Functional Manipulations of Acetylcholinesterase Splice Variants Highlight Alternative Splicing Contributions to Murine Neocortical Development

Proliferation and differentiation of mammalian central nervous system progenitor cells involve concerted, controlled transcriptional and alternative splicing modulation. Searching for the developmental implications of this programming, we manipulated specific acetylcholinesterase (AChE) splice variants in the embryonic mouse brain. In wild type mice, ‘synaptic’ AChE-S appeared in migrating neurons, whereas the C-terminus cleaved off the stress-induced AChE-R variant associated with migratory radial glial fibers. Antisense suppression of AChE-R reduced neuronal migration, allowing increased proliferation of progenitor cells. In contrast, transgenic overexpression of AChE-R was ineffective, whereas transgenic excess of enzymatically active AChE-S or inactive AChE-Sin suppressed progenitors proliferation alone or both proliferation and neuronal migration, respectively. Our findings attribute to alternative splicing events an interactive major role in neocortical development.

Keywords: alternative splicing, neurogenesis, neuronal migration, radial glia, readthrough acetylcholinesterase

Introduction

Neocortex development involves generation of projection neurons in the ventricular zone (VZ) in response to as yet incompletely understood cues, from a progenitor neuroepithelium that is mostly composed of radial glial cells (Tamamaki et al., 2001; Malatesta et al., 2003). These progenitor cells undergo interkinetic nuclear migration, whereby DNA replication occurs when the nucleus is in the basal (outer) portion of the VZ, and division adjacent to the ventricular surface give rise to two daughter cells (Fig. 1A) (Boulder Committee, 1970). Neurogenesis in mice commences on embryonic day (E) 11, with production of the first post-mitotic neurons and their exit from the VZ, while their sister cells re-enter the cell cycle and continue to proliferate (Takahashi et al., 1995). Clonally related proliferating cells coalesce in small, gap junction-coupled clusters that cycle synchronously (Bittman et al., 1997), giving rise to a clonally related output of neurons (Cai et al., 1997). As neurogenesis proceeds, proliferation slows under the influence of cell cycle-related proteins (Delalle et al., 1999), and cell divisions primarily yield post-mitotic neurons, while the proportion of daughter cells that continue to proliferate is greatly decreased (Takahashi et al., 1996).

Following mitosis, the neural progeny reach the developing cortex, i.e. the cortical plate (CP), either by somatic translocation, whereby a neuronal cell body migrates within a pial-contacting radial process (Nadarajah et al., 2001) or by locomotion, whereby newborn neurons develop elongated leading and trailing processes that are oriented in a radial direction, and migrate along radial glial fibers (Rakic, 1972).

Their adherence to nearby radial glial fibers activates multiple intercellular events, such as cell–cell recognition and transmembrane signal transduction, that facilitate their movement along the fibers (Rakic et al., 1994). Migrating neurons regulate and maintain the function of radial glia as migratory guides while radial glia regulate migration along their processes (Anton et al., 1997). Migrating neurons first pass through a subventricular zone (SVZ) occupied by a secondary proliferative population of cells adjacent to the VZ, and then through an intermediate zone (IZ). They enter the CP and reach its most superficial portion, adjacent to a marginal zone (MZ), where radial glial fibers arborize before terminating at the pial surface (Fig. 1A,B) (Gadisseux et al., 1989). Neurons are therefore guided by radially directed processes of their founder cells, reaching their appropriate destination in clonally related clusters (Noctor et al., 2001). They are positioned in the CP in an ‘inside-out’ sequence, with newly arriving cells settling superficial to those that arrived earlier (Rakic, 1974). Laminar fate, representing an aspect of neuronal phenotype, was shown to be specified by gene expression in the progenitor cells, accompanied by progressive restriction of multipotency (Frantz and McConnell, 1996).

