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The Molecular Mechanisms Regulating the Expression of Acetylcholinesterase in Cholinergic Neurons

by

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A thesis submitted to the School of Graduate Studies and Research

University of Ottawa

in partial fulfilment of the requirements for the degree of

Master of Science

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

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ABSTRACT

Acetylcholinesterase (AChE) is responsible for the rapid hydrolysis of acetylcholine into acetic acid and choline, thereby ensuring precise temporal control of synaptic transmission. The role of AChE has been well documented with respect to the neuromuscular junction where it performs this classic function. However, it has recently been discovered that AChE is also expressed in a variety of non-cholinergic tissues such as haematopoietic cells and selected populations of neurons. The role of the enzyme in non-cholinergic neuronal cells is currently unknown and its regulation in this population of cells has been generally overlooked. In this study, we established a cell model to study the developmental and trophic regulation of AChE in neuronal cells. In addition, we examined the regulation of the acetylcholine-synthesizing enzyme, choline acetyltransferase (ChAT) to elucidate the relationship between the two cholinergic enzymes.

In a first series of experiments, we tested several hybrid cell lines for AChE activity. We examined two septal-neuroblastoma hybrids (SN6 and SN56), and two spinal cord motoneuron-neuroblastoma hybrids (NSC-19 and NSC-34). Total AChE activity was highest in the NSC-34 cell line. We further characterized the NSC-34 motoneuronal cell line according to their morphology and determined that they resembled developing primary motoneurons. We also examined the effect of depolarizing agents such as potassium, veratridine and verapamil on AChE and

ChAT activities. The two cholinergic enzymes were differentially regulated by these various agents in a fashion similar to that observed in primary motoneurons. On the basis of these data, we determined that the NSC-34 motoneuronal cell line was an appropriate model for the study of AChE expression in neurons.

Next, we examined the developmental regulation of AChE and ChAT using NSC-34 motoneurons. During the course of neuronal development, we observed an overall increase in AChE expression which resulted mainly from an increase in the G_4 form of the enzyme. The differentiation-induced increase was accompanied by a shift from nonamphiphilic to amphiphilic species of G_4. The results obtained from our studies indicate that this increase was the result of a selective stabilization of AChE mRNAs, as opposed to an enhancement of transcript. In addition, ChAT activity and mRNA levels increased in parallel during development. The accumulation of ChAT mRNA was also due to stabilization of the transcript. We also demonstrated the potent effects of several neurotrophic factors on AChE and ChAT expression. The trkB ligands BDNF and NT-4 increased AChE and ChAT activities, whereas NT-3 had little effect. This may be attributed to the presence of inserted forms of the NT-3 receptor, trkC, in NSC-34 motoneurons. In addition, the neural cytokines bFGF and axokine dramatically increased both AChE and ChAT expression. When combined with the neurotrophins however, the effects were not found to be additive.

Together, these results demonstrate that AChE and ChAT are co-regulated during development and differentially regulated in response to electrical activity. The neurotrophins appear to have limited effects on uninjured neurons and may thus preferentially regulate expression of cholinergic enzymes in axotomized neurons. Conversely, neural cytokines appear to play a more important role in the maintenance of AChE and ChAT expression in healthy, uninjured cells. Thus several distinct factors appear to act together to regulate levels of AChE and ChAT in order to achieve precise control of cholinergic neurotransmission.

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ABBREVIATIONS

A forms	Asymmetric acetylcholinesterase molecular forms
ACh	Acetylcholine
AChE	Acetylcholinesterase
bFGF	Basic fibroblast growth factor
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium
ChAT	Choline acetyltransferase
cDNA	Complementary deoxyribonucleic acid
CNTF	Ciliary neurotrophic factor
DEPC	Diethylpyrocarbonate
E	Embryonic day
G forms	Globular acetylcholinesterase molecular forms
GPI	Glycophosphatidylinositol
H	Hydrophobic
NGF	Nerve growth factor
NSC	Neuroblastoma-spinal cord hybrid
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
R	Readthrough
T	Tailed
trk	Tyrosine kinase

CHAPTER 1

INTRODUCTION

Acetylcholinesterase (AChE) is an essential component of cholinergic synapses in both the central and peripheral nervous systems (Brimijoin, 1983; Massoulié et al., 1993; Fernandez et al., 1996). Within these specialized structures, AChE is responsible for the rapid hydrolysis of acetylcholine (ACh) released from the presynaptic nerve terminals, thereby ensuring precise spatial and temporal control of synaptic transmission. The proper functioning of the enzyme is of such importance that its inhibition leads rapidly to death of the organism. Following its discovery by Dale in 1914, AChE quickly became one of the most widely studied enzymes. The appeal of AChE lay in its complexity. AChE was demonstrated to be one of the fastest enzymes known, hydrolyzing ACh into acetate and choline at maximum theoretical limits of substrate hydrolysis (Quinn, 1987). The enzyme also displayed an unusual molecular polymorphism, reflecting various modes of association with the cell surface (Massoulié and Bon, 1982). In addition, the presence of AChE outside cholinergic synapses and the existence of a related enzyme, butyrylcholinesterase (BChE), whose function is unclear, suggest that AChE might also perform additional, non-catalytic functions, perhaps in growth and development (Massoulié et al., 1993). More recently, AChE has been associated with several pathological conditions including Alzheimer's disease, amyotrophic lateral sclerosis and various haematological disorders, thereby suggesting that expression of this enzyme may be even more complex than initially anticipated.

1.1 MOLECULAR FORMS OF AChE

Mammalian AChE is a glycoprotein of 70-80 kDa which exists as monomers

or oligomers of catalytic subunits bound together by disulfide bonds. These size isomers, or molecular forms, share similar catalytic properties but differ in their hydrodynamic properties and modes of association with the cell surface (Massoulié et al., 1993). The molecular forms of AChE can be divided into two classes: globular (G) forms and asymmetric (A) forms (see Figure 1).

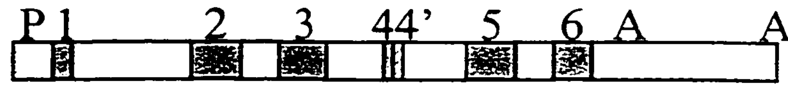
The globular forms exist as monomers (G_1), dimers (G_2) and tetramers (G_4) with sedimentation coefficients of 4.5S, 6S, and 10S, respectively. The globular forms may also be characterized as amphiphilic (G^a) and nonamphiphilic (G^{na}). Amphiphilic forms possess a hydrophobic domain which is believed to have a function in anchoring the enzyme to the membrane. Amphiphilic and nonamphiphilic forms may be easily distinguished through sedimentation analysis using the detergent Brij-96 which binds the amphiphilic elements and forms slow sedimenting complexes (Massoulié et al., 1993).

The globular monomer appears to be the fundamental building block of the enzyme, forming dimers via disulfide bonds, which in turn can aggregate into tetramers of catalytic subunits. Excess G_1 is destined for either secretion or intracellular degradation (Brockman and Younkin, 1986; Rotundo, 1988). As aforementioned, AChE globular forms may also associate with noncatalytic, structural subunits. A fraction of G_2 dimers have been shown to possess a glycophosphatidylinositol (GPI) anchor which is linked to the C-terminal amino acid residue of the catalytic subunit by an amide bond (Silman and Futerman, 1987; Low,

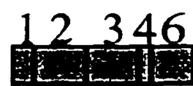
Figure 1. **The AChE Gene and Molecular Forms**

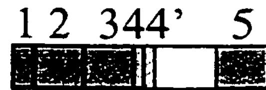
Schematic representation of splicing at the 3' end of the AChE coding sequence. The white bars represent introns, grey bars represent exons (numbered 1 - 6). The stippled bars represent the promoter region (P) and the PolyA tail (A). Also shown is the structure of the major forms of AChE in vertebrates. The H transcript encodes G_2 AChE which possesses a GPI anchor. The T transcript encodes G_1 and G_2, however with different anchoring subunits. It also encodes hydrophobic-tailed G_4 and the asymmetric forms. The protein product of the R transcript is a secreted monomer whose expression however, has yet to be demonstrated *in vivo*.

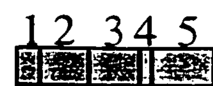
Mammalian AChE Gene



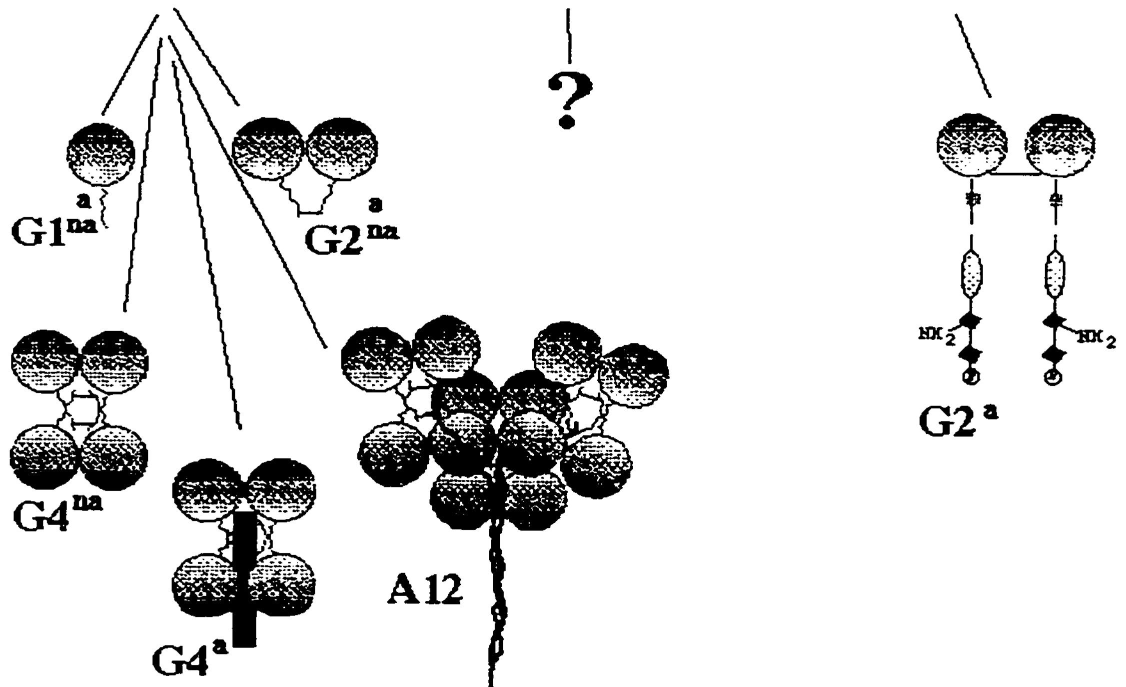
mRNAs







T (Tail) R (Readthrough) H (Hydrophobic)



1989). The GPI anchor allows for association of this molecular form with the plasma membrane. The GPI-linked dimers exist predominantly on the surface of mature erythrocytes and T lymphocytes (Ott et al., 1982; Rosenberry and Scoggin, 1984; Roberts et al., 1987), however they may also be found within the central and peripheral nervous systems (Skau and Brimijoin, 1980; Clark and Lenz, 1983). The predominant form of AChE in the mammalian central nervous system is an amphiphilic, hydrophobic-tailed tetrameric form (G_4^a) (Massoulié et al., 1993). This G_4 form is composed of two pairs of catalytic subunits, one of which is bound by disulfide bonds near the C-terminal end and the other which is bound through disulfide bonds to a 20 kDa non-catalytic hydrophobic subunit (Inestrosa et al., 1987; Fuentes and Inestrosa, 1988; Fuentes et al., 1988). This non-catalytic, structural subunit is designated P (Massoulié et al., 1993), and serves to anchor the tetrameric form to the cell membrane.

The asymmetric forms of AChE are characterized by their interaction with a collagen-like structural subunit, designated Q. The Q subunit is composed of a triple helical association of three collagenic subunits. The asymmetric forms are designated A_4, A_8 and A_{12}, and comprise 1, 2 or 3 tetramers, respectively, bound to the collagen-like tail via disulfide bonds. These forms are easily identified by their characteristic sedimentation coefficients: 16-20S (A_{12}), 14-15S (A_8) and 8.5S (A_4) (Massoulié et al., 1993). Asymmetric forms are also characterized by their ability to associate with polyanionic molecules such as glycosaminoglycans. It is this capacity which allows the asymmetric forms to attach to cellular matrices such as the

neuromuscular basal lamina (McMahan et al., 1978). Typically, these forms require high salt for efficient solubilization but they may also be solubilized by heparin suggesting that heparin sulfate proteoglycans play a role in anchoring the asymmetric forms to the basal lamina (Torres and Inestrosa, 1983; von Bernhardi and Inestrosa, 1990; Massoulié et al., 1993). However, it has been shown that several other unrelated polyanions can efficiently solubilize asymmetric AChE, thus rendering it difficult to designate a single glycosaminoglycan responsible for binding the asymmetric forms to the extracellular matrix (Pérez-Tur et al., 1991).

1.2 THE AChE GENE

Vertebrates possess a single gene encoding the catalytic subunit of AChE (Rachinsky et al., 1990; Li et al., 1991, 1993 a,b; Getman et al., 1995; Legay et al., 1995; Mutero et al., 1995). The mammalian AChE gene is approximately 7.0 kb in length and is located on chromosome 7, quadrant 22, in humans (Getman et al., 1992), and on chromosome 5 in mouse (Rachinsky et al., 1992). This single gene is responsible for directing the synthesis of all AChE molecular forms and it undergoes alternative splicing at its 3' end to generate several splice variants differing in their C-terminal regions . The first two exons (exons 2 and 3) and part of exon 4 within the open reading frame of the gene constitute the common coding region and encode the first 535 amino acids of the protein, including the catalytic domain and the signal peptide (Maulet et al., 1990) (see Figure 1). The remaining exons are subject to alternative splicing and give rise to three distinct transcripts designated T (tailed), H (hydrophobic) and R (readthrough) (Legay et al., 1993).

The T transcript arises from splicing of exon 4 to exon 6. Alternatively, the H transcript results from splicing of exon 4 to exon 5, whereas the R transcript results from reading through the splice donor site of exon 4 into the subsequent genomic sequence such that the transcripts are not spliced at that position (Massoulié et al., 1993).

The T transcript is the predominant species found in mammalian muscle and nervous tissues (Li et al., 1991). It participates in the formation of the asymmetric forms as well as the soluble and membrane-bound G_4, soluble G_2 and G_1. The T sequence (exon 6) encodes a C-terminal region which is maintained in the mature protein and participates in disulfide bonding of the catalytic subunit to the 20 kDa, noncatalytic P subunit of the hydrophobic-tailed G_4 and the collagenic Q subunit of the asymmetric forms (Massoulié et al., 1993; Legay et al., 1995). Alternatively, the H transcript is predominant in cells of haematopoietic origin, and encodes the GPI-linked dimer. The H sequence (exon 5) encodes a hydrophobic, C-terminal region which contains a GPI cleavage/attachment site. During post-translational processing, the distal region of exon 5 is cleaved and replaced by a glycophospholipid thus allowing for the formation of GPI-anchored dimers (Gibney et al., 1988; Li et al., 1991). Both the H and T catalytic subunits possess cysteine residues near their C-terminal ends which are believed to participate in intersubunit disulfide bonds (Lockridge et al., 1987). In contrast, the R transcript lacks the cysteine residues required for oligomer formation. Although the resulting protein does not appear necessary to account for the observed molecular forms of AChE,

it has been reported that the R transcript yields a hydrophilic monomer (Li et al., 1991, 1993b; Rachinsky et al., 1992). The R transcript has been detected *in vitro* in myogenic cell lines, both as myoblasts and myotubes (Legay et al., 1995),and in hematopoietic cells (Chan et al., 1998). Only recently has the transcript been identified *in vivo*, in the mouse diaphragm (Legay et al., 1995) and in the brain (Kaufer et al., 1998). It is possible that the R transcripts represent products of an immature splicing mechanism, without any real function. However, the forms resulting from this transcript may in fact play a transient role during muscle development (Legay et al., 1995).

1.3 LOCALIZATION OF AChE IN THE NERVOUS SYSTEM

AChE is present in high concentrations in mammalian brain (Siek et al., 1990). The enzyme is concentrated in cholinergic centers like the striatum, basal forebrain, superior colliculus, and motor nuclei of cranial nerves (Landwehrmeyer et al., 1993; Hammond et al., 1994; Bernard et al., 1995). These studies have also detected significant levels of both AChE activity and mRNA in the medial septum, thalamus and the spinal cord. However, in certain other areas such as the hippocampus, cerebellum and substantia nigra, noncholinergic cells express high levels of AChE mRNA in the absence of other cholinergic markers (Landwehrmeyer et al., 1993; Hammond et al., 1994; Bernard et al., 1995) thus questioning the role of the enzyme is these regions. Within the mammalian nervous system the predominant form of AChE is of the globular type, generated by the T transcript. In

fact, 80-90% of AChE in the nervous system is the G_4^a form, with the remainder occurring as G_1, G_2 and A_{12} (Grassi et al., 1982). Within the spinal cord, anterior and dorsal horn motoneurons contain significant amounts of AChE (Landwehrmeyer et al., 1993). The molecular form content however, varies according to the type of muscle which is innervated. Motoneurons innervating slow muscles, such as the soleus, contain much less G_4 than those innervating fast muscles like the extensor digitorum longus (EDL) (Gisiger and Stephens, 1982).

The subcellular localization of AChE molecular forms was characterized by Rotundo and Carbonetto (1987) in cultured sympathetic neurons. They reported that the dominant form was indeed a membrane-bound G_4 which was transported preferentially to the nerve fiber where it aggregated into clusters following its synthesis in the cell body. Earlier stop-flow experiments established that AChE moved by rapid axonal transport (Brimijoin, 1975). It was also established that although resting nerve had no detectable A_{12}, ligated nerves accumulated A_{12} at an astounding rate, whereas G_4 accumulation was disproportionately slow (Skau and Brimijoin, 1980; Fernandez et al., 1980). These experiments thus supported the concept that there were two separate streams of AChE, moving at different velocities, in neuronal cells. The fate of the G_4 was, presumably, insertion into the axolemma, whereas A_{12} appeared to be destined for the synapse (Brimijoin, 1983). Although AChE is secreted by neurons, it represents only a fraction of the enzyme found at the neuromuscular junction, thus the function of the secreted enzyme is still largely unknown (Di Giamberardino and Couraud, 1978).

In contrast to the nervous system, skeletal muscle contains a range of asymmetric forms, predominantly A_{12} and A_8. However, differences in the pattern of AChE molecular forms exist between fast and slow muscles. For example, the fast EDL muscle contains mostly G_1, G_4 and A_{12}, whereas the slow soleus presents a more complex distribution including G_2, A_4 and A_8 with significantly less G_4 (Groswald and Dettbarn, 1983). These forms are generally concentrated at the neuromuscular junction. However, significant amounts of AChE are retained outside of the synapse. Typically, the asymmetric forms are found in the synaptic cleft in association with the basal lamima (McMahan et al., 1978). At this location, the enzyme is believed to regulate the amount of acetylcholine reaching the acetylcholine receptors (AChRs) of the postsynaptic membrane (Massoulié et al., 1993). Conversely, membrane-bound G_4 is located perisynaptically, in the area surrounding the synapse (Gisiger and Stephens, 1988). Here, it may act to modulate the ambient levels of ACh molecules in the synaptic cleft. In contrast, G_1 monomers and G_2 dimers are almost entirely intracellular, in association with the sarcoplasmic reticulum (Donoso and Fernandez, 1985).

1.4 NON-CHOLINERGIC FUNCTION OF AChE

Although AChE has traditionally been studied in cholinergic tissues where it performs its classical function, it is well documented that the enzyme is also expressed in a variety of non-cholinergic tissues. For example, haematopoietic cells express significant levels of AChE and recent evidence suggests that in these cells, the enzyme may participate directly in the basic mechanisms underlying cellular

differentiation (Paoletti et al., 1992; Soreq et al., 1994; Chan et al., 1998). Moreover, as already mentioned, Northern blot analysis and *in situ* hybridization experiments have shown the presence of AChE mRNAs in non-cholinergic areas within the central nervous system, such as the hippocampus and cerebellum (Legay et al., 1993; Landwehrmeyer et al., 1993; Hammond et al., 1994; Bernard et al., 1995). The presence of significant levels of AChE in non-cholinergic neuronal tissues has led to the hypothesis that AChE may perform additional, non-catalytic functions similar to those proposed for haematopoietic cells.

In recent years, several studies have shown that AChE is expressed at early stages of neurogenesis. Interestingly, the pattern of AChE expression precedes the establishment of synaptic transmission and coincides with the period of neurite outgrowth (see Layĕr and Willbold, 1995 for review). In addition to these studies which examined expression of AChE during early stages of neurogenesis, several *in vitro* studies directly support the notion that AChE is involved in neurite outgrowth (Layer et al., 1993; Dupree and Bigbee, 1994, 1996; Jones et al., 1995; Inestrosa et al., 1996; Beeri et al., 1997; Holmes et al., 1997; Robitzki et al., 1997; Srivatsan and Peretz, 1997; Koenigsberger et al., 1997; Sternfeld et al., 1998; Keymer et al., 1999). Layer and colleagues (1993), for example, showed a profound reduction in neurite outgrowth in chick tectal cell cultures following treatment with the AChE-specific inhibitor BW 284C51. Similarly, Small et al. (1995) demonstrated that chick brain or sympathetic neurons grown on culture plates pre-coated with purified AChE exhibit a marked increase in neurite outgrowth. Pre-treatment of AChE with

diisopropylfluoro-phosphate (DFP), an inhibitor of AChE, failed to abolish this effect suggesting that the neurite promoting activity of AChE is independent of its well-known catalytic properties. Consistent with these observations is the demonstration that in cultures of chick sympathetic neurons, AChE localizes to the lamellopodia and filopodia which attach neurons to the substratum (Rotundo and Carbonetto, 1987).

Structural analysis of AChE has provided additional evidence for the role of the enzyme in the basic mechanisms underlying neurite outgrowth. Indeed, comparison of amino acid sequence revealed that AChE is homologous to the *Drosophila* cell adhesion molecules neurotactin and glutactin (Krejci et al., 1991), as well as neuroligins which are neuronal surface proteins (Ichtchenko et al., 1996; Grifman et al., 1998). Cell adhesion molecules are known to be essential for appropriate neuronal development, and a hallmark of these proteins is the presence of the HNK-1 carbohydrate epitope which plays a central role in cell adhesion (Keilhauer et al., 1985). Interestingly, AChE also bears the HNK-1 epitope (Bon et al., 1987). This therefore reinforces the concept that AChE may function in cell adhesion. Taken together, these data suggest that in addition to its role in the hydrolysis of ACh, AChE participates in the regulation of neurite outgrowth and consequently, influences neuronal cytoarchitecture.

