 2006:	Part-time professor, University of Ottawa Faculty of Health Science, ANP1703b Anatomie et physiologie des divers appareils II
2002-2006:	Teaching assistant, University of Ottawa Faculty of Health Science, ANP 2502 Anatomie de l'appareil locomoteur et sciences neurologiques
2001-2003:	Teaching assistant, University of Ottawa School of Medicine, MED 2504 Système nerveux (Anatomy laboratories)

AWARDS RECEIVED

Awards:

2000:	Outstanding Student Seminar Award (Doctoral program)
1999:	The Gerry Taichman Award for best research achievement by a graduate student (Master's program)
1999:	Outstanding Student Seminar Award (Master's program)

Scholarships:

2000-2002:	Ontario Neurotrauma Foundation
2000-2002:	University of Ottawa Excellence Scholarship
2000-2001:	Ontario Graduate Scholarship (declined)
2000-2001:	Ontario Graduate Scholarship for Science and Technology (declined)
2000:	University of Ottawa Admission Scholarship
1999-2000:	Ontario Graduate Scholarship for Science and Technology
1998-1999:	University of Ottawa Admission Scholarship