Proliferation, differentiation and programmed gene expression in the developing nervous system may all be subject to modulation by stress. Embryonic stress (e.g. ischemia–hypoxia) attenuates neuronal migration to the cerebral neocortex, resulting in morphological changes that are often accompanied by postnatal behavioral deficits (Tashima et al., 2001). Similarly, prenatal maternal stress impairs development of the offspring, reducing, for example, learning and behavioral performance (Kofman, 2002), and increasing the incidence of brain malformation and reduced head circumference (Mulder et al., 2002). Improved understanding of the molecular mechanisms underlying the effects of stress on brain development is therefore of considerable importance.

A notable cascade common to both brain development and stress responses involves alternative splicing of pre-mRNA transcripts, e.g. glutamic acid decarboxylase (Kuppers et al., 2000), G protein isoforms (Morishita et al., 1999), the transcriptional repressor ATF3 (Hashimoto et al., 2002) or potassium channels (Xie and Black, 2001). However, the relationships between these splicing modifications and the physiological changes occurring during development and under stress remained obscure.

One way to explore this question is to directly manipulate the embryonic expression levels and/or properties of specific variant mRNA transcripts of a neuronally-expressed gene that is subject to transcription and splicing changes under both development and stress, and observe the outcome with respect to subsequent developmental events. The acetylcholinesterase gene (ACHE) emerges as an appropriate example for such
AChE-R by alternative splicing of the AChE pre-mRNA (Fig. 1CD) (Kaufer et al., 1998). AChE overproduction acts in the short term to reduce available acetylcholine (ACh) and attenuate cholinergic neurotransmission (Soreq and Seidman, 2001), but subsequent accumulation may last weeks after exposure (Meshorer et al., 2002) and may induce vulnerability to head injury (Shohami et al., 2000). It is plausible, therefore, that changes in AChE gene expression are involved in both development- and stress-related responses of the mammalian brain.

To examine the involvement of alternative splicing in cortical development, we subjected mouse embryos to antisense oligonucleotide suppression or to transgenic overexpression of specific AChE splice variants, and quantified the effects on cortical development. Here, we report that both ‘synaptic’ AChE (AChE-S) and AChE-R mRNA are expressed by progenitor cells in the VZ and undifferentiated cells in the CP. However, while the membrane-associated AChE-S was detected in migrating neurons, embryonic brain AChE-R undergoes C-terminal cleavage, similar to the modification characterizing the AChE-R isoform found in blood (Grisaru et al., 2001), and appeared in radial glial fibers. Transgenic manipulations of AChE variants, moreover, induced changes in progenitor cell proliferation as well as neuronal migration, suggesting physiological and pathophysiological roles for alternative splicing of AChE in cortical development.

Materials and Methods

Animals

CD1 and FVB/N mice were used for antisense and transgenic experiments respectively. Vaginal plugs on post-mating morning designated E0. Pregnant dams were anesthetized by intra-muscular injection of a ketamin and xylazine mixture (50 and 10 mg/kg body wt, respectively). Embryos were removed and dissected in cold phosphate buffered saline (PBS). Heads (E11–15) and brains (E16–17) were immersed in 4% paraformaldehyde in PBS (48 h, 4°C), embedded in paraffin and sectioned at 4 μm in the coronal plane. Animal care followed institutional guidelines according to NIH published guidelines.

BrdU and Oligonucleotides Injections

Pregnant dams were injected intraperitoneally (i.p.) with bromodeoxyuridine (BrdU; 50 mg/kg in 7 mM NaOH–saline solution; Sigma, St Louis, MO). Post-injection time points at 1, 2, 4, 12 and 48 h served to detect labeled nuclei at S-phase, S+G2 (with a few mitotic cells in the VZ), S+G2+M, G1 and post-mitotic cells in the VZ, or a cohort of neurons ‘born’ on E14, that migrated and reached the CP respectively. EN101, a 20-mer antisense-oligodeoxynucleotide was previously shown to primarily suppress AChE-R mRNA (Cohen et al., 2002; Meshorer et al., 2002) to target exon-2 of mouse AChE mRNA. Its three 3′-terminal nucleotides 5′-CTGCAATATTCTTTGC (stars) were 2′-O-methylated for nuclease protection. Inversely oriented oligodeoxynucleotides (INV101) with the same sequence as the antisense, but oriented from 3′ to 5′ served as control. Oligonucleotides were dissolved in saline and injected i.p. three successive times at 12 h intervals, initiated 12 h following BrdU injection on E14 (40 or 100 μg/kg per injection). Animals were sacrificed 48 h after BrdU injection.