1.5 DEVELOPMENTAL REGULATION OF AChE EXPRESSION

During the course of development, the mechanisms regulating AChE expression remain largely unknown. The regulation of the enzyme has been studied predominantly at the protein level with a special focus on the activity-linked regulation (see below) (Inestrosa et al., 1983; Jasmin and Gisiger, 1990; Jasmin et al., 1991; Sveistrup et al., 1995; Boudreau-Larivière et al., 1997; Sketelj et al., 1997). It is only until recently that the molecular mechanisms underlying the expression of the AChE gene have begun to be elucidated. Despite the growing interest, there are relatively few studies that have examined this regulation during development. So far, most available studies have examined the regulation of AChE in vertebrate skeletal muscle.

In skeletal muscle, it has been demonstrated both *in vitro* and *in vivo*, that the T transcript predominates (Legay et al., 1995). In accordance with these data, myotubes have molecular form profiles consisting of mainly G_1 and G_4, with some A_{12}, A_8 and G_2 (Hubatsch and Jasmin, 1997). In skeletal muscle, the asymmetric forms of AChE accumulate preferentially at the synaptic basal lamina (McMahan et al., 1978), whereas membrane-bound G_4 tetramers are highly concentrated in the perijunctional compartments (Gisiger and Stephens, 1988). Later studies demonstrated that AChE mRNA accumulated in junctional regions of skeletal muscle fibers, whereas it was almost undetectable in extrajunctional segments (Jasmin et al., 1993).

The developmental expression of AChE has been studied much less extensively in neurons, and those studies that have been undertaken have focused mainly on regulation at the protein level. For example, Anselmet et al. (1994) examined the regulation of AChE *in vivo* in the central nervous system of the quail. Their study analyzed AChE mRNA, activity and molecular forms in the cerebellum, optic lobes and neuroretina of the quail during embryonic development and in the adult. The expression of AChE appeared to be temporally regulated in all three regions, with mRNA levels of the T transcript increasing towards adulthood. Specific AChE activity also increased dramatically during embryonic development (E10-E16), while a further increase was observed between E16 and adulthood in the cerebellum. Similar developmental increases have been observed in studies of mouse cortical neurons both *in vitro* and *in vivo* (Sawyer and Weiss, 1993; Robertson, 1987). AChE is expressed transiently in the developing cerebral cortex of mammals (Robertson, 1987; Schlaggar and O'Leary, 1994), and its expression increases steadily from birth (Robertson, 1987). Interestingly, this coincides with the development of thalamocortical projections (Kageyama and Robertson, 1993; Schlaggar and O'Leary, 1994). As the thalamocortical neurons are believed to be noncholinergic, this suggests a possible morphogenic role for the enzyme in this system.

Anselmet and colleagues (1994) did not however, see any correlation between the abundance of mRNAs and AChE activity. For example, the highest levels of mRNA were found in the cerebellum whereas the highest activity was

present in the optic lobes. This suggests that the efficiency of translation and stability of the protein may differ between various brain regions. Of the three regions analyzed, the major molecular forms of AChE were G_4 and G_1, with the G_4 predominating in both the embryo and the adult. In addition, the ratio of nonamphiphilic to amphiphilic G_4 forms decreased markedly between E10 and E16, such that the proportion of nonamphiphilic G_4 was relatively minor in the adult. The relative abundance of amphiphilic and nonamphiphilic G_4 molecules was thought to reflect the availability of the structural subunit. The amphiphilic forms are believed to associate with the 20 kDa P structural subunit. Conceivably, the P subunit may be limiting at early developmental stages, thus allowing only the production of the nonamphiphilic G_4 form.

In addition, the developmental expression of AChE was examined in embryonic rat dorsal root ganglia (Koenigsberger et al., 1998). The dorsal root ganglion (DRG) is of particular interest to the study of AChE as it contains only sensory nerve bodies with no synapses. Despite this, cholinergic markers such as AChE and ChAT are widely expressed, suggesting that the enzyme may have novel functions in this neuronal population (see Section 1.4). Within this system, both AChE and BChE protein and mRNA were detectable during early embryonic development (E11-E12) with BChE being the most prominent. At the onset of neural differentiation, there was a spatiotemporal change in expression of the enzymes such that from E13 on, AChE expression predominated, particularly in neuronal cell bodies. Expression of AChE increased steadily thereafter. These findings are

consistent with those obtained from a previous study using whole rabbit embryos (Jbilo et al., 1994). Taken together, this data indicates that AChE is temporally regulated in ganglionic neurons, and may serve in the development of sensory neurons.

1.6 MOLECULAR MECHANISMS UNDERLYING THE DEVELOPMENTAL REGULATION OF AChE EXPRESSION

During the process of muscle cell differentiation, both AChE activity and mRNA levels increase significantly. Fuentes and Taylor (1993) showed that the increase in mRNA levels was a result of increased mRNA stability, as opposed to an increase in the rate of transcription of the AChE gene. The observed post-transcriptional regulation of AChE mRNA levels is in sharp contrast to that of other synapse-specific proteins such as AChR, whose increased expression is due to enhanced transcription (Buonanno and Merlie, 1986; Wang et al., 1988). The stabilization of AChE transcripts was later shown to be dependent upon the influx of extracellular Ca^{2+} (Luo et al., 1994).

In addition to muscle, similar studies have been performed in haematopoietic cells (see Paoletti et al., 1992; Chan et al., 1998). Chan et al. (1998) examined the molecular mechanisms underlying AChE expression during the differentiation of murine erythroleukemia (MEL) cells. AChE activity and mRNA levels were shown to increase dramatically during differentiation. This increase was the result of a

preferential induction of the G_1 form, instead of the G_2 form which is normally expressed in mature erythrocytes. MEL cells predominantly expressed the T and R transcripts, which increased during differentiation due to enhanced stability, as opposed to an increase in the transcriptional rate. These results indicate that post-transcriptional mechanisms account for the increased expression of AChE in haematopoietic cells. Although the role of the enzyme in this population of cells is unknown, an understanding of its function and regulation may help to elucidate its role in other noncholinergic tissues of the nervous system.

In contrast, the molecular mechanisms underlying the developmental regulation of AChE in neurons have been generally overlooked. In fact, one of the only *in vitro* studies undertaken in this area was by Coleman and Taylor in 1996. In this study, the developmental expression of AChE was examined in P19 embryonic carcinoma cells. It was reported that AChE activity and mRNA were undetectable in undifferentiated cultures. Following differentiation with retinoic acid, AChE activity increased 8-fold while mRNA levels increased less dramatically. The predominant mRNA transcript in this culture system was the T transcript, in accord with the finding that the principal molecular form species was a membrane-bound tetramer. P19 cells also produced a soluble G_4 which was secreted upon differentiation. Coleman and Taylor (1996) also reported that there was no change in the rate of transcription of the AChE gene upon retinoic acid-induced differentiation. In fact, RNase protection assays, following transcriptional inhibition, showed that the differentiation-induced increase in AChE was due to increased stabilization of the

existing transcripts. Although this study shed some light concerning AChE regulation in neurons, it had serious limitations. The study was meant to address the regulation of AChE in cholinergic neurons. However, in retinoic acid-differentiated P19 cultures, AChE-positive cells constitute only 0.1-1% of the population, and choline acetyltransferase (ChAT), the ACh-synthesizing enzyme, is not detectable (Staines et al., 1994). The relevance of the data is thus limited by the heterogeneity of these cultures.

1.7 ACTIVITY-LINKED REGULATION OF AChE EXPRESSION

Although our knowledge of the developmental regulation of AChE remains elusive, it has been well documented that neural activity dramatically affects the expression of the enzyme. This concept has been extensively studied in skeletal muscle and to a smaller extent in neuronal populations. In rat muscles, for example, total AChE levels decrease 10-fold following surgical denervation (Michel et al., 1994). In particular, denervation causes the almost complete disappearance of the synaptic collagen-tailed form (Bacou et al., 1982; Collins and Youkin, 1982). Similar decreases in AChE are also observed following blockade of action potential propagation with tetrodotoxin and of neuromuscular transmission with botulinum toxin A (Boudreau-Larivière et al., 1997; Sketelj et al., 1993). Alternatively, enhanced neuromuscular activity leads to increases in total AChE activity, with prominent changes in the levels of the various molecular forms (Jasmin and Gisiger, 1990; Jasmin et al., 1991; Sveistrup et al., 1995). For example, exercise training results in selective increases or decreases in the levels of G_4 in fast muscle,

depending on whether the activity is phasic or tonic, respectively (Boudreau-Larivière et al., 1997). These data suggest the existence of a pool of G_4 whose function is separate from that of the synaptic A_{12} and which constantly adapts its level to the type and intensity of muscular activity.

In contrast, the effects of neural activity on the AChE content of neurons is less well understood. As aforementioned, the predominant species of AChE in nervous tissue is G_4^a, although low levels of G_1, G_2 and A_{12} are also present. Denervation of the mouse hippocampus results in a significant decrease in total AChE activity, with a selective decline in the content of G_4^a and a concomitant increase in the levels of G_4^{na}, G_1 and G_2 (Schegg et al., 1990). Similarly, denervation of sympathetic and parasympathetic ganglia results in a decrease in ganglionic AChE (Massoulié and Bon, 1982). These data are supported by several *in vitro* studies which used high potassium conditions to mimic the effects of depolarization. In primary cultures of rat superior cervical ganglia, it was demonstrated that the A_{12} form of AChE is preferentially decreased following potassium depolarization. This effect was partially blocked by the action of the calcium-channel blocker methoxyverapamil (Verdière et al., 1984). Similarly, in cultures of dissociated spinal cord neurons, potassium depolarization results in a suppression of AChE levels with an increase in ChAT. Veratridine, a Ca^{2+} agonist, also increases ChAT and decreases AChE activity. However, these effects are blocked by the Ca^{2+} antagonist verapamil (Ishida and Deguchi, 1983). Taken together, these data suggest that electrical activity plays a crucial role in the regulation of AChE in neurons and that

this effect is mediated by the entry of Ca^{2+} into neuronal cells.

1.8 NEUROTROPHIC FACTORS AND NEURONAL SURVIVAL

As discussed above, AChE expression is regulated developmentally and by electrical activity. In addition, recent evidence suggests that trophic factors may also regulate the survival of neuronal populations and expression of cholinergic enzymes, including AChE. This theory was initially advanced by studies which demonstrated that motoneurons innervating skeletal muscle were dependent upon the presence of their targets for proper development and survival (Oppenheim, 1991). Moreover, it was shown that conditioned media from skeletal muscle extracts supported the survival of motoneurons (Dohrman et al., 1986; Bloch-Gallego et al., 1991; Jeong et al., 1991). This evidence formed the basis for what is now called the "neurotrophic theory". This theory provides some explanation for the effect of target tissues on their innervating neurons. It suggests that a target-derived factor is retrogradely transported to the neuronal cell body and acts as a survival factor for the innervating neuron. The limited availability of the factor in the target field thus permits the survival of the required number of neurons (Purves, 1988; Thoenen and Barde, 1980). The first putative survival factor to have been identified was the nerve growth factor (NGF) (Levi-Montalcini and Angeletti, 1968). Initially, the neurotrophic theory applied specifically to NGF. However, the recent purification of several other target-derived factors has extended the generality of the theory. Presently, the neurotrophic theory applies to a family of NGF-related factors known collectively as the neurotrophins.

1.8.1 Neurotrophins and their Receptors

The neurotrophins are a group of functionally and structurally related polypeptides that regulate the growth and maintenance of vertebrate neurons in both the peripheral and central nervous systems. The neurotrophin family consists of four proteins approximately 120 amino acids in length - NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (referred to hereafter as NT-4). Most recently NT-6 and NT-7 have been cloned in fish (Gotz et al., 1994; Lai et al., 1998). The proteins share approximately 50% sequence homology (Leibrock et al., 1989; Hohn et al., 1990; Maisonpierre et al., 1990; Ip et al., 1992) and trigger a wide variety of biological responses including proliferation, differentiation and survival.

Neurotrophins signal through a family of receptors which are encoded by the Trk family of proto-oncogenes, designated trkA, trkB and trkC (for review see Maness et al., 1994). The receptors are believed to be activated by dimerization, in which the binding of the ligand causes a pairing of the tyrosine kinase receptors and a subsequent activation of the intrinsic tyrosine kinase activity (Jing et al., 1992). Within the receptor dimer, the individual trk subunits then catalyze the phosphorylation of the other subunit (Heldin, 1995). The phosphorylated trk receptor then serves to recruit a variety of linker proteins and enzymes which ultimately transduce the signal to the nucleus of the responsive neuron.

One of the most interesting features of neurotrophin signal transduction is the

great degree of redundancy that exists between the neurotrophins and their receptors. Although each receptor displays a high affinity for a specific neurotrophin, there is an unparalleled amount of cross-reactivity. trkB not only binds BDNF and NT-4, but also NT-3 (Soppet et al., 1991). Similarly, trkA, which binds NGF with high-affinity, is also bound and activated by NT-4 (Cordon-Cardo et al., 1991; Berkemeier et al., 1991). Although our knowledge of this system remains rudimentary, it is likely that this redundancy has some biological significance. For example, temporal differences in the expression of a factor and/or its receptor could allow a single neurotrophin to have multiple functions throughout development. Alternatively, by allowing a single receptor to be activated by several factors, developing neurons could utilize various neurotrophins expressed along their growth pathways (Eide et al., 1993).

1.8.2 The Effects of Neurotrophins on Neurons

The effects of NGF in the central nervous system (CNS) remain largely unclear. Within the brain, NGF is produced in the pyramidal cells of the hippocampus (Whittemore et al., 1988). It is then transported retrogradely to the septal cholinergic neurons (Schwab et al., 1979) which do not synthesize the trophic factor (Korsching et al., 1985). However, convincing data demonstrating a role for NGF in the development of this cholinergic system are lacking. It has nonetheless, been shown that intraventricular injections of NGF increase the levels of ChAT (Gnahn et al., 1983) and also increase survival of septal cholinergic neurons (Hatanaka et al., 1988). Despite the lack of knowledge concerning the role of NGF

in the CNS, it is known that sympathetic (Levi-Montalcini and Angeletti, 1968) and sensory (Hamburger et al., 1981) neurons of the peripheral nervous system respond to NGF with an increased survival rate.

In contrast, NGF has very little, if any, biological effect on motoneurons (Oppenheim et al., 1982; Yan et al., 1988). For example, NGF fails to upregulate the cholinergic phenotype in cultures of enriched embryonic rat motoneurons (Wong et al., 1993). It was recently discovered that, unlike the other neurotrophins, NGF is not retrogradely transported in spinal cord motoneurons (DiStefano et al., 1992) and that this population of cells does not express the high-affinity NGF receptor trkA (Ernfors et al., 1992; Henderson et al., 1993).

The effects of BDNF and NT-4 appear to have been studied most extensively in motoneurons, which have intimate contact with the target muscles that synthesize them. Within skeletal muscle, BDNF and NT-4 are expressed at E15, at the beginning of the period of naturally occurring motoneuron cell death (Funakoshi et al., 1993). In addition to skeletal muscle, BDNF mRNA is also produced in the hippocampus, amygdala, neocortex, cerebellum and superior colliculus (for review see Maness et al., 1994). Interestingly, BDNF and NT-4 are also found within the sciatic nerve itself, and their levels appear to increase following axotomy (Funakoshi et al., 1993; Kobayashi et al., 1996; Cho et al., 1997). These data suggest that not only are BDNF and NT-4 ideal trophic factors present in the target-tissues at appropriate developmental stages and locales but also, that they function in an

autocrine fashion, providing support to injured neurons.

The physiological importance of BDNF and NT-4 was examined by studying mice with mutated neurotrophin and neurotrophin receptor genes. Mice lacking the functional trkB tyrosine kinase receptor showed a 60-70% loss in the trigeminal ganglion and a similar loss of facial motoneurons (Klein et al., 1993). In a separate experiment, mice lacking both trkB and trkC where found to have severe sensory deficits in the vestibular and cochlear ganglia (Silos-Santiago et al., 1997). Despite the dramatic loss of sensory neurons in these mice, CNS development was normal suggesting that trkB and trkC are essential for the development of sensory ganglia but not for CNS neurons. Conversely, mice lacking the gene for BDNF showed a loss of trigeminal neurons but no significant loss of facial or spinal cord motoneurons (Klein et al., 1993; Davies, 1994; Klein, 1994). Interestingly, BDNF/NT-4 double mutants were no more seriously affected than mice lacking BDNF alone, and NT-4-deficient mice appeared normal (Conover et al., 1995; Liu et al., 1995). Therefore, the discrepancy between the phenotypes of trkB-deficient and BDNF-deficient mice could not simply be explained by the actions of NT-4 on trkB. This suggests either that NT-4 works through a separate pathway or that another trkB ligand functions in the regulation of neuronal survival.

Soon after the discovery of BDNF, speculation on the possible existence of other members of the family of neuronal growth factors began to emerge. Mature NT-3 was found to share over 50% homology with BDNF and NGF. However, it is

present in skeletal muscle at a level 15 times greater than that of BDNF (Griesbeck et al., 1995). Although levels of NT-3 decrease towards adulthood, it remains the most abundant neurotrophin found in adult muscle. High levels of NT-3 are also found in the CNS, particularly in the cortex, hippocampus and cerebellum (Ernfors et al., 1990; Hohn et al., 1990). It has been demonstrated that NT-3 is a specific survival factor for proprioceptive neurons within the DRG (for review see Lindsay, 1994). It also has trophic effects on neurons of the hippocampus and locus coeruleus (Collazo et al., 1992; Friedman et al., 1993). In addition, NT-3 has been shown to rescue facial motoneurons after lesion, although less efficiently than BDNF and NT-4 (Sendtner et al., 1996), and to promote partial functional recovery following spinal cord injury (Grill et al., 1997).

1.8.3 The Effects of Neurotrophins on the Cholinergic Phenotype of Neurons

Within adult, cholinergic neurons, AChE is co-expressed with the acetylcholine-synthesizing enzyme ChAT. ChAT is currently the most specific marker for the functional state of cholinergic neurons. Initial production of the enzyme is target independent (Phelps et al., 1991). However, it has been shown that ChAT expression declines in the absence of target contact *in vivo*, in young and mature motoneurons (Chiu et al., 1993; Yan et al., 1994). Like AChE, ChAT is encoded by a single gene. The ChAT gene comprises several exons (designated M, N and R) including a number of alternatively spliced 5'-noncoding exons (Kengaku et al., 1993; Lönnerberg et al., 1996). Transcription of the mouse ChAT

gene initiates at multiple promoters, resulting in the production of five different 5'-mRNA sequences (Misawa et al., 1992; Kengaku et al., 1993). The most abundant species in the mouse nervous system is the M-type exon which contains elements that regulate neuron-specific expression of ChAT activity (Misawa et al., 1992).

On the basis of our current knowledge, it is expected that ChAT and AChE would be differentially regulated. Understandably, increases in ACh levels would require an increase in ChAT activity with a concomitant decrease in AChE activity. Indeed, evidence of differential regulation is seen throughout development. AChE mRNA expression occurs early in neuronal development, ie. prior to synaptogenesis, whereas levels of ChAT mRNA reach detectable levels later in embryonic development (Ibáñez et al., 1991). Similarly, AChE can be expressed in the absence of ChAT in various regions of the nervous system (Legay et al., 1993; Landwehrmeyer et al., 1993; Hammond et al., 1994). However, both AChE and ChAT activity decrease dramatically following denervation in mammalian muscle (Boudreau-Larivière et al., 1997; Friedman et al., 1995) and ChAT mRNA levels are augmented in AChE-transgenic mice (Andres et al., 1997). This suggests that the two enzymes may in fact be co-regulated in response to certain stimuli.

It is presently known that certain neurotrophic factors are able to regulate ChAT expression. Both BDNF and NT-4 have been shown to prevent the axotomy-induced decrease in ChAT protein and mRNA levels (Alderson et al., 1990; Yan et al., 1994; Chiu et al., 1994; Friedman et al., 1995; Tuszynski et al., 1996; Wang et

al., 1997). They have also been shown to increase ChAT activity in enriched cultures of embryonic motoneurons (Wong et al., 1993; Henderson et al., 1993), as well as other cholinergic cell types (Martinez et al., 1987; Raynaud et al., 1988; Alderson et al., 1990; Ojika et al., 1994). Based on the effects of neurotrophins on ChAT expression, these factors are plausible candidates for regulators of AChE expression. In fact, it has recently been demonstrated that BDNF and NT-4 can increase the number of AChE-positive cells in hippocampal cultures (Ip et al., 1993) and cultures of embryonic septal cells (Alderson et al., 1990). Finally, exogenously supplied BDNF and NT-4 have been shown to prevent the axotomy-induced decrease in AChE activity in facial motoneurons (Fernandes et al., 1995, 1998). These data provide evidence that the neurotrophins have important effects in the differentiation and cholinergic phenotype of several populations of neurons.

1.9 NEURAL CYTOKINES

Neural cytokines are a group of heterogeneous peptides including the ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF). These compounds and their receptors are expressed ubiquitously and share many properties of neurotrophins in preventing neuronal cell death. CNTF was originally characterized for its effects on chick ciliary ganglia (Barbin et al., 1984). Since then, several studies have demonstrated the survival-promoting effects of CNTF on various neuronal populations (Arakawa et al., 1990; Sendtner et al., 1990; Oppenheim et al., 1991; Gurney et al., 1992). In addition, mice lacking the CNTF receptor exhibit profound motoneuron deficits at birth (DeChiara et al., 1995).

Studies involving CNTF have been hampered however, by its rapid degradation at the application site (Clatterbuck et al., 1994; Rosenberg and Lucas, 1997). To circumvent this problem, more stable variants of CNTF have been developed. In particular, a second generation CNTF molecule known as axokine (Regeneron Pharmaceuticals, Tarrytown, NY) has proven useful in the study of its trophic actions.

Similarly, bFGF is also capable of supporting neuronal survival. Basic FGF prevents the death of lesioned cholinergic neurons *in vivo* (Anderson et al., 1988) and can stimulate ChAT expression in rat spinal cord cultures (McManaman et al., 1989). It has also been demonstrated to stimulate the development of central cholinergic and dopaminergic neurons *in vitro* (Knüsel et al., 1990). In a recent study, it has been shown that bFGF can protect spinal motoneurons *in vivo* after experimental spinal cord injury (Teng et al., 1998). This suggests that bFGF may contribute to the recovery of function following acute spinal cord injury. Together, these data suggest that both CNTF and bFGF have profound survival-promoting effects on several neuronal populations and may influence the cholinergic properties of neurons.

1.10 PURPOSE

The regulation of AChE has been extensively studied at the protein level in skeletal muscle and brain. However, the molecular mechanisms regulating the

expression of the AChE gene have only recently begun to be elucidated. In addition, the developmental regulation of AChE has yet to be addressed in populations of purely cholinergic neurons. In this study, we wish to establish a model system for studying the regulation of AChE expression in cholinergic neurons. This model could then serve to examine the regulation of the enzyme during neuronal differentiation, as well as the effects of various trophic factors on AChE expression. In addition, we wish to examine the developmental and trophic regulation of ChAT to further elucidate the co-regulation of the two cholinergic enzymes. It is our intent to begin characterizing the molecular mechanisms underlying the developmental and trophic regulation of AChE in neurons as an initial approach to develop a better understanding of the enzyme in health, and subsequently as it relates to neurodegenerative disease states.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

NSC-34 and NSC-19 cells were kindly provided by Dr. Neil Cashman, Montreal Neurological Institute, McGill University. SN6 and SN56 were kindly provided by Dr. Bruce Wainer, Wesley Woods Health Center, Emery University. BDNF, NT-4, NT-3, bFGF (Sigma) and axokine (Regeneron) were provided by Dr. David J. Parry, University of Ottawa. Verapamil (RBI) was provided by Dr. Jean-Marc Renaud (University of Ottawa) and veratridine (Sigma) was provided by Dr. Anthony Krantis (University of Ottawa). All cell culture supplies were from Nalge Corp. (Mississauga, ON), and components of culture medium were from Gibco Life Technologies Inc. (Burlington, ON). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

2.2 CULTURE OF NSC-34 AND NSC-19 CELL LINES

NSC-34 and NSC-19 (Cashman et al., 1992) are neuroblastoma-spinal cord motoneuron hybrid cell lines. The cells were grown in suspension at 37°C in 5% CO_2 in 25 cm^2 NUNC EasY Flasks (Nalge Corp.). The growth media consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated (56°C for 45 min) fetal bovine serum (Cansera; Rexdale, ON), 1% L-glutamine and 1% penicillin-streptomycin. All components were filtered through 0.22 µm cellulose acetate filters (Millipore Millex GP; Bedford, MA). Differentiation was induced by plating the cells on 35 mm^2 NUNC 6 well tissue culture plates at a density of 100,000 cells/ well. Media was changed every 24-48 hr.

Developmental studies required NSC-34 and NSC-19 cells to be maintained in culture for a period of six days prior to harvesting. Day 1 cells were harvested 12 hr after plating. To study the effects of trophic factors, NSC-34 cells were incubated for six days in the presence of any one of the following: 10 ng/ml NT-3 or bFGF, 50 ng/ml BDNF or NT-4, and 100 ng/ml axokine. Trophic factors were added every 24 hr for the first 48 hr and every other day thereafter for the remainder of the experiment (Hughes et al., 1993; Wong et al., 1993). The effects of electrical activity were determined by incubating the cells with either 55 mM KCl , 5 μM verapamil or 3 μM veratridine (Ishida and Deguchi, 1983). Cells were treated with these agents every 24 hr for two days in culture prior to harvesting at day six..

2.2.1 Storage of NSC Hybrid Cell Lines

NSC hybrid cell lines were harvested and centrifuged at 4°C for 8 min at 1,200 rpm. The supernatant was discarded and the pellet was resuspended in 0.5 ml of cell media (80% heat-inactivated fetal bovine serum, 20% DMEM with 1% penicillin-streptomycin) and transferred to CryoNUNC vials (Nalge Corp.). An equivalent amount of freezing media (70% heat-inactivated fetal bovine serum, 10% DMEM with 1% penicillin-streptomycin, 20% dimethylsulfoxide - DMSO) was layered on top of the cell suspension. All components, except DMSO, were filtered through 0.22 μm cellulose acetate filters and chilled. The cells were stored at -80°C overnight and then transferred to a liquid nitrogen cryostorage tank (Locator 4 Plus Cryostorage Vessel; Mississauga, ON) the following day.

2.2.2 Thawing of NSC Hybrid Cell Lines

The contents of one CryoNUNC vial were thawed quickly in a 37°C water bath. The cell suspension was added to a 15 ml conical Falcon tube (Becton Dickinson; Franklin Lakes, NJ) and the vial was washed with 2 ml of growth media to remove any extra cells. The cells were pelleted at 4°C for 8 min at 1,200 rpm. The supernatant was aspirated and the pellet was resuspended in 1 ml of growth media and plated or grown in suspension.

2.3 CULTURE OF OTHER CELL LINES

SN6 and SN56 are cell lines derived from fusion of septal cholinergic neurons and a parent neuroblastoma cell line, N18TG2 (Lee et al., 1990; Hammond et al., 1990b). All three cell lines were grown in 60 mm tissue culture plates at 37°C with 5 ml of culture medium per plate. The growth medium consisted of 88% DMEM, 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin solution. All components were filtered through a 0.22 μm acetate filter. Cells were fed when the media became slightly orange, approximately every 24-48 hr. Cells typically divided every 24 hr and were passaged when they reached 90% confluency. The cells were harvested by adding 0.25% trypsin and squirting them off the culture dish with a pipette into conical tubes. They were then centrifuged at 4°C for 10 min at 1,200 rpm. The supernatant was subsequently removed and the pellet resuspended in an appropriate amount of growth medium. Cells were frozen in CryoNUNC vials at -80°C. The freezing media was similar to the growth media with the addition of

10% DMSO. Differentiation of the SN6 and SN56 cell lines was achieved by adding retinoic acid to the growth medium, to a final concentration of 10 μM.

2.4 ACETYLCHOLINESTERASE EXTRACTION AND ANALYSIS

Cells were washed twice with cold phosphate-buffered saline (PBS), and harvested in 500 μl of a high-salt detergent buffer containing antiproteolytic agents: 10 mM tris(hydroxymethyl) aminomethane (Tris-HCl) (pH 7.0), 10 mM EDTA, 1 M NaCl , 1% Triton X-100, 1 mg/ml bacitracin, and 2.5 mg/ml aprotinin (Boehringer Mannheim; Laval, PQ) (modified from Michel et al., 1994). The cells were homogenized for 30s with a Polytron (Kinematica; Littan, Switzerland) set to level 6 and then centrifuged at 14,000 rpm for 15 min at 4°C. The resulting supernatants were transferred to fresh tubes and stored at -80°C for further analysis (Michel et al., 1994). To assay for secreted AChE, cell media was removed, centrifuged at 14,000 rpm for 15 min at 4°C to remove any cellular debris and stored at -80°C for further analysis.

Total AChE activity was determined using a modified version of the spectrophotometric method of Ellman (Ellman et al., 1961). Aliquots of cell lysate (25 μl) were incubated in 1 ml of phosphate buffer solution (pH 7.0) containing 7.5×10^{-4} M acetylthiocholine as substrate, 5×10^{-4} M 5,5'-dithiobis(2-nitrobenzoic acid), and 10^{-5} M tetraisopropyl pyrophosphoramide (iso-OMPA), a nonspecific cholinesterase inhibitor. Specific AChE activity was monitored by comparing substrate hydrolysis in the absence or presence of the AChE-specific inhibitor 1,5-

bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51). Protein concentration was determined using a bicinchoninic (BCA) assay kit (Pierce Laboratories; Rockford, IL) according to the instructions supplied by the manufacturer.

2.5 MOLECULAR FORMS

The content of AChE molecular forms was determined by velocity sedimentation as described in detail by Jasmin and Gisiger (1990) and Chan et al (1998). Five and 20% sucrose solutions were prepared in Tris salt buffer (TSB) which consisted of 40 mM Tris-HCl (pH 7.0-7.2), 1 M NaCl, 0.1 M $MgCl_2$, 1 % Triton x-100 or 0.5 % Brij-96 and 1 mg/ml bacitracin. Combinations of these two sucrose solutions were used to generate 20, 17.5, 15, 12.5, 10, 7.5 and 5% sucrose solutions. One and a half ml of the solutions were serially layered into centrifuge tubes (Beckman; Palo Alto, CA) and chilled at 4°C for 1 hr. Subsequently, 100 µl aliquots of cell extract were layered onto the sucrose gradients which were then centrifuged in a Beckman SW41 rotor at 40,000 rpm for 16 hr at 4°C. Approximately 45 fractions were collected from the bottom of the tubes and assayed for AChE activity. The molecular forms of AChE were identified according to the nomenclature of Bon et al. (1979) on the basis of their apparent sedimentation coefficients.

2.6 CHOLINE ACETYLTRANSFERASE ENZYME ASSAY

ChAT activity was measured using a modification of the method of Fonnum

(Fonnum, 1975). Ten μl aliquots of cell extract were incubated with a mixture containing 100 mM $NaPO_4$ (pH 7.4), 600 mM NaCl, 40 mM EDTA (pH 7.4), 0.2 mM acetyl coenzyme A, 0.1 mM eserine, 8 mM choline chloride (pH 7.4) and 1 μCi (^3H) acetyl-coenzyme A (Amersham; Baie d'Urfé, PQ) for 2 hr at 37°C. The reaction was terminated by the addition of 2 ml tetraphenylboron in acetonitrile (BDH) and the amount of radioactivity in the organic phase was determined by liquid scintillation using toluene scintillant containing 4 g of 2,5-diphenyloxazole (PPO) (Kent Laboratories Ltd.) and 150 mg of 1,4-Bis(5-phenyloxazol-2-yl)benzene (POPOP) in 1 L of toluene.

2.7 RNA EXTRACTION AND REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Cells were washed twice with cold PBS and suspended in 1 ml of Trizol (Boehringer Mannheim; Laval, PQ). Following phenol/chloroform extraction, RNA pellets were washed with 70% ethanol, air-dried and resuspended in an appropriate volume of diethylpyrocarbonate (DEPC)-treated water (ICN; Montreal, PQ). RNA concentrations were determined using a GeneQuant RNA/DNA calculator (Pharmacia; Baie d'Urfé, PQ). Fifty to 500 ng of RNA was subsequently reverse transcribed to generate cDNAs. The reverse transcription mixture consisted of 5 mM $MgCl_2$, 1 X PCR buffer II (50 mM KCl; 10 mM Tris-HCl, pH 8.3), 1 mM dNTPs, 20 units of RNase inhibitor, 50 units of Moloney murine leukemia virus reverse transcriptase, and 2.5 μM random hexamer primers (GeneAmp RNA PCR kit, Perkin

Elmer; Mississauga, ON). Negative controls consisted of reverse transcription mixtures in which the RNA was replaced by DEPC-treated water.

To amplify AChE and other cDNAs, specific synthetic primers based on mouse sequences were used (Förster et al., 1993; Misawa et al., 1993; Marsh et al., 1993; Legay et al. 1995; Fernandes et al., 1998) (see Table 1). The AChE primers were denoted m for mouse; R, in the reverse direction; C, in the common coding region and F, in the forward direction. R, H and T were used to denote the alternatively spliced variants of AChE. Amplification of the selected cDNAs was performed in a DNA thermal cycler (Perkin-Elmer) by adding 5 µl of the reverse transcribed sample to 20 µl of a PCR mixture containing 0.025 units/µl AmpliTaq DNA polymerase, 1.5 mM of each appropriate 5'- and 3'-primer, 2 mM $MgCl_2$ and 1X PCR buffer II. PCR cycling conditions are listed in Table 1. Under these conditions, all measurements were made in the linear phase of amplification (see for example Michel et al., 1994; Chan et al., 1998). Initially, all RT-PCR conditions (i.e. primer concentration, RNA input) were optimized and maintained constant for all subsequent analyses. This was ensured by using RT and PCR master mixes and by running control and experimental samples in parallel. Precautions were taken to avoid contamination and RNA degradation (i.e. use of gloves, filtered tips, dedicated sets of pipettes for sample preparation, etc.). PCR products were visualized in1.5% agarose gels containing ethidium bromide and quantitated with agarose gels containing VistraGreen (Amersham). The relative amounts of PCR products were quantitated with a Storm PhosphorImager (Molecular Dynamics; Sunnyvale, CA).

Quantitation of the absolute abundance of AChE transcripts was performed using an external standard control plasmid, C5, which contains a mutated AChE cDNA (kindly provided by Dr. Claire Legay). PCR analysis of reverse transcribed samples was performed in parallel with serial dilutions of the C5 plasmid, thus generating a standard curve of the control plasmid. Amounts of AChE transcripts were determined by comparing the intensity of the PCR product from unknown samples to those of the C5 standard curve (see Legay et al., 1995; Chan et al., 1998).

2.8 mRNA STABILITY ASSAY

To assess the stability of the various AChE transcripts, day 0 and day 5 cells were incubated with the transcription inhibitor actinomycin D (2.5 μM) (Gibco) (Chan et al., 1998). Total RNA was extracted from these samples at 0, 2, 4, 8, 12, and 24 hr and levels of specific mRNAs were analysed by RT-PCR.

2.9 NUCLEAR RUN-ON ASSAY

Day 1 and day 6 cells were collected by centrifugation (1,500 rpm, 15 min) and washed twice with ice-cold, sterile PBS. Cells were resuspended in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.2% Nonidet P-40 (ICN), vortexed for 10 s and incubated on ice for 10 min. Nuclei were then sedimented by centrifugation at 1,500 rpm for 5 min at 4°C and subsequently washed once with pre-chilled lysis buffer. Nuclei were then resuspended in 200 μi of freezing buffer containing 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 40% glycerol,

0.1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) and stored in liquid nitrogen (Chan et al., 1998).

Run-on assays were performed using a modified version of a procedure described elsewhere (Ray et al., 1995, Chan et al., 1998). Fifty µl of nuclei were incubated with 200 µl of transcription buffer containing 5X reaction buffer (1.5 M $(NH_4)_2SO_4$, 0.5 M Tris-HCl (pH 7.9), 20 mM $MgCl_2$, 20 mM $MnCl_2$, 1 M NaCl, 2 mM EDTA (pH 8.0), 0.5 mM PMSF), 1 mM dithiothreitol (DTT), 10 mM creatine phosphate, 1 mM each of GTP, ATP and CTP (Perkin-Elmer), 10 units/ml RNase inhibitor (Perkin-Elmer), 50% glycerol, 1 mM PMSF and 250 µCi of [α-^{32}P]UTP (Amersham). RNA was transcribed at 28°C for 30 min. Following RQ1 DNase (Promega; Madison, WI) treatment and addition of yeast tRNA, labelled RNA was isolated using Trizol. Samples were then hybridized for 48 hr with 10 µg each of linearized rat AChE, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, tubulin, and mouse ChAT cDNA plasmids, as well as genomic DNA, immobilized on Genescreen Plus nylon membrane (DuPont; Mississauga, ON). Following hybridization, membranes were washed once with 1X sodium citrate buffer (SSC) and 0.1% sodium dodecyl sulfate (SDS) (BDH) at 42°C, and exposed for autoradiography at -80°C for 3 -7 days with intensifying screen using Kodak BioMax film (Rochester, NY). Quantitation was performed using a Storm Phosphorimager.

2.10 IN SITU HYBRIDIZATION AND ACETYLCHOLINESTERASE HISTOCHEMISTRY

C57B1/6 mice and Sprague-Dawley rats were anaesthetized with sodium pentobarbitol, their spinal cord and brain were removed quickly. They were placed in OCT embedding compound and frozen in melting isopentane cooled by dry ice. Tissues were stored at -80°C. Serial sections (10 μm) were obtained and placed on alternate Superfrost plus slides (Fisher Scientific Co.; Toronto, ON) and stored at -80°C until further analysis. In situ hybridization was performed according to a modified version of Schalling et al. (1988). Sections were fixed and incubated with a synthetic AChE-specific oligonucleotide (5'- CAAGTCAATGTGGAG-GCACGGTGTTCAAAGATGTAGGCATAGACCCGAGC -3'). Oligonucleotides were 3' end-labelled by incubation with 5 X Co reaction buffer (0.7 mM potassium cacodylate (pH 7.2), 150 mM Tris base, 5 mM $CoCl_2$ and 0.5 mM DTT), TdT terminal transferase (Gibco) and 125 μCi [^{35}S]-ATP (Amersham) for 2 hr at 37°C. The reaction was terminated with the addition of Tris/EDTA (TE) buffer (pH 8.0). Following phenol-chloroform extraction and DNA precipitation, the pellet was washed with 70% ethanol and air dried. The pellet was subsequently resupended in an appropriate volume of TE buffer with DTT and stored at -20°C.

Following prehybridization, sections were incubated overnight at 42°C with hybridization buffer (0.2 M $NaPO_4$, 60% deionized formamide, 20X SSC, 50X Denhardt's solution, 0.1 g/ml dextran sulfate and 20% sarcosyl) containing 10 μg/μl

of salmon sperm DNA, 5 M DTT and 10^6 cpm of labelled probe per slide. Sections were washed thoroughly, air dried and subsequently coated with photographic emulsion (Kodak NTB2, diluted 1:1). Slides were exposed for 2-3 weeks and developed using Kodak D-19 Developer. Computer-assisted image analysis was performed using NIH Image 4.0 software.

AChE histochemistry was performed according to the method of Karnovsky and Roots (1964). Samples were fixed in 4% paraformaldehyde and incubated for 15 min to 1 hr in a solution containing 5 mg/ml acetylthiocholine as substrate, 70 mM sodium acetate buffer (pH 5.2), 10 mM sodium citrate, 3 mM $CuSO_4$, 0.5 mM potassium ferricyanide and 10^{-5} M iso-OMPA to inhibit nonspecific cholinesterase activity. Sections were photographed using a Zeiss Axiophot microscope and Kodak T-Max 100 film.

2.11 STATISTICAL ANALYSIS

To evaluate the effects of the experimental conditions, student's t-tests were performed. Paired t-tests were employed in all cases. The level of significance was set at $P<0.05$. The data were also analysed using a one-way analysis of variance with least significant difference and the results were comparable. Data are expressed as mean ± SEM throughout. The profiles of AChE molecular forms and ethidium bromide-stained gels of PCR products presented in the Figures are representative examples.

Table 1. PCR Primers and Cycling Conditions

Primer	Sequence	PCR Conditions	Reference
mRT	5' -TCACAGGTCTGAGCAGCGCTCCCTG- 3'	94°C, 1 min 70°C, 3 min 38-50 cycles	Legay et al., 1995
mRR	5' -TTACACCTGTGTTTTGCACCCCAT- 3'	as above	
mRH	5' -TTAGAGCCACGGAAGCCCGGAGTG- 3'	as above	
mFC	5' -CTGGGGTGCGGATCGGTGTACCCC- 3'	as above	
ChAT	5' -AGAGAGGTGTGGCTGGTTTGCTTG- 5' 5' -GGCCAGGGTTTGCTGCAGTGGGGGCACTGG- 3'	94°C, 1 min 72°C, 3 min 38-50 cycles	Misawa et al., 1993
TrkB	5' -TGAAGGACGCCAGCGACAATGCACGCAAGG- 3' 5' -GGTGAATTTCCTATACATGATGCTCTCTC- 3'	94°C, 1 min 55°C, 1 min 72°C, 2 min 35-40 cycles	Marsh et al., 1993
TrkC	5' -GCCAAGGGGGAGCTAGGACT- 3' 5' -CTCCACACATCACTCTCTGT- 3'	94°C, 1 min 55°C, 1 min 72°C, 2 min 35-40 cycles	Fernandes et al., 1998
S12 rRNA	5' -GGAAGGCATAGCTGCTGG- 3' 5' -CCTCGATGACATCCTTGG- 3'	94°C, 1 min 54°C, 1 min 72°C, 2 min 30 cycles	Förster et al., 1993

CHAPTER 3

RESULTS

3.1 CHARACTERIZATION OF A MODEL FOR THE REGULATION OF ACETYLCHOLINESTERASE IN NEURONS

3.1.1 Localization of AChE in the Spinal Cord and Brain

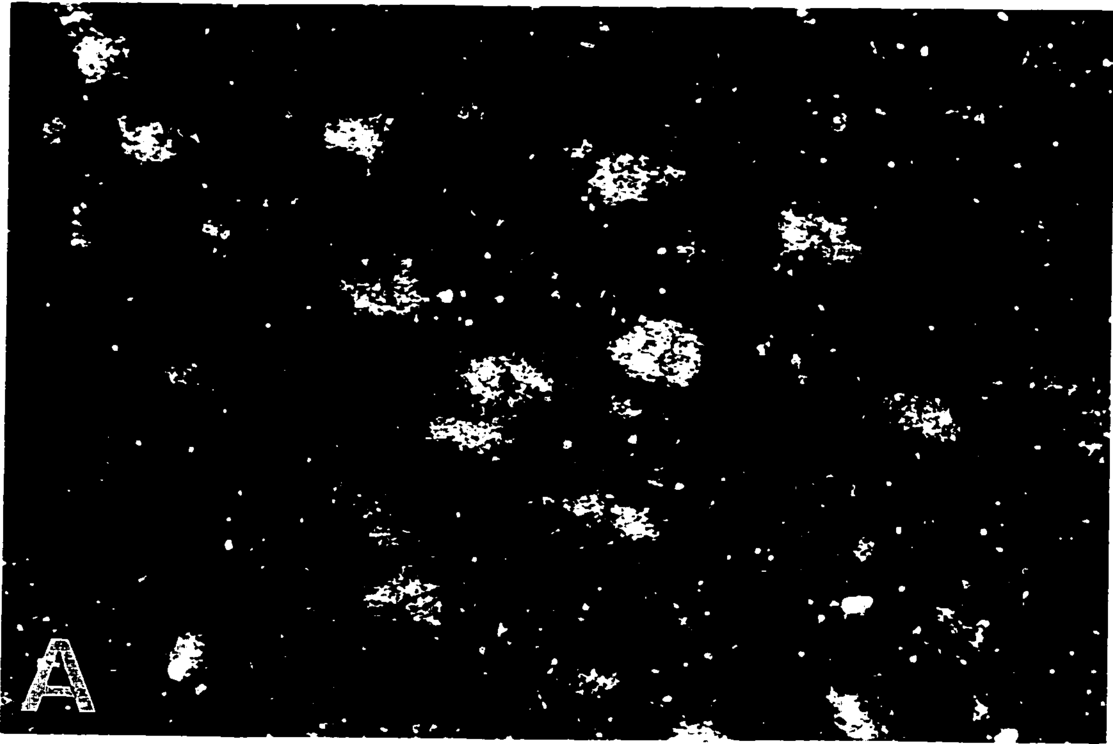
In order to study the regulation of AChE in the nervous system, it was necessary to first find an appropriate model. Preferably, the model system should express the enzyme endogenously and at relatively high levels. Two areas within the nervous system known to express high levels of AChE mRNA and activity are the spinal cord (Landwehrmeyer et al., 1993) and the septum (Bernard et al., 1995). The presence of AChE mRNA in the rat spinal cord was confirmed by in situ hybridization using a synthetic probe complementary to the common coding region of AChE transcripts. We found that AChE transcripts were localized to the cell bodies of spinal cord motoneurons as evidenced by the accumulation of silver grains over the cell somas (Figure 2A). This was further confirmed by performing AChE histochemistry on the serial sections. This showed a definite correlation between the presence of AChE transcripts and activity to the motoneuronal cell bodies (Figure 2B and C). AChE histochemistry experiments were also performed using cryostat sections of rat septum. As seen in panels D and E, there was a much weaker staining pattern in the septum indicating a relatively lower level of AChE activity.

3.1.2 NSC-34 Motoneurons Express High Levels of AChE

Following the localization of AChE in neurons of the spinal cord and septum,

Figure 2. Localization of AChE in Rat Spinal Cord and Brain

Panel A shows a representative dark-field photomicrograph of a longitudinal section of rat spinal cord processed for *in situ* hybridization. Silver grains, indicating selective accumulation of AChE transcripts, are localized to motoneuronal cell bodies. Panel B shows a representative bright-field photomicrograph of a serial section from a rat spinal cord processed for *in situ* hybridization to localize AChE transcripts. Panel C shows a serial section processed for AChE histochemistry. Note the expression of AChE transcripts in motoneurons as well as the co-localization of cell bodies and AChE mRNA (arrows). Panels D and E show representative bright-field photomicrographs of sections of rat brain processed for AChE histochemistry. Note the relatively lower level of AChE activity in the septum (arrows). Bar = 120 μm.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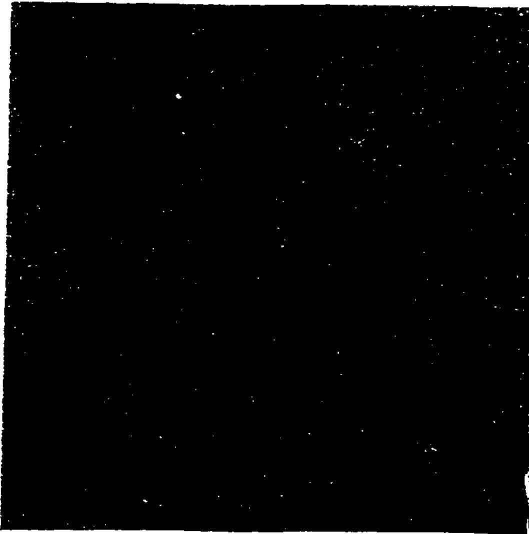
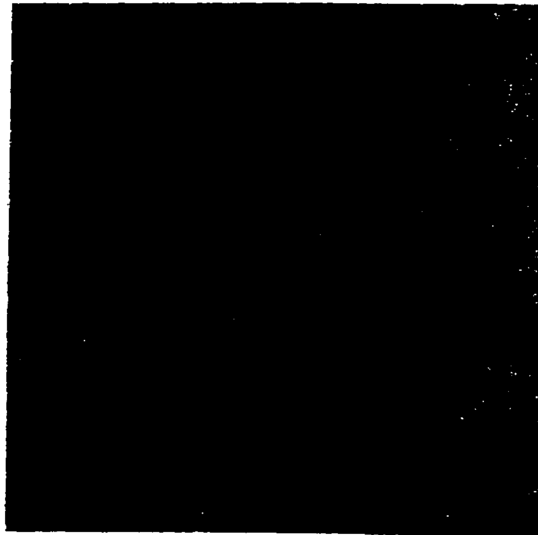
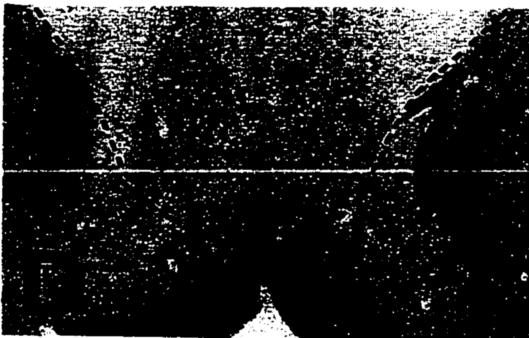






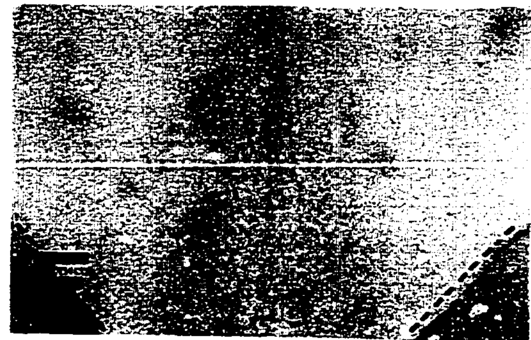
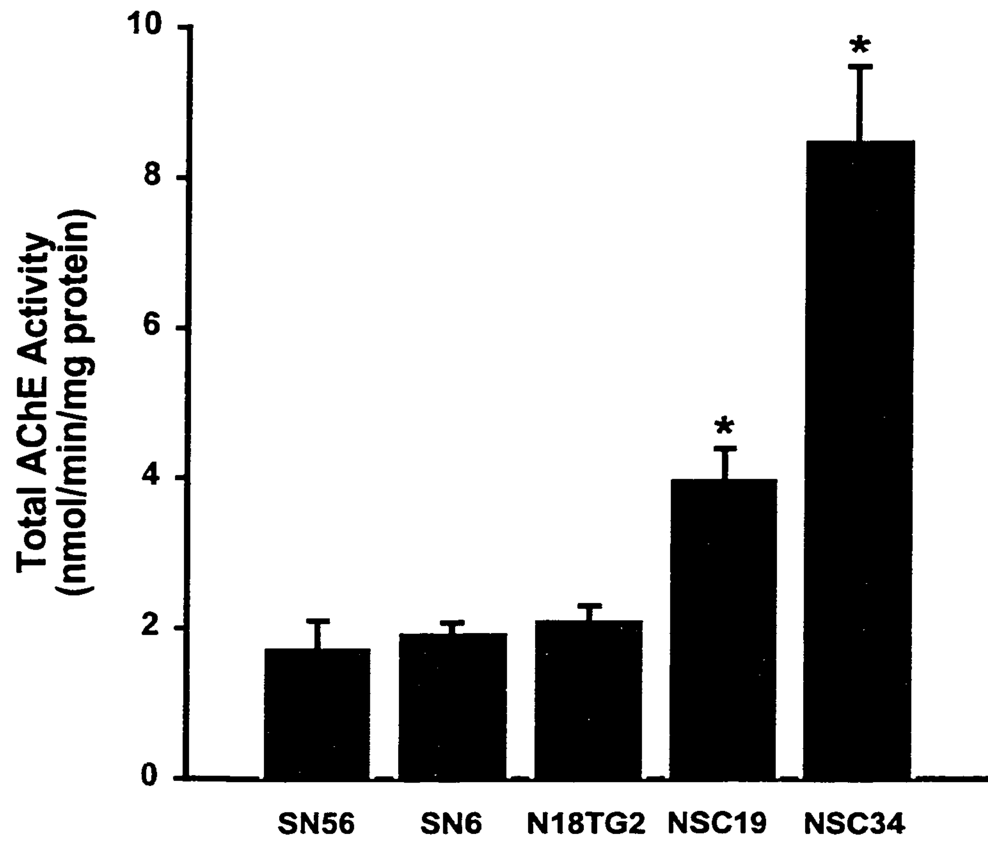


Figure 3. Initial Screening of Cholinergic Cell Lines

Shown are the AChE activities of several cell lines following differentiation in culture. SN56 and SN6 are septum-neuroblastoma hybrids, whereas NSC-19 and NSC-34 are spinal cord motoneuron-neuroblastoma hybrids; N18TG2 is the parent neuroblastoma for all four cell lines. SN56 and SN6 cells were differentiated with 10 uM retinoic acid whereas the NSC and N18TG2 cells were differentiated by plating on tissue culture plates. Note the NSC-34 cell line expressed the highest AChE activity of differentiated cells ($P<0.05$). Results are from 6 independent experiments performed with duplicate samples. Asterisks denote significant differences.


Total AChE Activity
(nmol/min/mg protein)
10
8
6
4
2
0
*
*
SN56
SN6
N18TG2
NSC19
NSC34

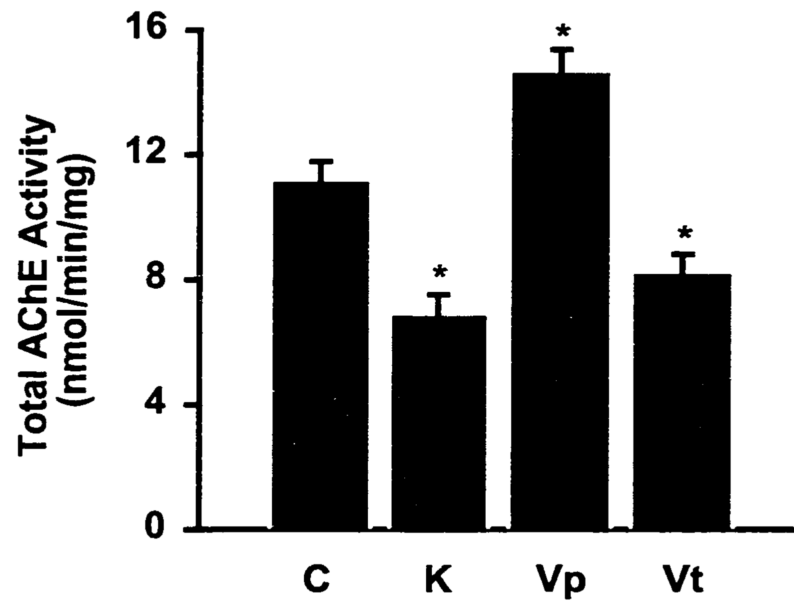
we aimed to find a model with which to study the regulation of AChE in cultured cells. Although in general, primary cell culture has been useful as an approach to study the molecular basis of neuronal development, it is nonetheless limited by the heterogeneity of the cell preparations. Even distinct areas of the nervous system contain neurons with different phenotypes, in addition to non-neuronal cells. Advances in somatic cell fusion have allowed the immortalization of neuronal cells by fusion with tumor cells. As part of our experiments, we obtained two cell lines derived from the septum, SN6 and SN56 (Hammond et al., 1990b), and two derived from spinal motoneurons, NSC-19 and NSC-34 (Cashman et al., 1992). All four cell lines were fused with the same parent neuroblastoma, N18TG2. We therefore analyzed these cell lines for the level of AChE activity. Figure 3 shows the expression of AChE in differentiated cells. SN56 and SN6 were differentiated with 10 uM retinoic acid whereas the NSC and N18TG2 lines were differentiated by plating a cell suspension on tissue culture plates. As shown, the NSC-34 displayed the highest level of AChE specific activity ($P<0.05$). Previous studies using the NSC-34 cell line have shown that they express many of the morphological and physiological characteristics of primary motoneurons (see Discussion) (Cashman et al., 1992). Given this information, we therefore chose the NSC-34 cell line as our model system for the study of the regulation of AChE in neurons.

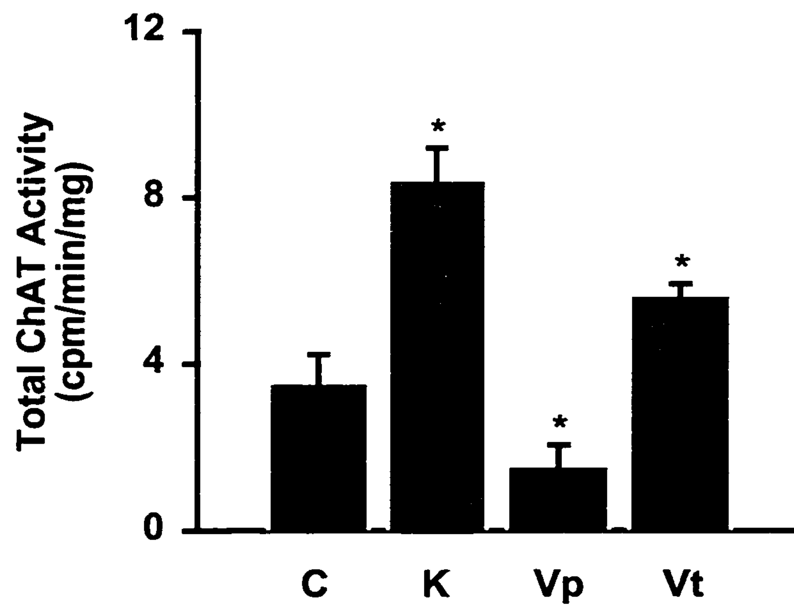
3.1.3 NSC-34 Motoneurons Respond to Depolarizing Agents

To further verify that NSC-34 cells represent a good model system for studying the regulation of AChE, we examined the effects of depolarizing agents on

Figure 4. The Effects of Depolarizing Agents on AChE and ChAT Activity in NSC-34 Motoneurons

Panels A and B show the effects of high potassium (K), verapamil (Vp) and veratridine (Vt) on AChE and ChAT activity, respectively, in comparison to control levels (C). See text for details. Results represent 2 separate experiments performed in triplicate. Asterisks denote a significant difference.


(A)
Total AChE Activity (nmol/min/mg)
16
12
8
4
0
*
*
*
C
K
Vp
Vt


(B)
Total ChAT Activity (cpm/min/mg)
12
8
4
0
*
*
*
C
K
Vp
Vt

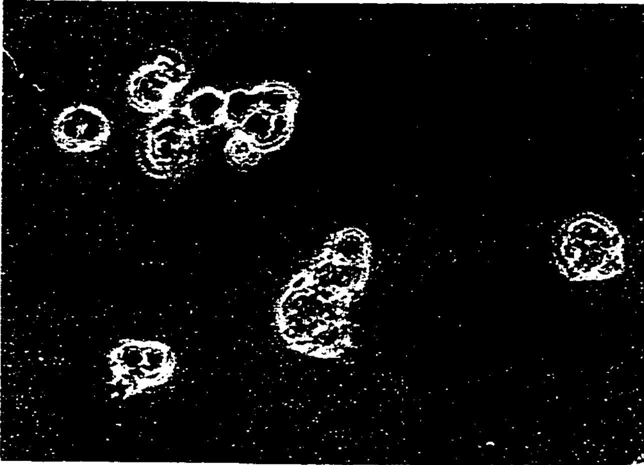
AChE and ChAT activity and compared the results to those obtained previously. High concentrations of KCl (55 mM) increased ChAT activity 2-fold ($P<0.02$) and suppressed AChE activity to approximately half of control levels ($P<0.02$) (Figure 4A and B). Veratridine, which depolarizes the neural cell membrane, had similar effects. Conversely, the Ca^{2+} channel antagonist verapamil significantly increased AChE activity ($P<0.05$) while reducing ChAT activity to 50% of control levels ($P<0.01$). These results are consistent with those obtained previously with primary cell cultures of spinal cord motoneurons (Ishida and Deguchi, 1983).

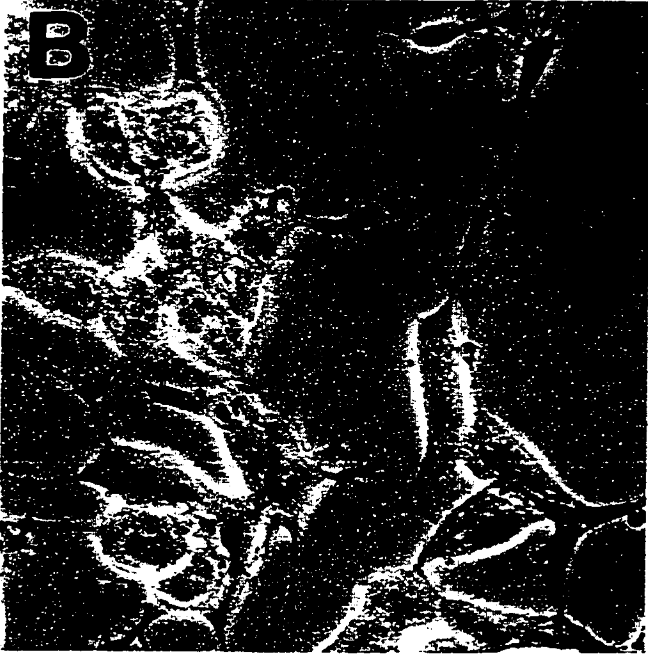
3.1.4 Morphological Features of NSC-34 Motoneurons

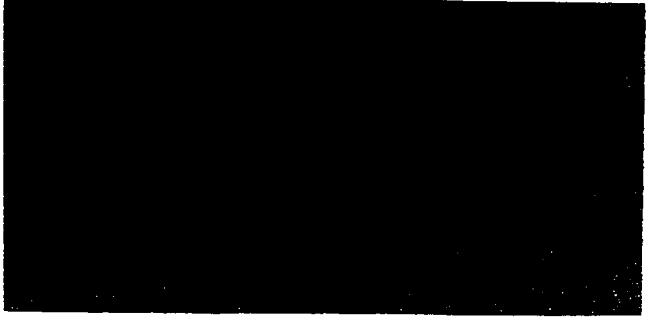
Next, we examined the morphological features of motoneurons derived from the NSC-34 cell line. Figure 5A is an example of cells cultured in suspension for 12 hr. As shown, they have a round appearance characteristic of undifferentiated cells. Figures 5B and C represent cells grown for 6 days on tissue-culture plates. Multiple neurites extend from most cells indicating that they have undergone morphological differentiation. The cells were also stained for AChE histochemistry (Figure 5D and E) further illustrating that differentiated NSC-34 motoneurons express high levels of AChE activity. Approximately 80% of NSC-34 motoneurons were positive for AChE staining.

Figure 5. **Morphological Features of NSC-34 Motoneurons**

Panel A shows cells cultured for 24 hours. Note the round appearance of these cells characteristic of undifferentiated neurons. Panels B to E represent cells grown for 6 days in culture. Note the multiple neurites extending from most cells indicating that they have undergone differentiation. Panels A to C are phase contrast photomicrographs. In panels D and E, the cells have been histochemically stained for AChE. Note that differentiated NSC-34 motoneurons express significant levels of AChE. Bar = 120 μm.




B



3.2 DEVELOPMENTAL REGULATION OF CHOLINERGIC ENZYMES IN NSC-34 MOTONEURONS

3.2.1 Differentiation of NSC-34 Motoneurons Results in Increases in AChE and ChAT Expression

NSC-34 cells were plated for 6 days to induce differentiation, which was evidenced by the extension of neurites. Compared to undifferentiated cells, which were plated for 12 hr, expression of AChE was greatly increased in differentiated cultures. In differentiated cells, AChE activity increased approximately 4-fold ($P<0.001$) (Figure 6A). As shown in other studies (Chan et al., 1998), a significant portion of the total enzyme activity appeared in the growth media indicating that these cells secrete large amounts of AChE. In fact, secreted AChE activity also increased by approximately 4-fold ($P<0.004$) (Figure 6B). Differentiation also induced a 4-fold increase in the expression of cell-associated ChAT ($P<0.001$) (Figure 6C). Since ChAT is not a secreted enzyme (Mautner, 1977, 1986), we did not examine ChAT levels in the growth media.

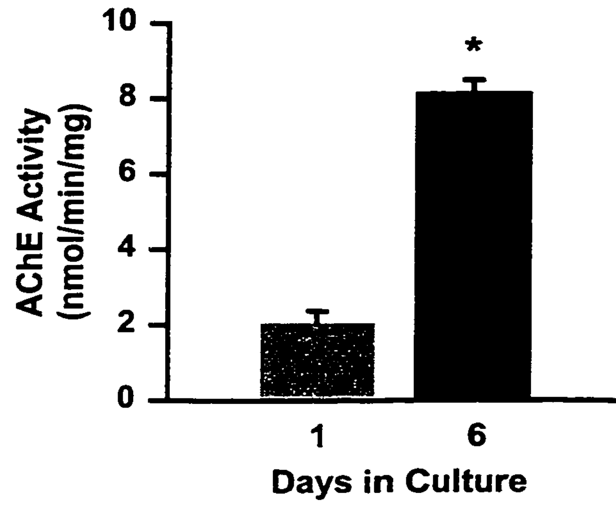
3.2.2 Changes in AChE Molecular Forms with Differentiation

To determine whether differentiation induced any changes in specific molecular forms, aliquots of cell extract and growth media from undifferentiated and differentiated cultures were separated by velocity sedimentation using sucrose gradients. As shown in Figure 7A, undifferentiated cell extracts contained a small

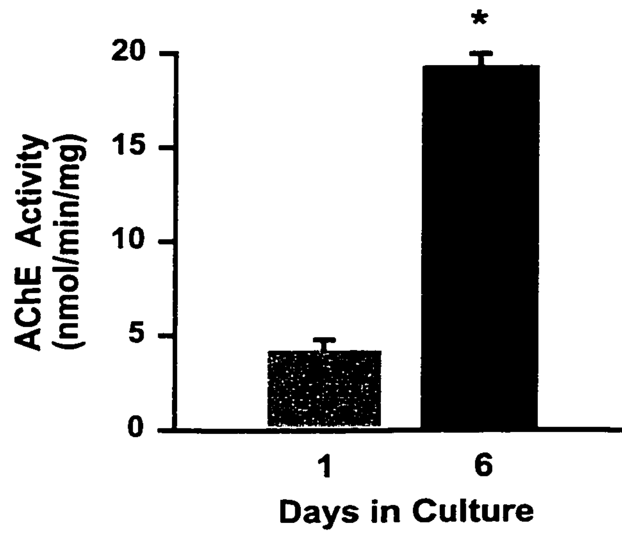
Figure 6. Differentiation of NSC-34 Motoneurons Leads to Increases in Total AChE and ChAT Activity

Panel A shows total AChE activity in NSC-34 cells at day 1 (undifferentiated) and 6 (differentiated) in culture. Note the 4-fold increase in enzyme activity in differentiated vs. undifferentiated neurons ($P<0.001$; $n = 7$, in duplicate). Panel B shows total AChE activity in the media of day 1 and day 6 cells. Note the parallel increase in AChE activity of ~4-fold ($P<0.004$; $n = 7$). Panel C shows total ChAT activity in undifferentiated and differentiated NSC-34 motoneurons. As seen with AChE, there was an increase in ChAT activity during differentiation ($P<0.001$; $n = 5$, in duplicate). Asterisks denote a significant difference.

(A)



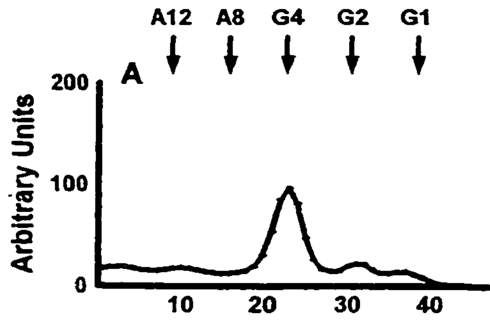
(B)

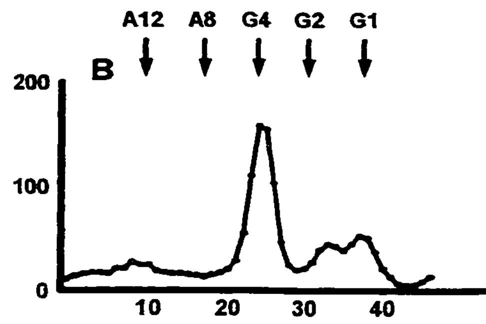


(C)

Figure 7. Comparison of AChE Molecular Forms in Undifferentiated and Differentiated NSC-34 Motoneurons

Shown are representative AChE molecular form profiles (n = 10) of extracts from undifferentiated (panel A) and differentiated (panel B) NSC-34 motoneurons, as well as from the growth media of undifferentiated (panel C) and differentiated (panel D) cells obtained following velocity sedimentation in sucrose gradients containing Triton X-100. Note the predominant peak of the tetrameric (G_4) form of the enzyme, which increases during differentiation. Occasionally a G_1 peak can also be detected. Undifferentiated (panel E) and differentiated (panel F) cell extracts were further analyzed in sucrose gradients containing Triton x-100 or Brij-96 (n = 6). Note the right shift of the G_4 peak in differentiated cells demonstrating the amphiphilic nature of the enzyme. A_{12} and A_8 refer to the asymmetric forms; G_4, G_2 and G_1 refer to the globular forms.


A12
A8
G4
G2
G1
A
Arbitrary Units
200
100
0
10
20
30
40


A12
A8
G4
G2
G1
B
200
100
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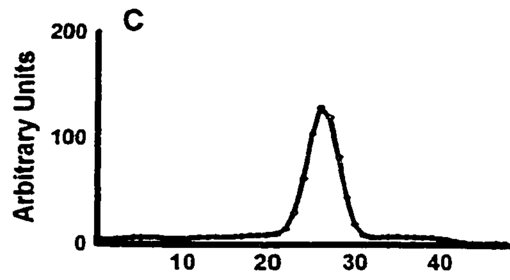
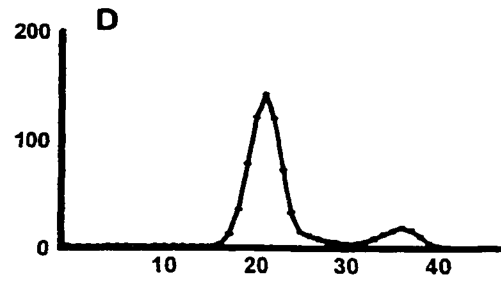
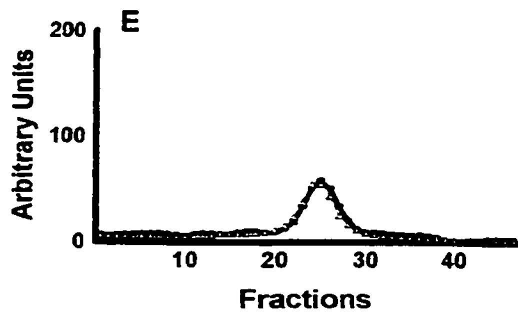
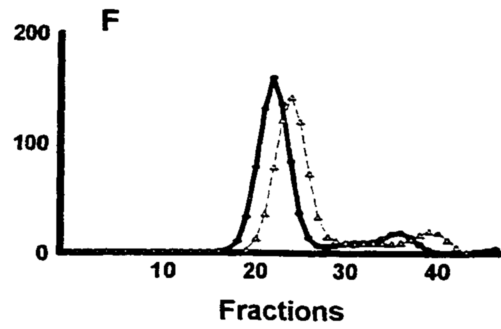
10
20
30
40


C
Arbitrary Units
200
100
0
10
20
30
40


D
200
100
0
10
20
30
40


E
Arbitrary Units
200
100
0
10
20
30
40
Fractions


F
200
100
0
10
20
30
40
Fractions

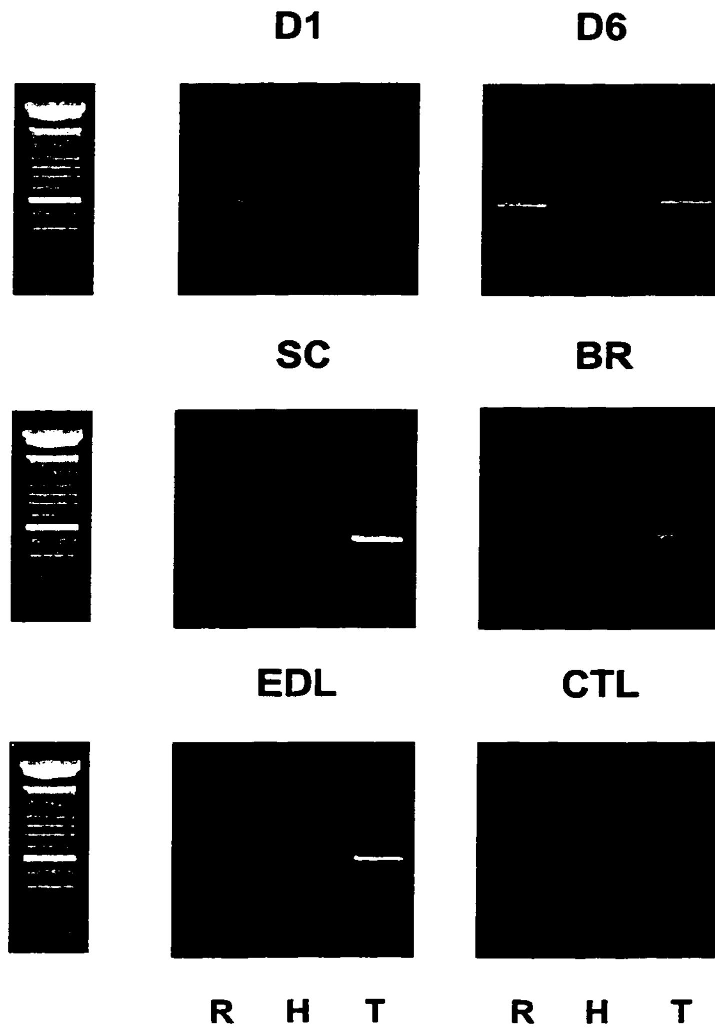
amount of G_4 and, occasionally, traces of G_1 and G_2. In contrast, differentiated cell extracts contained high levels of G_4 as well as greater amounts of G_1 and G_2 (Figure 7B). Figures 7C and D show the molecular forms present in extracts of growth media from undifferentiated and differentiated cell cultures, respectively. Consistent with the cell extract data, the media contained mainly G_4 and occasionally G_1 in differentiated samples. These results indicate that the increases in total AChE enzyme activity induced by differentiation result from increases in the levels of AChE globular forms. In separate experiments, undifferentiated (Figure 7E) and differentiated (Figure 7F) cell extracts were further analyzed in sucrose gradients containing Triton x-100 or Brij-96 to determine the amphiphilic character of the G_4 enzyme species expressed by these cells. It was observed that the G_4 displayed a reduced sedimentation coefficient in differentiated cells, thus demonstrating the amphiphilic nature of the enzyme.

3.2.3 Differentiation Increases AChE and ChAT Transcript Levels in NSC-34 Motoneurons

We next examined expression of the various AChE transcripts found in NSC-34 cells. Although AChE is encoded by a single gene, the immature mRNA undergoes alternative splicing to yield three potential transcripts termed R, H and T. We thus used RT-PCR with specific primers to amplify individual transcripts in order to determine their relative abundance. Messenger RNAs from undifferentiated (D1) and differentiated (D6) cultures were analyzed for each of the three transcripts. In

Figure 8. Expression of AChE Transcripts in NSC-34 Motoneurons

Shown are representative examples of ethidium bromide-stained agarose gels of AChE PCR products for day 1 cultures of NSC-34 cells (D1), day 6 (D6), whole mouse spinal cord (SC), whole mouse brain (BR), mouse extensor digitorum longus muscle (EDL) and a negative control (CTL). Note the increase in T transcript from day 1 to day 6 in NSC-34 cells as well as a more moderate increase in the R transcript. All three transcripts are present in NSC-34 motoneurons, mouse spinal cord and brain. However, the T transcript appears to be dominant. EDL muscle expresses strictly the T transcript. Results are representative of 3 independent experiments.


D1
D6
SC
BR
EDL
CTL
R H T
R H T

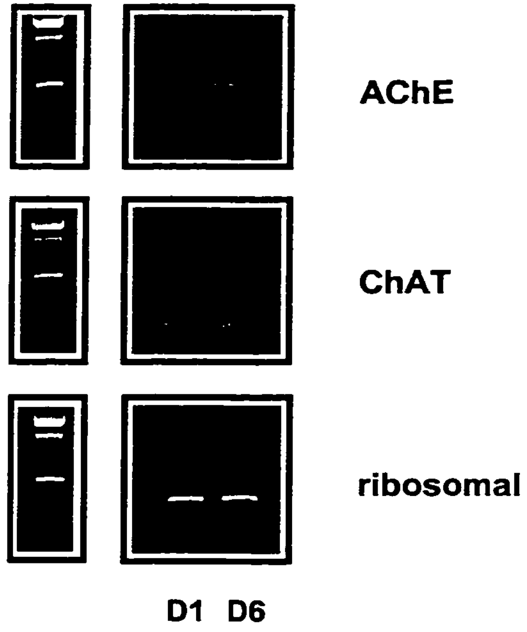
parallel, mRNAs from mouse spinal cord (SC), brain (BR) and extensor digitorum longus muscle (EDL) were also analyzed (Figure 8). The various samples were processed in parallel with a negative control in which the mRNA had been replaced with DEPC-treated water. All three transcripts appeared to be expressed in both undifferentiated and differentiated NSC-34 cells. As shown in Figure 8, it also appeared that the levels of R and T transcripts increased with differentiation. The three transcripts were also present in mouse spinal cord and brain although the T transcript, as expected, was clearly dominant. By contrast, the muscle sample expressed solely the T transcript.

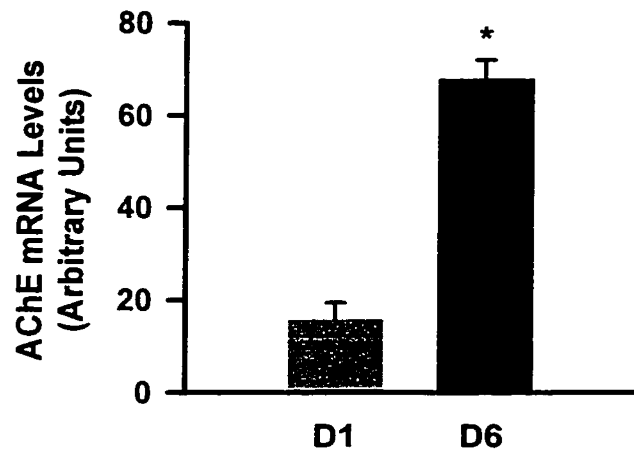
Next, we examined whether the increase in AChE enzyme activity which occurred during differentiation was accompanied by an increase in the levels of AChE mRNA. Since the T transcript appeared to be the most abundantly expressed in the NSC-34 cell line, and since our molecular form analysis revealed little if any of the soluble monomer that would be expected from expression of the R transcript, we used specific PCR primers to selectively amplify the T transcript. AChE mRNA levels were examined in both undifferentiated and differentiated NSC-34 cells (Figure 9A). During differentiation, levels of the T transcript increased approximately 4-fold ($P<0.001$) (Figure 9B).

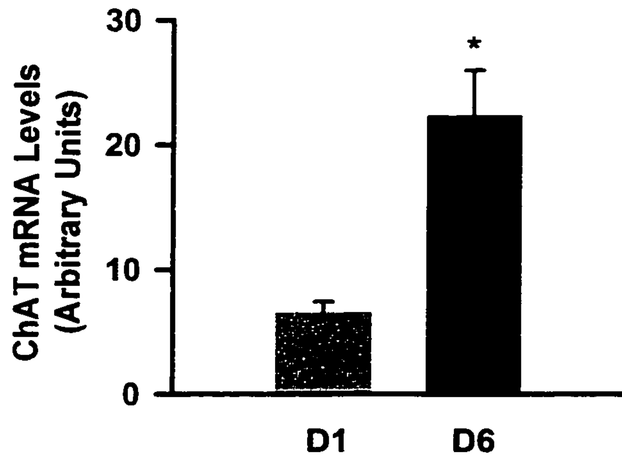
We also examined the levels of the M-type ChAT mRNA, a transcript which is abundantly expressed in mouse spinal cord (Misawa et al., 1992). Figures 9A and C show that differentiation induced a 3-fold increase in ChAT mRNA expression

Figure 9. AChE and ChAT mRNA Levels Increase During Differentiation of NSC-34 Motoneurons

Panel A shows ethidium bromide-stained agarose gels of PCR products corresponding to the AChE T transcript, ChAT mRNA and S12 rRNA in NSC-34 motoneurons grown for 1 (D1, undifferentiated) and 6 (D6, differentiated) days in culture. Panels B and C represent the quantitation of AChE and ChAT mRNA levels, respectively. Note the approximate 4-fold increase in both AChE ($P<0.001$; $n = 6$) and ChAT ($P<0.002$; $n = 4$) transcript levels which parallels the differentiation-induced increase in activity of both enzymes. Levels of S12 ribosomal RNA serve as an internal control and remain constant with differentiation. Asterisks denote a significant difference.


(A)
AChE
ChAT
ribosomal
D1 D6


(B)
AChE mRNA Levels
(Arbitrary Units)
80
60
40
20
0
*
D1
D6


(C)
ChAT mRNA Levels
(Arbitrary Units)
30
20
10
0
*
D1
D6

(P<0.002). Thus, differentiation leads to increases in both AChE and ChAT mRNA levels which parallel the differentiation-induced increases in enzyme activity (see Figure 6).

3.2.4 Quantitation of the Abundance of AChE T and R Transcripts in NSC-34 Motoneurons

As indicated in Figure 8, the R transcript was also expressed in differentiated NSC-34 cells. To compare the relative abundance of the T and R transcripts in undifferentiated and differentiated NSC-34 cultures, we determined the absolute amount of each transcript by quantitative RT-PCR using the C5 control plasmid (see Legay et al., 1995). We observed that in undifferentiated NSC-34 cells, there were approximately 2.4×10^5 copies of AChE T mRNA and 0.2×10^5 copies of AChE R per 0.5 µg of total RNA (Figure 10A and B). This indicates that the T transcript is more abundant in undifferentiated NSC-34 cultures. Although differentiation induced an overall increase in the absolute values for both transcripts, it did not significantly alter the ratio of T versus R mRNAs. These results also indicated that both the T and R mRNAs are rare to moderately abundant transcripts, as expected (Jasmin et al., 1993; Chan et al., 1998).

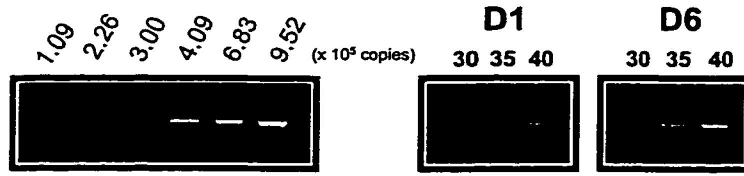
3.2.5 Differentiation of NSC-34 Motoneurons Does Not Affect the Transcriptional Activity of the AChE and ChAT Genes

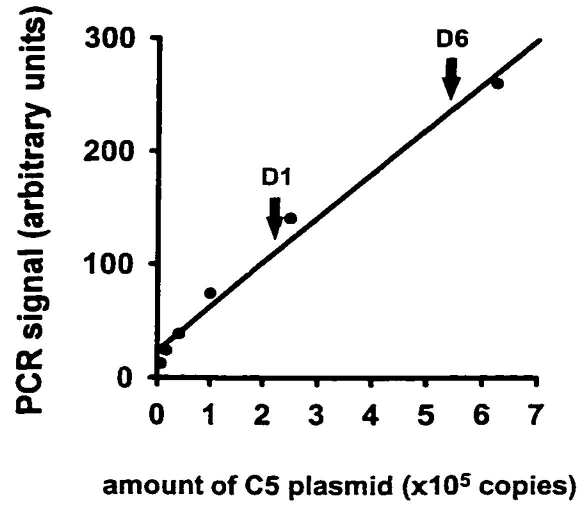
To determine whether the accumulation of AChE mRNA and increased

Figure 10. Quantitation of AChE T and R Transcript Levels by RT-PCR

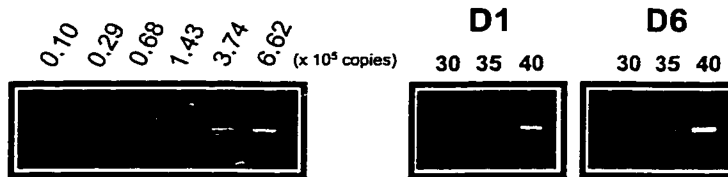
To determine the absolute number of AChE transcripts in NSC-34 samples, we performed a quantitative PCR study for the two most abundant AChE transcripts, T and R. Serial dilutions of the control plasmid (C5) that contained an engineered AChE cDNA were amplified in parallel with reverse-transcribed cDNAs from undifferentiated and differentiated NSC-34 cells for the quantitation of the T (panel A) and R (panel B) transcripts. Shown are ethidium bromide-stained agarose gels of PCR products amplified from the C5 plasmid (numbers denote the number of copies used) and from undifferentiated (D1) and differentiated (D6) NSC-34 samples amplified for 30, 35 or 40 cycles. For quantitation, aliquots of the same samples were electrophoresed in agarose gels containing VistraGreen and scanned using a Storm Phosphorimaging System. Data obtained from the serial dilutions were platted as standard curves (amount of C5 plasmid vs. PCR signal); arrows indicate the levels of the transcripts in undifferentiated and differentiated NSC-34 cells. Note the level of both transcripts increased by approximately 3-fold. Measurements shown were taken after 35 cycles of amplification. These results are representative examples obtained from 5 independent experiments.

(A)





(B)



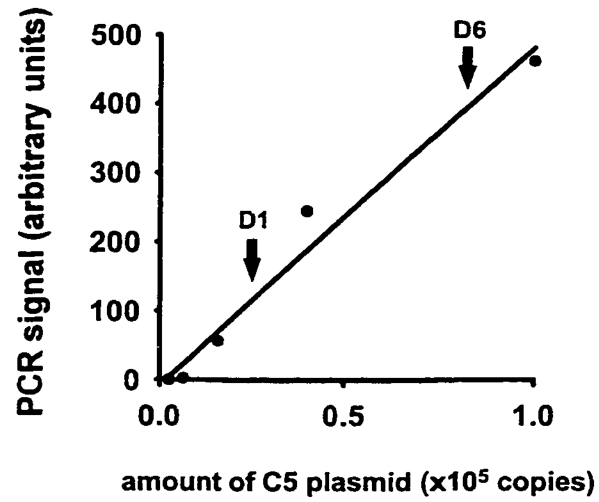
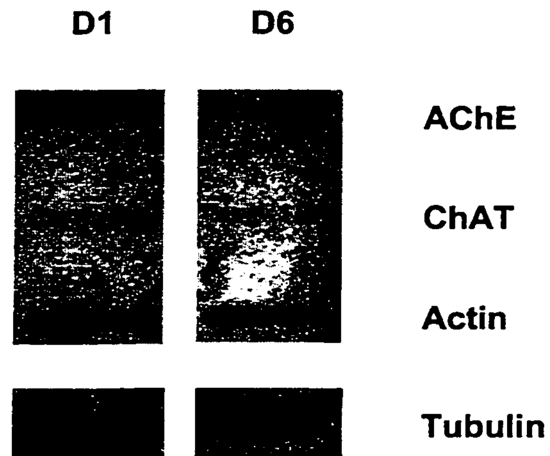


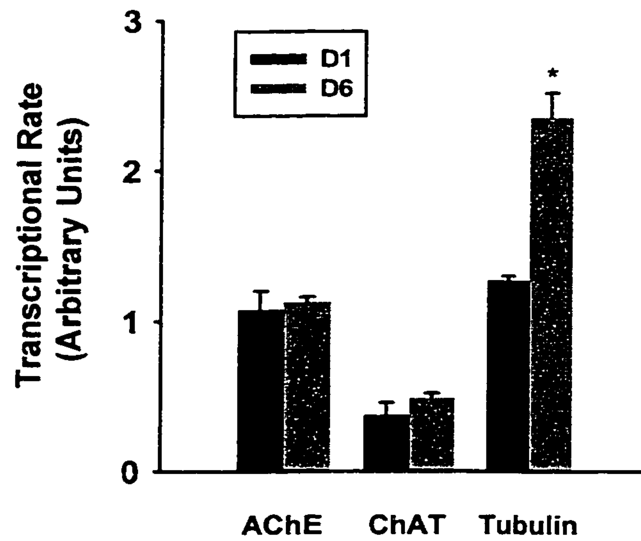
Figure 11. Differentiation of NSC-34 Cells Does Not Affect the Transcriptional Activity of the AChE or ChAT Genes

Nuclear run-on assays were performed to determine whether the increases in AChE and ChAT mRNA levels were due to changes in the transcriptional activity of their respective genes. Panel A shows an example of an autoradiogram of a nuclear run-on assay for undifferentiated (D1) and differentiated (D6) NSC-34 cells. Panel B shows the quantitation of newly synthesized AChE, ChAT, β-actin and α-tubulin transcripts in NSC-34 cells. Note that in contrast to the increase in the transcriptional activity of the α-tubulin gene seen in differentiated NSC-34 cells ($P<0.01$), the transcriptional rates of the AChE and ChAT genes remained unaffected. Values for these three transcripts were normalized to the internal standard β-actin and are expressed as arbitrary units. Results are from 3 separate experiments performed in duplicate.

(A)



(B)



enzyme activity reflected a change in the rate of transcription of the AChE gene, we performed nuclear run-on assays with nuclei isolated from undifferentiated and differentiated NSC-34 cells. As seen in Figure 11A, the AChE gene is actively transcribed even in undifferentiated cells. Over the course of differentiation, no change in transcription rate was observed. Similar results were observed for transcription of the ChAT gene. In contrast, the transcriptional rate of the α-tubulin gene increased significantly with differentiation ($P<0.02$) (Figure 11B). These experiments indicated that the observed increases in AChE and ChAT mRNAs resulted from post-transcriptional regulation. This suggested that the differentiation-induced enhancement of AChE and ChAT activities was due to stabilization of pre-existing AChE and ChAT mRNAs.

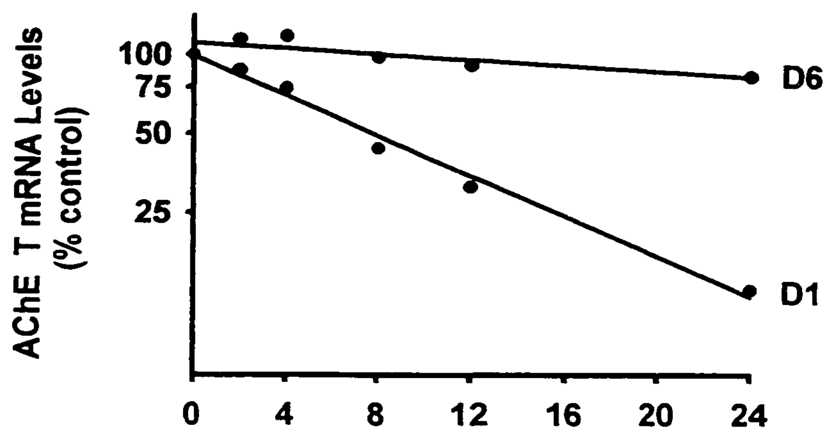
3.2.6 Differentiation Increases the Stability of AChE and ChAT Transcripts

Since differentiation of NSC-34 motoneurons did not have a detectable effect on the transcriptional rates of either the AChE or the ChAT genes, we verified that the increase in mRNA levels was caused by a stabilization of AChE and ChAT transcripts. Therefore, day1 and day 6 NSC-34 cultures were treated with actinomycin D to inhibit the activity of the RNA polymerase II and thus to block transcription. Total RNA was extracted at various time intervals thereafter and the levels of AChE and ChAT transcripts were determined by RT-PCR. Both T and R AChE transcripts displayed an increase in stability during differentiation of NSC-34 cells (Figure 12A and B). The half-lives of the T and R transcripts in undifferentiated NSC-34 cells were 7.68 hr ± 1.15 (n = 4) and 8.13 hr ± 0.16 (n = 4), respectively.

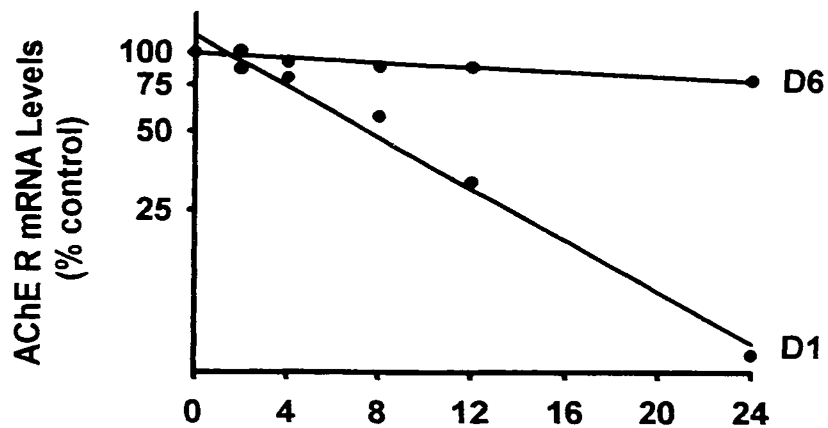
Figure 12. Stabilization of AChE and ChAT mRNA in Differentiated NSC-34 Motoneurons

Undifferentiated (D1) and differentiated (D6) NSC-34 cultures were treated with actinomycin D to block transcription. Total RNA was extracted at various time intervals thereafter and the levels of AChE and ChAT transcripts were determined by RT-PCR. Both T (panel A) and R (panel B) AChE transcripts displayed increased stability during differentiation of NSC-34 motoneurons. Panel C shows that ChAT mRNA stability also increased in differentiated motoneurons. Data are expressed as a percent of control levels (i.e. time = 0). These results are representative of 4 independent experiments performed in duplicate.

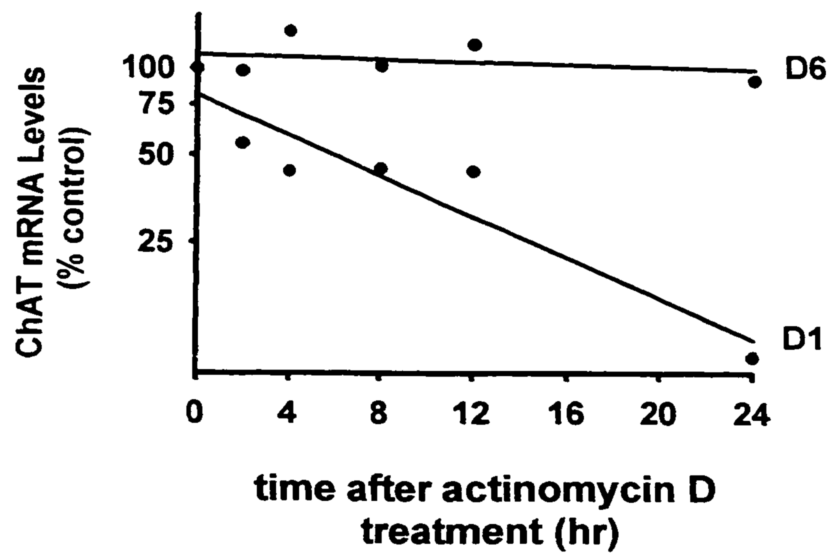
(A)



(B)



(C)



As illustrated, the half-lives of the two transcripts increased markedly with differentiation such that accurate measurements could not be determined since they appear to extend well beyond the duration of the experimental time course. Figure 12C shows that the stability of ChAT mRNA also increased upon differentiation. The half-life of ChAT transcripts in undifferentiated cultures was 4.57 hr ± 0.34 (n = 4). Again due to a dramatic increase in stability induced by differentiation, we were unable to determine accurately the half-life of ChAT transcripts in differentiated cultures of NSC-34 cells. Therefore, as expected on the basis of our nuclear run-on assays, the stability of AChE T and R transcripts, as well as that of ChAT transcripts, increased markedly in differentiated NSC-34 cells.

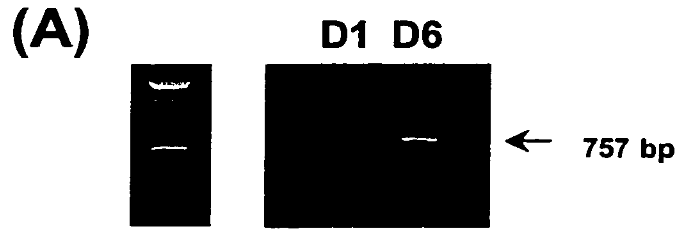
3.3 REGULATION OF CHOLINERGIC ENZYMES BY NEUROTROPHINS

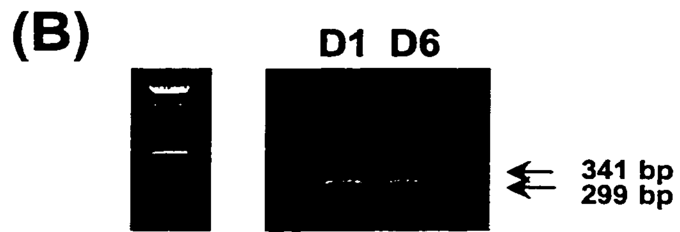
3.3.1 NSC-34 Motoneurons Express TrkB and TrkC Tyrosine Kinase Receptors

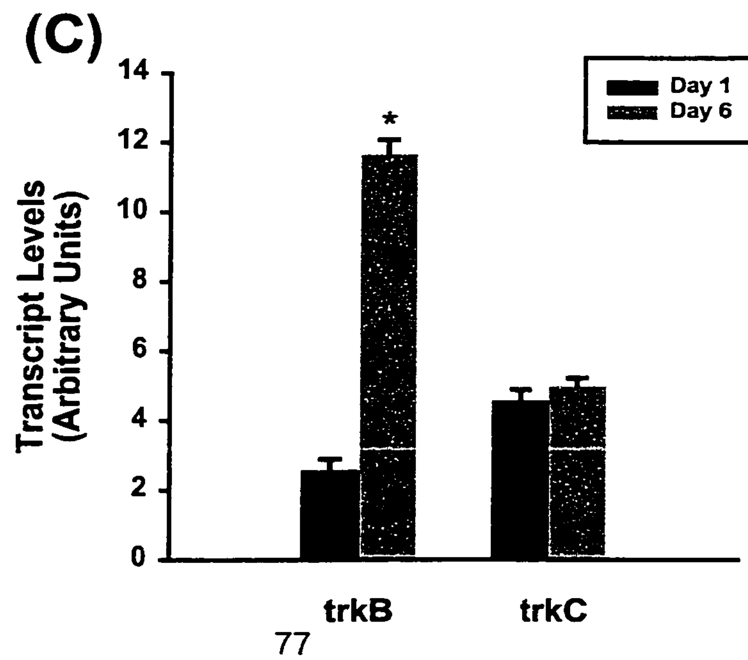
To begin studying the regulation of AChE in motoneurons, we initiated a series of experiments in which we examined the effects of neurotrophins. However, prior to examining this, it was necessary to determine whether NSC-34 motoneurons expressed the neurotrophin receptors trkB and trkC. In our experiments we used specific RT-PCR primers to selectively amplify trkB and trkC transcripts. As shown in Figure 13A, NSC-34 motoneurons express full-length trkB tyrosine kinase receptors. Furthermore, the levels of trkB mRNA increase approximately 6-fold with

Figure 13. NSC-34 Motoneurons Express trkB and trkC Tyrosine Kinase Receptors

Panels A and B show representative ethidium bromide-stained agarose gels of PCR products corresponding to trkB and trkC mRNAs, respectively, in undifferentiated (D1) and differentiated (D6) NSC-34 motoneurons. Note the ~6-fold increase in trkB mRNA from day 1 to day 6 ($P<0.001$; $n = 5$). In contrast, trkC mRNA did not change from day 1 to day 6 (panel B). Note also that two different isoforms of trkC are expressed in NSC-34 motoneurons. Panel C shows the quantitation of trkB and trkC transcripts at days 1 and 6. Asterisk indicates a significant difference. These results are representative examples obtained from 5 independent experiments.


(A)
D1 D6
757 bp


(B)
D1 D6
341 bp
299 bp


(C)
Transcript Levels
(Arbitrary Units)
0
2
4
6
8
10
12
14
*
Day 1
Day 6
trkB
trkC

differentiation ($P<0.001$) ($n = 5$). In contrast, trkC mRNAs were already expressed in undifferentiated cultures and their levels did not change with differentiation (Figure13B). In addition, both non-inserted (299 bp) and inserted (341 bp) isoforms of trkC mRNAs were detected in NSC-34 cells. The inserted isoforms contain amino-acid inserts which interfere with the signaling capabilities of the receptor thus rendering it nonfunctional. The non-inserted form corresponds to the functional trkC receptor. Figure 13C shows the quantitation of trkB and C transcripts during differentiation of NSC-34 motoneurons.

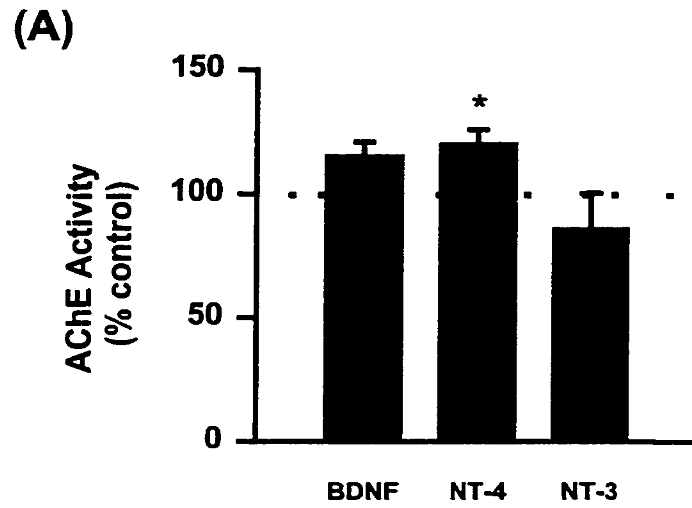
3.3.2 Effect of Neurotrophins on AChE and ChAT Expression

Previous studies have shown that BDNF and NT-4 are able to maintain AChE and ChAT expression in axotomized motoneurons and prevent the axotomy-induced reduction in AChE activity (Yan et al., 1994; Freidman et al., 1995; Tuszynski et al., 1996; Fernandes et al., 1998). We therefore treated cultures of NSC-34 motoneurons with BDNF and NT-4 to determine their effects on AChE and ChAT activity in normal, cholinergic neurons. Our results showed that cell-associated AChE activity was increased by 25% following NT-4 treatment ($P<0.05$) (Figure 14A). By contrast, the levels of secreted AChE were not affected by neurotrophin treatment (Figure 14B). As expected on the basis of the above data, BDNF and NT-4 significantly increased ChAT activity by approximately 50 - 70% ($P<0.002$) (Figure 14C). NT-3 treatment did not appear to affect AChE activity but resulted in a significant decrease in ChAT levels ($P<0.002$).

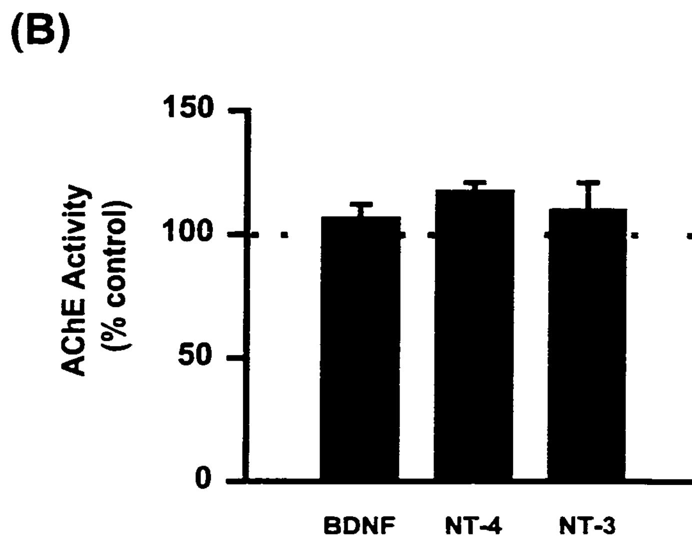
Figure 14. Effects of Neurotrophins on AChE and ChAT Activity in NSC-34 Motoneurons

Panels A and B show cell-associated and secreted AChE activity, respectively, in response to BDNF, NT-4 and NT-3 in NSC-34 motoneurons. Only NT-4 was able to increase cellular AChE activity to a significant level ($P<0.05$). Panel C shows the effects of neurotrophin treatment on ChAT activity. Note the 50 - 70% increase in ChAT activity in response to both BDNF ($P<0.001$) and NT-4 ($P<0.002$). Data are expressed as a percentage of the levels seen in non-treated cells. Results are from 4 separate experiments performed in duplicate. Asterisks denote a significant difference.

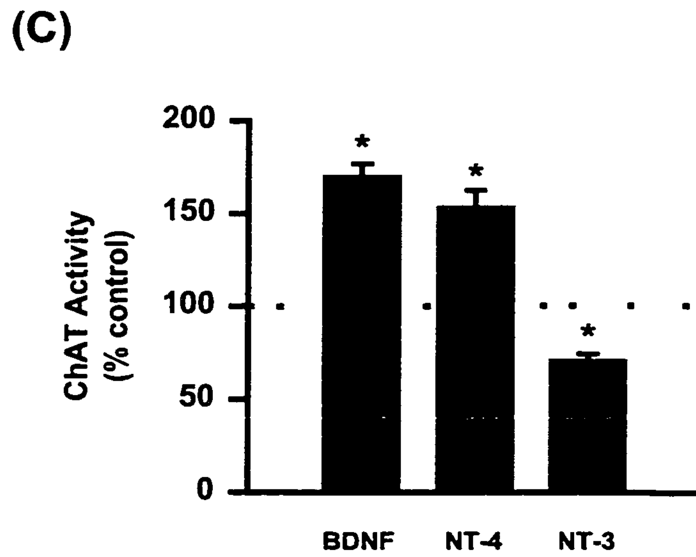
(A)



(B)



(C)



3.3.3 Basic FGF and Axokine Increase AChE and ChAT Expression in NSC-34 Motoneurons

In addition to classic neurotrophins, several other target-derived trophic factors have also been shown to affect the cholinergic phenotype of motoneurons, particularly bFGF and CNTF (McManaman et al., 1989; Hughes et al., 1993; Kato and Lindsay, 1994; Teng et al., 1998). We therefore treated NSC-34 motoneurons with bFGF and axokine (see Materials and Methods) to determine their effects on AChE and ChAT expression (Figure 15). We found that axokine increased both cell-associated (Figure 15A) and secreted (Figure 15B) AChE activity by approximately 2.5-fold ($P<0.01$). bFGF treatment resulted in a 1.5-fold increase in total AChE activity ($P<0.01$). However, the most dramatic effects of bFGF and axokine were on ChAT activity. bFGF increased ChAT activity by 3.5-fold ($P<0.02$) while axokine caused an approximate 5.5-fold increase in ChAT activity ($P<0.01$). Thus, it appears that of all the trophic factors tested, bFGF and axokine had the greatest effect on AChE and ChAT expression in developing spinal cord motoneurons.

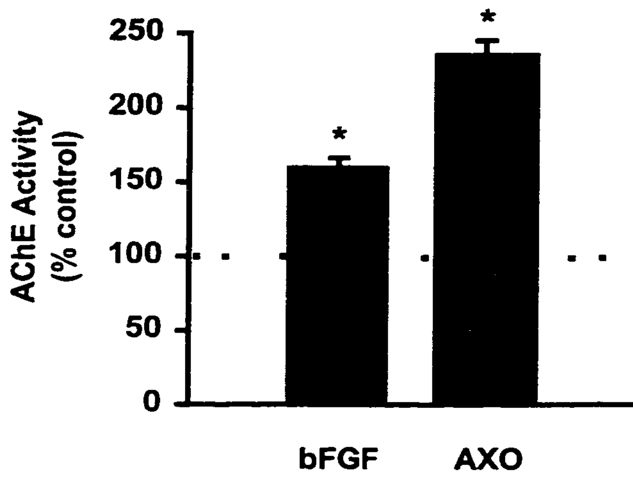
3.3.4 Effects of Axokine-Neurotrophin Combinations on AChE and ChAT Activity

Previous studies have shown that both CNTF and certain members of the neurotrophin family (ie. BDNF, NT-4) promote the survival and differentiation of developing motoneurons (Arakawa et al., 1990; Henderson et al., 1993; Wong et al., 1993). Moreover, synergistic interactions between CNTF and BDNF have also been

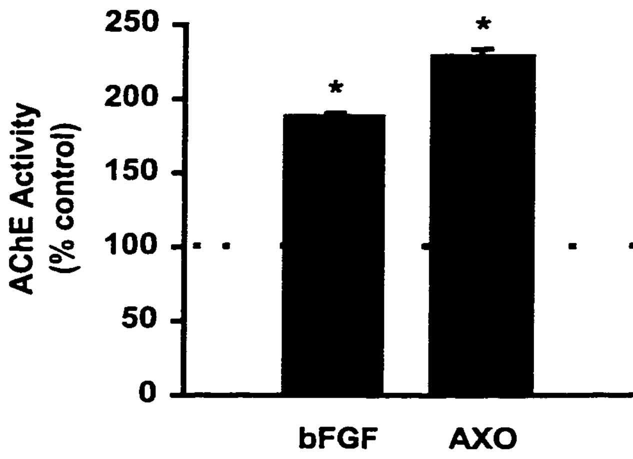
Figure 15. Effects of bFGF and Axokine on AChE and ChAT Activity in NSC-34 Motoneurons

Panels A and B show cell-associated and secreted AChE activity, respectively, in response to bFGF and axokine in NSC-34 motoneurons. Note that both bFGF and axokine increased AChE activity significantly ($P<0.01$). Panel C shows ChAT activity in response to bFGF and axokine. The two factors increased ChAT activity by 350 and 500%, respectively ($P<0.02$). Data are expressed as a percentage of the levels seen in non-treated cells. Results are representative of 4 separate experiments performed in duplicate. Asterisks denote a significant difference.

(A)



(B)



(C)

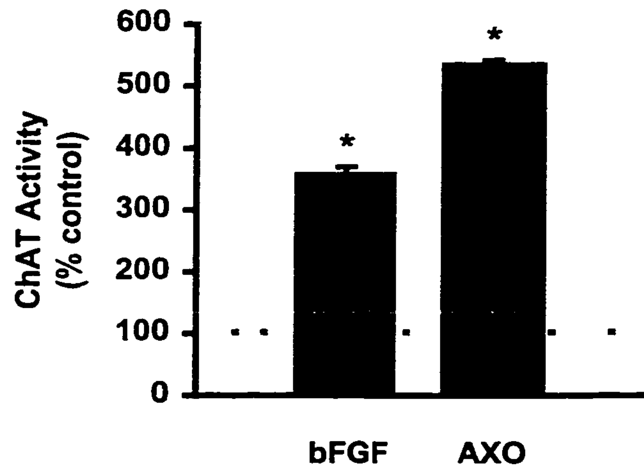
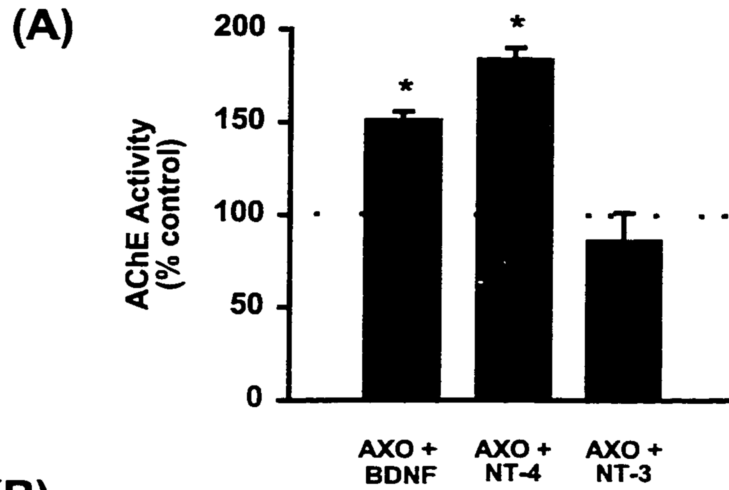


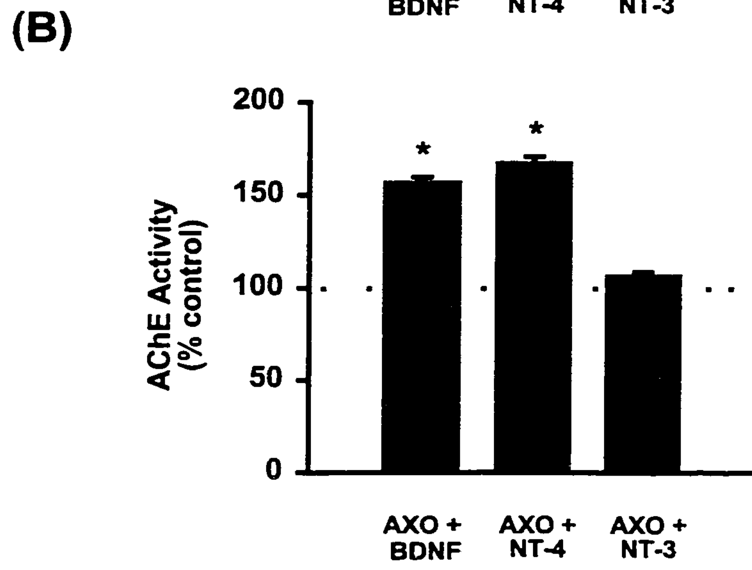
Figure 16. Effects of Axokine-Neurotrophin Treatment on AChE and ChAT Activity

NSC-34 cells were treated with axokine and either BDNF, NT-4 or NT-3. Panel A shows the effects of these treatments on cell-associated AChE. Note that the combinations of both axokine and BDNF ($P<0.02$) and axokine and NT-4 ($P<0.01$) increased AChE activity by more than 50%. The same result was observed in the media of treated NSC-34 cultures (panel B) ($P<0.02$). Panel C shows the effects of the three treatments on ChAT activity. Data are expressed as a percentage of the levels seen in non-treated cells. Results are representative of 3 separate experiments performed in duplicate. Asterisks denote significant difference.

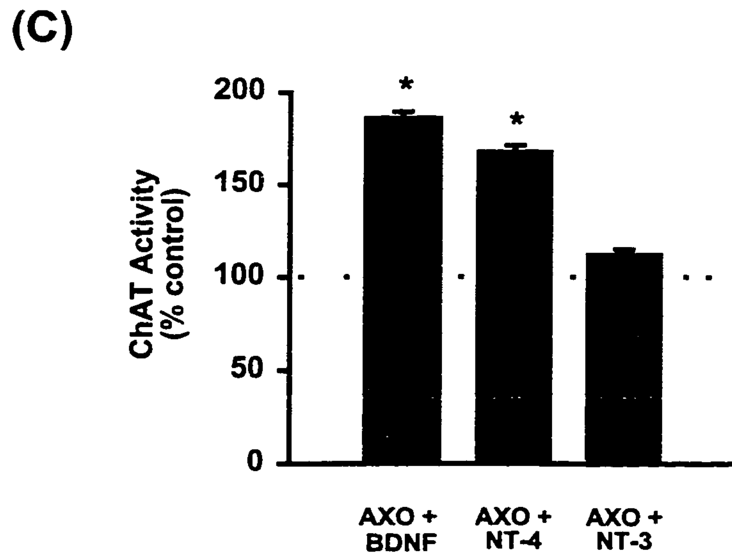
(A)



(B)



(C)



reported (Wong et al., 1993; Mitsumoto et al., 1994). However, the combinatorial effects of trophic factors remain poorly understood. We therefore treated NSC-34 cells with combinations of neurotrophins and axokine (Figure 16). We found that the combination of axokine and BDNF increased cell-associated AChE activity 1.5-fold (P<0.02) (Figure 16A). Similar results were obtained with the combination of axokine and NT-4 (P<0.01). NT-3 and axokine did not affect AChE activity. Secreted AChE activity was also increased with axokine and BDNF (P<0.02) and NT-4 (P<0.02) (Figure 16B). Again, NT-3 and axokine did not affect the activity of the secreted enzyme. In agreement with the above data, both BDNF (P<0.01) and NT-4 (P<0.02) in combination with axokine significantly increased ChAT activity in NSC-34 motoneurons (Figure 16C).

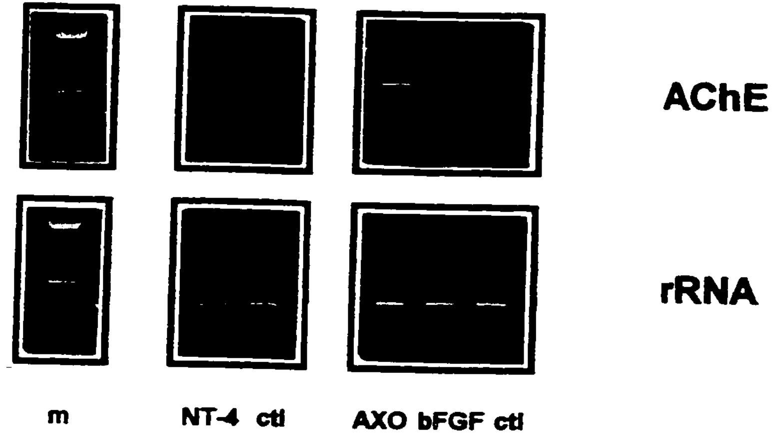
3.3.5 Neurotrophins Increase AChE mRNA Levels

Based on the observed increases in AChE activity caused by NT-4, bFGF and axokine, we examined the effects of these treatments on AChE mRNA levels (Figure 17). AChE mRNA levels were increased approximately 2-fold by NT-4 (P<0.02) and 4-fold by both bFGF and axokine (P<0.001). These preliminary results indicate that the trophic factor-induced increase in AChE activity is the result of an accumulation of AChE transcripts. Based on our data, it is likely that this accumulation is due to an enhanced stability of AChE mRNA, as opposed to an increase in the rate of transcription of the AChE gene.

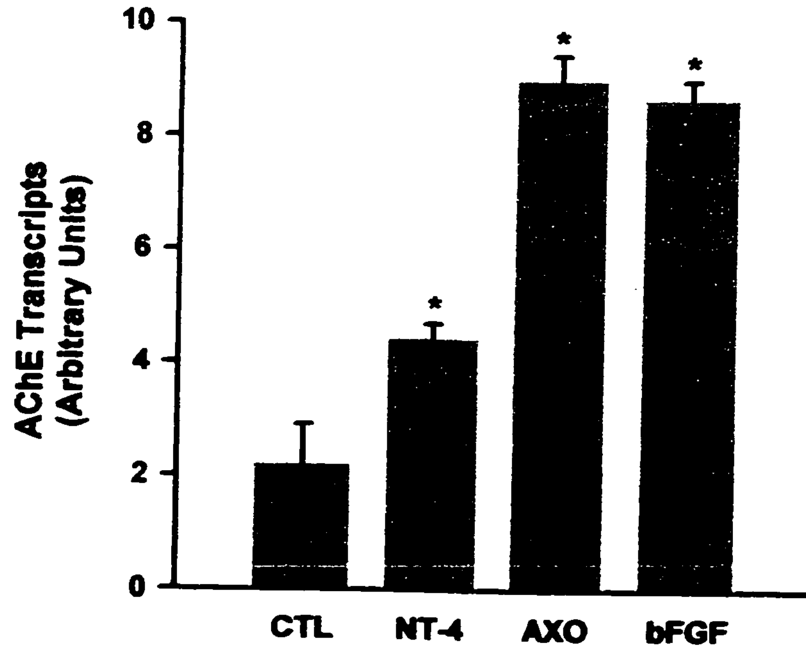
Figure 17. Neurotrophins Increase AChE mRNA Levels

The effects of neurotrophic factor treatment on AChE transcript levels are consistent with the effects observed on enzyme activity. Shown are representative ethidium bromide-stained agarose gels showing AChE mRNA levels in response to NT-4, bFGF and axokine. Note that all three factors increased AChE transcript levels. S12 ribosomal RNA served as an internal control and remained constant throughout. Results are from 3 independent experiments performed in duplicate. m, 100 bp marker (Gibco BRL). ctl, control. Asterisks denote a significant difference.

(A)



(B)



CHAPTER 4

DISCUSSION

Generally, the role of AChE has been well documented with respect to the neuromuscular junction. Within this area of nerve-muscle contact, AChE performs its traditional function of terminating neurotransmission by catalyzing the hydrolysis of ACh. The regulation of AChE in neurons has been an area of interest not only for its essential role in cholinergic transmission but also because the enzyme is found in non-cholinergic regions of the central and peripheral nervous systems. The presence of AChE in non-cholinergic tissues has led to the hypothesis that AChE may perform additional, non-catalytic functions in these areas. This hypothesis led to various reports in which the enzyme was found to regulate neuronal morphogenesis and differentiation (see Introduction). This concept has gained much interest over the years and has been studied in various systems including haematopoietic cells, the cholinergic system of the brain and spinal cord motoneurons. In the present study, we characterized a model with which to study the molecular mechanisms underlying the regulation of AChE expression in neurons. Using this model, we examined the developmental and trophic regulation of the enzyme. In addition, we also examined the regulation of ChAT during development and in response to certain neurotrophic factors. By examining the regulation of AChE and ChAT in parallel, we therefore developed a better understanding of the relationship between the two cholinergic enzymes in neuronal cells.

4.1 DEVELOPMENT OF A MODEL SYSTEM TO STUDY THE REGULATION OF AChE EXPRESSION IN NEURONS

The study of AChE regulation in neurons has been limited to date by a lack of satisfactory experimental models. An appropriate model system would be one in which AChE was endogenously expressed at detectable levels and where its expression did not require the use of inducing agents. Although primary cell culture has been used to study AChE in motoneurons (Bataille et al., 1998) and hippocampal cells (Schegg et al., 1986; Shingai et al., 1990), the isolation procedures are complicated and the resulting cultures are heterogeneous. Previous studies have attempted to examine the regulation of AChE in fibroblasts and COS cells (Getman et al., 1995; Li et al., 1993 a,b). The use of fibroblasts and COS cells is however, limited since they do not express AChE endogenously and must be transfected. They do not mimic any of the characteristics of neuronal cells and thus do not serve as adequate models for the study of AChE in neuronal populations. Studies conducted with embryonic chick retinal cells are similarly limited by the necessity of transfection for the expression of detectable AChE activity (see for example Robitzki et al., 1997). The regulation of AChE during neuronal differentiation has also been examined in P19 embryonic carcinoma cells (Coleman and Taylor, 1996). These cells are limited by the need for differentiating agents such as retinoic acid to induce the expression of AChE. Furthermore, only 0.1% - 1.0% of retinoic acid-treated P19 cells actually stain for AChE activity and ChAT is virtually undetectable (Staines et al., 1994). These cells therefore do not express the two

cholinergic enzymes at levels appropriate for proper detection and analysis.

4.1.1 Characterization of a Model for the Study of AChE in Neurons

In the present study, we sought to examine the regulation of AChE in a cholinergic cell line in which AChE was endogenously expressed, in an attempt to elucidate the role and regulation of the enzyme in neurons. We therefore examined AChE activity in several hybrid cell lines including two septal-neuroblastoma hybrids (SN6 and SN56), two motoneuron-neuroblastoma hybrids (NSC-19 and NSC-34), as well as the parent neuroblastoma for all four hybrid lines, N18TG2. We found that the NSC-34 line expressed the highest level of AChE, followed by the NSC-19. The septal-neuroblastoma hybrids also expressed AChE but at significantly lower levels and the enzyme was only detectable when the cells were differentiated with retinoic acid or dbcAMP (Lee et al., 1990; Blusztajn et al., 1992). Moreover, the differentiation of these cells was unpredictable and somewhat heterogeneous. The N18TG2 parent neuroblastoma expressed the lowest levels of AChE and most importantly, did not express any detectable ChAT activity (data not shown). Therefore, due to their endogenous expression of AChE and ease of culture, we chose the NSC-34 line as a suitable model for the study of both the developmental and trophic regulation of AChE and ChAT.

Motoneurons have long been regarded as a distinct neuronal population due to their large size and selective involvement in neurodegenerative diseases. They have been studied most extensively in primary cell culture. However, these studies

have been limited by a lack of understanding with respect to the molecular basis for their distinction from other neuronal populations (Cashman et al., 1992). Since the cells obtained from primary tissue are post-mitotic they cannot be serially passaged and require days or weeks to develop in culture. Primary culture is also limited by low yields and heterogeneity of neuronal preparations. An immortal, homogeneous line would therefore surmount many of the inherent difficulties encountered with cells derived from primary tissue. Nonetheless, there have not been any reports of a spontaneously arising motoneuron tumor cell line. This problem has been circumvented however, with the advent of somatic cell fusion.

Somatic cell fusion involves the fusion of end-mitotic, differentiated primary cells with tumor lines that proliferate but do not express the desired characteristics themselves (Cashman et al., 1992; Hammond et al., 1986). Hybridization of primary cells with, for example, neuroblastoma lines has been successful with embryonic mouse septal neurons (Hammond., 1989; Hammond et al., 1990 a, b; Blusztajn et al., 1992), sympathetic ganglia (Greene and Rein, 1977), dorsal root ganglia (Platika et al., 1985) and most recently, motoneuron-enriched embryonic mouse spinal cord cells (Cashman et al., 1992). In particular, Cashman and colleagues performed fusions with E12-14 spinal cord cells and N18TG2 neuroblastoma cells to produce several NSC (neuroblastoma-spinal cord) hybrid cell lines. Further characterization revealed that these cells extended processes, expressed ChAT and formed synapses when co-cultured with myotubes. In addition, they were also able to express choline uptake mechanisms and to synthesize, store and release ACh. The

two most promising lines selected for further study were the NSC-19 and NSC-34. These cell lines supported action potentials and expressed neurofilament proteins such as vimentin. In addition, the NSC-34 was able to induce clustering of AChR in myotubes. Thus, both NSC-19 and NSC-34 cells were found to express many of the morphological and physiological features of primary motoneurons.

The suitability of the NSC-34 cell line has also been acknowledged by various other researchers (Hunter et al., 1991; Durham et al., 1993; Matsumoto et al., 1995; Moscoso and Sanes, 1995; Porter and Sanes, 1995; Nagy et al., 1995; Usuki et al., 1999). For example, NSC-34 cells have been used to investigate the importance of the LRE motif in the binding of motoneurons to S-laminin (Hunter et al., 1991). The cell line was shown to bind S-laminin in the basal lamina in an LRE-dependent manner suggesting that the LRE comprised a motoneuron-selective adhesion site which served to inhibit neurite outgrowth. Furthermore, the cells have also been used to characterize the ganglioside compositions of motoneurons in an attempt to identify the target antigens in amyotrophic lateral sclerosis-like disease (Matsumoto et al., 1995). Finally, NSC-34 motoneurons were used to demonstrate the requirement of the hyaluronan receptor RHAMM in neurite motility and migration (Nagy et al., 1995).

4.1.2 Morphological Features of NSC-34 Motoneurons

In our experiments, we examined the NSC-34 according to their morphological characteristics to determine if indeed they mimicked primary spinal

cord motoneurons (Cashman et al., 1991, 1992). Following subculture, most NSC-34 cells had a rounded appearance, but within a few days many had extended multiple neurite-like processes, resembling primary motoneurons (Cashman et al., 1991). Adhesion to the substratum was poor compared to primary cultures (Durham et al., 1993) and the cells could be detached mechanically with Pasteur pipettes. Regardless, the cells began to detach spontaneously after about 10 days in culture, possibly due to apoptotic cell death (Cashman et al., 1991). Staining of NSC-34 motoneurons for AChE histochemistry revealed that the enzyme was found in the cell bodies of the neurons as well as along the neurites. This is in contrast to sympathetic neurons in which AChE is found clustered along the neurites but is essentially absent from the cell somas (Rotundo and Carbonetto, 1987). Previous studies performed in cultured motoneurons however, have shown that AChE staining is more intense in the cell bodies as opposed to the dendritic or axonal processes (Kim, 1972). In our study, enzyme activity was also found lining the surface of some cell bodies and axons which are otherwise devoid of any intracellular reaction. The significance of this distribution is unknown although we may tentatively suggest that the cells containing intracellular enzyme activity represent cholinergic neurons whereas those without intracellular AChE represent cholinoceptive neurons.

4.1.3 Effects of Depolarizing Agents on NSC-34 Motoneurons

In a separate series of experiments, we compared the effects of depolarizing agents on AChE and ChAT expression in NSC-34 to those seen with primary motoneurons. To this end, we treated NSC-34 cells with depolarizing agents such

as veratridine and high potassium, as well as agents that block calcium influx (verapamil) for two days, and subsequently measured AChE and ChAT activities.

Our results are consistent with those obtained by Ishida and Deguchi (1983) who have used cultures of primary motoneurons. Specifically, we demonstrated that AChE activity was depressed by both veratridine and potassium, whereas ChAT activity was increased significantly by these depolarizing agents. Conversely, AChE activity was increased by the calcium-channel blocker verapamil, whereas ChAT activity was reduced. Our results show that NSC-34 cells mimic primary motoneurons in their response to these agents and indeed constitute a useful model for the study of cholinergic regulation.

These results indicate that the activities of both AChE and ChAT are regulated by the influx of extracellular calcium, a result which has been observed in several other cell populations (Bursztajn et al., 1991; Luo et al., 1994, 1996). The above observations suggest that AChE and ChAT activities are inversely regulated by depolarizing agents in cholinergic neurons. Indeed, this would allow the enzymes to properly exert their cholinergic functions since modulations in ACh levels require parallel but inverse changes in AChE and ChAT expression.

4.2 REGULATION OF AChE EXPRESSION DURING NEURONAL DEVELOPMENT

4.2.1 Differentiation of NSC-34 Motoneurons Increases AChE Expression

The developmental regulation of AChE has been well documented in muscle where it performs its traditional role in neurotransmission. However, little is known about the cellular and molecular mechanisms underlying the expression of AChE during neuronal development and differentiation. We thus employed the NSC-34 motoneuronal cell line as a model system to address this issue. Differentiation of NSC-34 motoneurons resulted in large increases in both cell-associated and secreted AChE activity. The observed changes in AChE activity were accounted for mostly by increases in the levels of G_4.

The G_4 form of the enzyme is the predominant form found in mammalian nervous tissue, followed by the G_1 monomer (Grassi et al., 1982; Tsim et al., 1997). Particularly abundant is the amphiphilic form of G_4 which is anchored to the cell membrane via a 20 KDa hydrophobic tail (Inestrosa et al., 1987; Fuentes and Inestrosa, 1988; Fuentes et al., 1988). It was previously demonstrated that this G_4 form is preferentially transported into the nerve fibers and appears as clusters on the surface of the neurites (Rotundo and Carbonetto, 1987). In our experiments, the amphiphilic nature of the enzyme was examined in NSC-34 extracts by comparing its sedimentation coefficients in sucrose gradients containing either Triton x-100 or

Brij-96. As expected, differentiation induced a shift from the nonamphiphilic G_4 to an amphiphilic species. The amphiphilic G_4 found in mature nervous tissue is believed to be linked to the P subunit. It is thus possible that in immature NSC-34 cells the availability of the P subunit is limited thereby allowing the formation of only nonamphiphilic species. Once the P subunits become available, the cell switches to producing amphiphilic G_4 molecules. Similar observation were made by Anselmet et al. (1994) who showed that embryonic quail brain contained a significant proportion of nonamphiphilic forms whereas adult brain contained mostly amphiphilic species.

Unlike skeletal muscle (Massoulié et al., 1993) and other neuronal populations such as sympathetic neurons (Rotundo and Carbonetto, 1987), there were no detectable asymmetric forms in NSC-34 cells. These results are consistent with those obtained by Tsim et al. (1997) who failed to demonstrate the presence of asymmetric forms of AChE in chick spinal cord motoneurons. As expected, the NSC-34 cells also secreted a G_4 form of AChE and the changes observed during differentiation were due to increases in the G_4 form, as well as the G_1. Unlike the cell-associated AChE activity, all tetramers present in the media appeared nonamphiphilic.

During NSC-34 cell differentiation, the increases in AChE activity were accompanied by significant increases in the levels of AChE transcripts, as revealed by our RT-PCR analysis. Differential splicing of the AChE gene gives rise to three

transcripts designated T, H and R. Previous studies have shown that the T transcript, which arises from splicing of exon 4 to exon 6, is the only splice variant found in adult muscle (Legay et al., 1995) and the predominant transcript found in mammalian brain (Li et al., 1993b; Karpel et al., 1994). Alternatively, the H transcript is found almost exclusively in haematopoietic tissue and results from splicing of exon 4 to exon 5 (Chan et al., 1998). The R transcript results from reading through the splice donor site of exon 4 into the subsequent genomic sequence (Massoulié et al., 1993; Legay et al., 1995).

Our analysis revealed that the predominant species in undifferentiated NSC-34 cells was the T transcript. Differentiation led to an increase of approximately 3-4-fold. The R transcript was also present in both undifferentiated and differentiated cultures albeit, at slightly lower levels. However, the expected molecular form arising from translation of the R transcript was not detectable in our analysis. Although in transfected cultures, the R cDNA has been demonstrated to produce a soluble, secreted monomer (Li et al., 1993b), its protein product has never been unequivocally identified *in vivo*. It is therefore possible that the R transcript simply represents the product of an immature splicing mechanism found in immortalized cell lines (Legay et al., 1995; Chan et al., 1998). However, in our study we also demonstrated the presence of the R transcript in the mouse spinal cord and brain, thus confirming a previous report (Karpel et al., 1994). It is unlikely that our results were due to contamination by haematopoietic cells since the tissues were perfused prior to processing. It is therefore conceivable that the R transcript is normally

expressed in nervous tissue and its protein product subjected to translational and/or post-translational regulation. Interestingly, Kaufer et al. (1998) demonstrated the presence of the R transcript in the mouse cortex following acute stress suggesting that certain stimuli modify the alternative splicing of the AChE gene.

In contrast, the H transcript was barely detectable by RT-PCR analysis. Amphiphilic dimers of AChE have been shown to possess a GPI anchor linked to the C terminal amino acid residue of the catalytic subunit (for review see Massoulié et al., 1993). Such molecules exist on the surface of erythrocytes and other haematopoietic cells in vertebrates (Bon et al., 1988), but are also found exclusively in the CNS of *Drosophila* (Incardona and Rosenberry, 1996 a,b). In *Drosophila* the GPI-linked form of AChE is required for survival. AChE mutants show degenerative changes in CNS neurons which appear to be the result of the unopposed action of ACh (Incardona and Rosenberry, 1996b). Furthermore, flies expressing chimeric transmembrane forms of AChE are viable but show reduced locomotor activity and poor coordination. This suggests that the enzyme performs its traditional role in the termination of neurotransmission and may perhaps have other morphogenic functions. Nonetheless, the role of GPI-anchored AChE in the vertebrate CNS remains elusive. It is conceivable that the enzyme may in fact function as a regulator of proliferation and differentiation as has been suggested in haematopoietic cells (Paoletti et al., 1992; Soreq et al., 1994; Stephenson et al., 1996).

4.2.2 Differentiation of NSC-34 Motoneurons Increases the Stability of AChE Transcripts

As aforementioned, the molecular mechanisms regulating AChE gene expression have been studied most extensively in muscle cells in culture (Fuentes and Taylor, 1993; Luo et al., 1994; Hubatsch and Jasmin, 1997), and *in vivo* (Jasmin et al., 1993; Michel et al., 1994; Sveistrup et al., 1995; Chan et al., 1999). In the C2 myogenic cell line it has been demonstrated that the increase in the levels of AChE mRNA during the transition from myoblasts to myotubes results from an increase in the stability of the existing transcripts as opposed to a transcriptional activation of the AChE gene (Fuentes and Taylor, 1993). Similarly, post-transcriptional mechanisms have also been suggested to account for the induction of AChE mRNA in P19 embryonic carcinoma cells (Coleman and Taylor, 1996) and murine erythroleukemia cells (Chan et al.,1998). Using NSC-34 cells as a model to study the mechanisms underlying the increase in AChE expression during neuronal development, we show that the increase in AChE mRNA levels also results from increases in the stability of presynthesized transcripts. Indeed, our nuclear run-on assays demonstrated that there was no increase in the transcriptional rate of the AChE gene during differentiation of NSC-34 neurons despite an increase in the rate of transcription of the tubulin gene.

In differentiating muscle cells it has been determined that the influx of Ca^{2+} via L-type channels is linked to the stability of AChE transcripts (Luo et al., 1994).

Although L-type Ca^{2+} channels have not been detected in cultures of NSC-34 cells, slow inward calcium currents have been demonstrated, as well as responses to chemicals that affect voltage-gated ion channels (Durham et al., 1993). However, it remains to be established whether Ca^{2+} plays a direct role in controlling AChE mRNA stability in motoneurons. In differentiating C2 cells, it has also been observed that AChE mRNA levels increase following protein synthesis inhibitor treatment, therefore suggesting that certain labile proteins may be involved in the destabilization of AChE mRNAs (Fuentes and Taylor, 1993). The stability of mRNA in eukaryotes is known to be regulated by cis-acting sequences in the 3' untranslated region and trans-acting factors which stabilize or destabilize the transcript (see Sachs, 1993). Although in general little is known about these factors and regulatory sequences, it is apparent that post-transcriptional mechanisms operating at the level of mRNA stability represent a major regulatory step dictating the levels of AChE mRNA in cells that express the enzyme at significant levels.

In addition to the increases in AChE activity and transcript levels observed during differentiation, ChAT activities and mRNA levels also increased in parallel. Moreover, the stability of the ChAT transcript increased significantly with differentiation. These results suggest that expression of the two enzymes is co-regulated during development. Within adult cholinergic neurons, AChE and ChAT are co-expressed, but their expression patterns appear to be differentially regulated. For example, AChE mRNA expression occurs early during neuronal differentiation (i.e. between E10 and E12 in the mammalian nervous system) (Koenigsberger et al.,

1998) while ChAT expression begins just as the axons are reaching their targets (Phelps et al., 1984). Furthermore, AChE may also be expressed in the absence of ChAT in various regions of the nervous system such as the hippocampus (Landwehrmeyer et al., 1993; Hammond et al., 1994). Together, these findings suggest that despite differential regulation under certain circumstances, the two enzymes may in fact be co-regulated in response to certain stimuli.

4.3 REGULATION OF AChE AND ChAT BY NEUROTROPHINS

The survival of spinal motoneurons is thought to be dependent on factors secreted by their target tissues. These neurotrophic factors (NTFs) belong to a family of polypeptides that are required for the survival of discrete neuronal populations both *in vitro* and *in vivo* (Levi-Montalcini, 1987; Barde, 1989). Recently, the theory of target regulation of motoneuronal survival has spread to include neurotransmitter enzymes such as ChAT. Consistent with this theory are the findings that certain NTFs are able to restore ChAT immunoreactivity to axotomized cholinergic neurons (Yan et al., 1994; Friedman et al., 1995). Undoubtedly, AChE is also required for proper cholinergic neurotransmission. As aforementioned, the relationship between AChE and ChAT is poorly understood as their expression appears to be co-regulated under certain conditions and differentially regulated in others. Thus, given our current understanding, co-regulation of the two enzymes can not be assumed.

Based on the findings that motoneurons express the neurotrophin receptors trkB and trkC (Yan et al., 1988) and that both BDNF and NT-4 can restore the cholinergic phenotype of axotomized motoneurons (Chiu et al., 1994; Tuszynski et al., 1996; Fernandes et al., 1998), neurotrophins are thus candidate target-derived factors for the regulation of AChE expression in motoneuronal cell populations. In fact, application of neurotrophins *in vitro* induces an increase in AChE activity in several populations of embryonic neurons (Alderson et al., 1990; Ojika et al., 1994), including motoneurons (Wong et al., 1993). We therefore assessed the ability of certain NTFs (ie. BDNF, NT-4 and NT-3) to regulate the expression of AChE and ChAT in motoneurons using the NSC-34 cell line as a model system.

Although the prototypical neurotrophin is the nerve growth factor (NGF), we did not study the effects of NGF in our system since trkA, the NGF receptor, has not been found in motoneuronal cell populations (Ernfors et al., 1992; Henderson et al., 1993). Moreover, previous studies have shown that NGF is unable to prevent the loss of the cholinergic phenotype in adult motoneurons (Tuszynski et al., 1996) or support survival of cultured CNS neurons (Ip et al., 1993). We therefore focused on the remaining members for our studies.

4.3.1 NSC-34 Motoneurons Express TrkB and TrkC Tyrosine Kinase Receptors

Prior to examining the effects of neurotrophins on AChE and ChAT

expression, we ascertained, via RT-PCR, that NSC-34 cells expressed trkB and trkC. Mature motoneurons express the high-affinity trkB and trkC receptors, which mediate signaling by BDNF/NT-4 and NT-3, respectively (Fernandes et al., 1998). In our assays, we detected both trkB and trkC mRNAs in both undifferentiated and differentiated NSC-34 motoneurons suggesting that these cells are susceptible to the effects of certain NTFs. We also found that the levels of trkB mRNA increased approximately 6-fold during differentiation whereas levels of trkC mRNA remained relatively constant. Assuming that these mRNA levels reflect expression of the protein, the increases in trkB transcript levels indicate that NSC-34 cells may be responsive to the effects of BDNF and NT-4.

Detection of the trkC receptor revealed the presence of two isoforms, one noninserted and another carrying an insertion of 14 amino acids within the tyrosine kinase domain. The presence of such insertions appears to interfere with the signaling capabilities of the receptor, by inhibiting the mitogen-activated protein kinase pathway (Tsoulfas et al., 1996; Gunn-Moore et al., 1997). However, the presence of the full-length non-inserted isoform suggests that these cells have the ability to respond to the trkC ligand NT-3. Nonetheless, differentiation did not induce any changes in the levels of trkC transcripts or in the relative abundance of the two isoforms.

4.3.2 Effects of Neurotrophins on AChE Expression

In the present study, we found that both NT-4 and BDNF had a slight effect

on AChE activity and mRNA levels in differentiated NSC-34 cells. Levels of secreted AChE activity were not affected by NTF treatment at all. This implies that the trophic factors may only affect a specific pool of AChE which is destined to become localized to the cell surface. Indeed, our molecular form studies detected a nonamphiphilic pool of AChE in the media of differentiated NSC-34 cells, as opposed to the amphiphilic molecules found in the cell extract. In addition, the possibility that the NTFs were inactive can be ruled out since both BDNF and NT-4 increased ChAT activity and transcript levels significantly in differentiated cells. It is also possible that BDNF and NT-4 do not affect normal (i.e. uninjured) cholinergic neurons and act mainly as lesion factors in axotomized motoneurons.

The effects of NT-3 are somewhat more heterogeneous. NT-3 is the most abundant muscle-derived trophic factor and its effects are seen in embryonic motoneurons (Wong et al., 1993). However, few effects have been reported in adult motoneurons. Consistent with these findings, our studies showed no effect of NT-3 on AChE activity, whether cell-associated or secreted. Again, assuming that mRNA levels reflect the expression of the protein itself, one possibility for the minor NT-3 effects is the presence of the inserted isoforms of the trkC receptor tyrosine kinase. The limited signaling capabilities of the inserted trkC isoforms has been shown using *in vitro* studies with PC12 cells, where the cells responded to NT-3 with neurite outgrowth following transfection with full-length trkC but not with inserted full-length trkC variants (Lamballe et al., 1993; Tsoulfas et al., 1993; Valenzuela et al., 1993). The use of PCR primers that bracket the insertion site allowed the detection of both

noninserted and inserted isoforms of trkC and allowed us to demonstrate that the levels of both isoforms remained constant during differentiation. This suggests that the cells may simply not be responsive to NT-3 or that NT-3 simply does not affect the expression of AChE. It is possible that the inserted isoforms exert a dominant negative effect on the noninserted trkC signaling pathways, contributing to the limited responsiveness of NSC-34 motoneurons to NT-3. On the other hand, NT-3 decreased the levels of ChAT slightly indicating that perhaps other unknown mechanisms specific to the ChAT gene may be responsible for this regulation.

According to the theory of target-derived regulation, cholinergic differentiation is regulated by factors present in the target tissue. This was first demonstrated by Giller et al. (1973) in cultures of dissociated spinal cord. They showed that the development of ChAT activity in mouse spinal cord cultures was enhanced by co-culture with myotubes. It has since been demonstrated that myotube conditioned medium also enhanced ChAT activity and synthesis of ACh (Kaufman et al., 1985; McManaman et al., 1988). Attempts to isolate these muscle-derived neurotrophic factors has led to the discovery of BDNF, NT-4 and NT-3. However, the effects of the above neurotrophins have been shown to be quite varied and the search continued for other factors potentially responsible for the regulation of motoneuronal survival.

4.3.3 Effects of Neural Cytokines on AChE Expression

The neural cytokine CNTF has also been shown to enhance the survival of

motoneurons *in vivo* and *in vitro* (Arakawa et al., 1990; Sendtner et al., 1990; Oppenheim et al., 1991; Gurney et al., 1992). Indeed, endogenous CNTF has been shown to blunt the progression of several mouse models of motoneuron disease (Sendtner et al., 1992; Mitsumoto et al., 1994; Sagot et al., 1995) suggesting that CNTF may be of therapeutic value for motoneuron diseases in humans. However, the mechanism by which CNTF achieves this enhanced motoneuronal survival remains poorly understood. In fact, there is evidence that it is the CNTF receptor, not the CNTF molecule itself, that is responsible for the survival-promoting effects. DeChiara and coworkers (1995) recently demonstrated that the lack of CNTF in embryonic mice did not result in any notable abnormalities. However, mice lacking the CNTF receptor died shortly after birth and had severe motoneuron deficits. Thus it is likely that the CNTF receptor is critical for the development of the nervous system, and serves as the receptor for another CNTF-like ligand. Notwithstanding the effects of CNTF on the survival of motoneurons, very little is known of its effect on AChE or ChAT. In skeletal muscle, CNTF failed to increase AChE expression in intact muscle and failed to prevent the decrease normally observed following denervation (Boudreau-Larivière et al., 1996). Despite these findings, we showed dramatic increases in AChE following treatment with the stable, CNTF derivative, axokine. We observed 2.5-fold increases in both cell-associated and secreted AChE activity as well as 5.5-fold increases in ChAT expression. These were all accompanied by similar increases in mRNA levels. These results are in accord with those obtained by Di Marco and colleagues (1996) who showed dramatic increases in ChAT activity following treatment of SN56 cells with CNTF. Taken together with

the findings of Boudreau-Larivière et al. (1996) this suggests that unknown, neuron-specific mechanisms may be involved in the regulation of AChE and ChAT by CNTF.

Among the other factors suspected to regulate the cholinergic phenotype of neurons is bFGF, a neural cytokine present in skeletal muscle extracts which is upregulated following spinal cord injury (Mocchetti et al., 1996). Although bFGF is known as a potent mitogen, it has previously been shown to enhance the *in vitro* survival of cortical, hippocampal and ciliary ganglia neurons (Walicke et al., 1986; Morrison et al., 1986; Unsicker et al., 1987). Recently, it has been demonstrated that bFGF can also induce ChAT activity *in vitro* in cultures of cholinergic and dopaminergic neurons (Knüsel et al., 1990), as well as rat spinal cord motoneurons (McManaman et al., 1989). Furthermore, bFGF can spare axotomized cholinergic neurons from cell death (Anderson et al., 1988; Teng et al., 1998). The effects of bFGF on AChE however, have not been reported. In our study, we showed that bFGF increased both cell-associated and secreted AChE activity by approximately 2.5-fold. We also confirmed previous reports that bFGF increases ChAT activity significantly (McManaman et al., 1989; Teng et al., 1998). These results lend further evidence to the hypothesis that bFGF derived from skeletal muscle can regulate the cholinergic phenotype of spinal cord motoneurons. Although the mechanism by which bFGF stimulates AChE expression remains to be elucidated, its ability to stimulate ChAT expression does not appear to be related to its mitogenic activity. Treatment of neuronal cultures with blockers of mitogenic activity does not affect the ability of bFGF to enhance ChAT expression (McManaman et al., 1989). Thus the

effects of bFGF on the cholinergic phenotype of motoneurons may be the result of direct interactions with the neurons themselves. We have shown that the increase in AChE activity following bFGF treatment is due to an increase in AChE transcript levels. Whether this accumulation is the result of an increase in transcriptional activity or an enhanced stability of the AChE mRNA remains to be determined, but based on our developmental data, it is likely that stabilization of the transcript is involved.

4.3.4 Effects of Axokine-Neurotrophin Combinations on AChE Expression

To further our observations, we examined the effects of neurotrophins and axokine on cultures of NSC-34 motoneurons. In agreement with other studies (Wong et al., 1993; Kato and Lindsay, 1994), we observed enhanced ChAT activity in cultures co-treated with axokine and BDNF or NT-4. Similar results were obtained for AChE expression. In both cases, co-treatment with axokine and NT-3 did not result in any significant changes. The effects seen with axokine were only apparent with neurotrophins whose actions are thought to be mediated by trkB (ie. BDNF and NT-4). Neurotrophins whose action is preferentially mediated by trkC, had no apparent effects on AChE or ChAT activity. Although the combinatorial effects were greater than those observed with neurotrophin treatment alone, they were less dramatic than those seen with axokine. Despite these findings, previous reports have shown synergistic effects between neurotrophins and CNTF (Mitsumoto et al., 1994). The role of CNTF in motoneuron survival is still poorly understood and there is conflicting data concerning its effects on the cholinergic status of neuronal cells

(Mitsumoto et al., 1994; Clatterbuck et al., 1994). There may, therefore be mechanisms regulating its function and interaction with other neurotrophic factors which have not yet been elucidated.

4.3.5 Mechanisms Underlying the Neurotrophic Regulation of AChE Expression

Our preliminary data demonstrated that NT-4, bFGF and axokine increased AChE mRNA in comparison to control levels. However, the mechanisms by which neurotrophins affect the cholinergic character of neurons are unknown. It is currently well established that activation of neurotrophin receptors triggers a series of biochemical events that culminate in alterations in gene expression within the nucleus of target cells (for review see Bonni and Greenberg, 1997). Based on our findings, the effects of neurotrophic factors on NSC-34 motoneurons may result from an increase in the stability of the AChE mRNA. This suggests that the application of neurotrophic factors directly influences the abundance of RNA-binding proteins which in turn, affect the stability of existing AChE transcripts.

4.4 CHOLINERGIC REGULATION IN NSC-34 MOTONEURONS

AChE and ChAT must be specifically regulated in neurons in order to properly exert their cholinergic function. In the present study, we have shown that during neuronal development expression of the two enzymes appears to be co-regulated. Specifically, the expression of both enzymes increase during the course of

development. This increase appears to result from enhanced mRNA stability as opposed to an increased rate of transcription. In fully differentiated neurons, the enzymes exhibit a differential regulation in response to depolarizing agents such that the levels of ACh can be precisely controlled according to electrical activity. In addition, neurotrophic factors also elicit a differential regulation of the enzymes. It appears that the neurotrophins BDNF and NT-4 regulate levels of ChAT in uninjured neurons whereas they have little effect on AChE expression. These factors may then serve as lesion factors to upregulate AChE expression in axotomized neurons. Conversely, the neural cytokines bFGF and axokine appear to regulate AChE and ChAT in a co-ordinate fashion. It is possible that these factors play a more significant role in the maintenance of the cholinergic phenotype in uninjured neurons. Thus, AChE and ChAT appear to be regulated such that levels of ACh can be quickly and precisely altered, producing a dynamic system which is able to respond to various environmental stimuli.

4.5 FUTURE DIRECTION

In our study we have shown that mRNA stability plays an important role in the regulation of AChE expression. To further these findings we would like to determine the mechanisms underlying this increase in stability. Specifically, we would like to examine the role of RNA binding proteins and tissue-specific cis-acting sequences involved in the stabilization of AChE mRNA. Despite the role of post-transcriptional mechanisms in the control of AChE expression, it is still of interest to examine the contribution of specific AChE promoter elements to the transcriptional regulation of

the AChE gene. Of particular interest would be identifying tissue-specific promoter elements that regulate the expression of the AChE gene in motoneurons. This would require transfection of cultured neurons with AChE promoter constructs and analysis of transcriptional activity. In fact, we have successfully transfected NSC-34 cultures with several AChE promoter-reporter gene constructs thus providing a model for future analysis of the tissue-specific regulation of the AChE gene. In addition, we would like to elucidate the mechanisms involved in the trophic regulation of AChE expression. Specifically, we would investigate the existence of regulatory sites on the AChE gene which might be involved in the regulation of AChE expression by trophic factors such as CNTF and bFGF. An understanding of the mechanisms regulating expression of the AChE gene is necessary not only for the comprehension of its vital function(s) in different tissues, but also for the elucidation of the underlying pathology in various disease states. Indeed, deregulation of AChE expression has been implicated in several diseases including haematopoietic disorders such as leukemia, and in Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS).

4.6 CLINICAL IMPLICATIONS

Although the role of AChE in the haematopoietic system is unknown, it remains an area of interest since the AChE gene maps to a region involved in the development of myelodysplastic disorders and acute myeloid leukemias (Ehrlich et al., 1992). It has been suggested that AChE could serve as a tumor suppressor and regulate the proliferation of haematopoietic cells (Soreq et al., 1994; Stephenson et

al., 1996). Similar findings have been reported in the study of neurodegenerative diseases such as Alzheimer's. The cholinergic deficit in AD involves a loss of both AChE, particularly the G_4 form, and ChAT in the affected brain regions (Layer and Willbold, 1995; Siek et al., 1990). Despite the overall decrease in AChE activity in AD brains, there is however, a localized accumulation of AChE surrounding the characteristic amyloid plaques. This localization does not correlate with the density of cholinergic innervation, suggesting that the deposition of AChE is unrelated to its function in neurotransmission (Small et al., 1995). Finally, AChE has also been implicated in the pathophysiology of ALS. ALS is a motoneuron disease characterized by neuronal degeneration of large alpha motoneurons, as well as some large sensory fibres. It is known that most ALS patients have autoantibodies directed towards AChE and that this is associated with the fasiculations which are so characteristic of the disease (Sindhupak et al., 1988).

Despite a rapidly growing interest in this area of study, very little is known concerning the relationship between AChE and neuronal cells. It is however, clear that there are serious clinical implications involved in this relationship and an understanding of the regulation of AChE in health could advance treatments and even cures in many harmful disease states.

4.7 CONCLUSIONS

In this study, we examined the molecular mechanisms underlying the

developmental and trophic regulation of AChE in cholinergic neurons. We established and characterized a model system, the NSC-34 cell line, in which to examine this regulation . We demonstrated that the NSC-34 cell line models many aspects of the developmental maturation of spinal cord motoneurons. Morphologically, NSC-34 cells resemble developing motoneurons and do not present any of the complications encountered with primary tissue culture. They express AChE and ChAT at highly detectable levels and respond to depolarizing agents in a manner similar to primary motoneurons. Given these results we suggest that the NSC-34 motoneuronal cell line was a suitable model for the study of the developmental regulation of AChE expression in cholinergic neurons.

During the course of neuronal development, we observed an overall increase in AChE expression which resulted mainly from an increase in the G_4 form of the enzyme. The differentiation-induced increase was accompanied by a shift from nonamphiphilic to amphiphilic species of G_4. The results obtained from our studies indicate that this increase was the result of a selective stabilization of the AChE mRNA, as opposed to an enhancement of transcriptional activity. The mechanism of this stabilization has yet to be elucidated but is most likely regulated by extracellular calcium. We also demonstrated the potent effects of several neurotrophic factors on AChE and ChAT expression. The trkB ligands BDNF and NT-4 had the greatest influence on AChE and ChAT activities whereas the trkC ligand NT-3 had only limited effects. This may be attributed to the presence of inserted forms of the NT-3 receptor, trkC, in NSC-34 motoneurons. In addition, the

neural cytokines bFGF and axokine had dramatic effects on neurotransmitter enzyme expression.

Together, these results demonstrate that AChE and ChAT are regulated so as to achieve precise control of cholinergic neurotransmission. During development, the two enzymes are co-regulated and their expression increases in parallel. These increases are mediated by enhanced mRNA stability. Co-regulation thus allows for adequate amounts of both enzymes to be present during the formation of the neuromuscular synapse. Conversely, electrical activity results in a differential regulation of AChE and ChAT expression. This pattern of regulation is ideal since modulations in ACh levels require parallel, yet inverse changes in AChE and ChAT. Finally, the various neurotrophic factors differ in their regulation of the two enzymes. Despite increasing ChAT activity, the neurotrophins BDNF and NT-4 have little effect on AChE expression. We tentatively suggest that these neurotrophins do not affect AChE in uninjured cells, but may act as lesion factors to modulate AChE levels in axotomized neurons. The neural cytokines bFGF and axokine regulate AChE and ChAT coordinately and thus may be involved in the maintenance of the cholinergic phenotype in healthy, uninjured neurons.

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