Immunohistochemistry

Sections were deparaffinized, microwave-treated (750 W, 15 min) in 0.01 M citric buffer, pH 6.0, and blocked (30 min) in 5% normal goat, rabbit or horse serum in PBS with 0.5% Tween-20 (PBST) for AChE Readthrough Peptide (ARP), AChE Synaptic Peptide (ASP) or AChE N-terminus (N-trm), and nestin, respectively (Fig. 1D). Immunoreactions (90 min, room temperature) were with rabbit anti-ARP (Sternfeld et al., 2000), goat anti-ASP [Santa Cruz Biotechnology, Santa Cruz, CA; AChE (C-16)] or goat anti-AChE [Santa Cruz N-terminal AChE (N-19)], 1:100 in

420 ‘Readthrough’ AChE Variant in Murine Neocortical Development · Dori et al.
PBST containing 2.5% serum. Immunoreactivity for ARP was eliminated by incubation of the antiserum with synthetic ARP (Sternfeld et al., 2000) at a molar ratio of 1:5, attesting to specificity of the antiserum (not shown). TUJ1 antibody (Lee et al., 1990) (generously provided by Dr A. Frankfurter) and mouse anti-nestin (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) were 1:500 in PBST. Secondary IgG were biotin-conjugated goat anti-rabbit for ARP, donkey anti-goat for ASP or N-trm, and horse anti-mouse for nestin detection (Vector), 1:200 in PBST containing 2.5% serum (1 h). TUJ1 detection involved goat anti-mouse Cy3-conjugated IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:200 in PBST). Biotinylated antibodies were incubated with avidin-biotin peroxidase complex (ABC Elite, Vector Laboratories) for 1 h, rinsed with 0.05 M Tris, pH 7.6, and reacted for 90 s with 0.05% diaminobenzidine (Sigma) and 0.006% H2O2 in 0.05 M Tris, pH 7.6, with 0.05% nickel ammonium sulfate. Selected sections were counterstained with Gill-2 hematoxylin (Sigma).

Immunocytochemistry for the nuclear antigen Ki67 was used to monitor cell proliferation. Ki67, previously used to label dividing cells in the human embryonic VZ (Weissman et al., 2003), is expressed by proliferating cells during late G1, S, M and G2 phases of the cell cycle (Gerdes et al., 1984; Scholzen and Gerdes, 2000), and is often used to evaluate the proliferative fraction of solid tumors (Scholzen and Gerdes, 2000). The utility of Ki67 as a proliferative marker that is comparable to BrdU labeling was previously tested for neurogenesis in the adult dentate gyrus of the hippocampus, where its expression mimicked that of BrdU when examined soon after exogenous BrdU administration. Experimental increases in the number of mitotic cells by ischemia, or their reductions by radiation produced parallel changes in BrdU and Ki67 labeling (Kee et al., 2002). Ki67 staining increases during S-phase, reaches a peak during metaphase (du Manoir et al., 1991) and decreases during ana- and telophase (Starborg et al., 1996). Quantification of Ki67 expression was compiled by measuring the mean sum of pixel values in a 50 × 100 μm rectangle at the apical portion of the VZ, positioned 100 μm lateral to the dorsomedial to medial cortical border, similar to that done for detection of AChE.

BrdU Labeling
Sections were treated with 100 μg/ml deoxyribonuclease in PBST (30 min), incubated with mouse anti-BrdU (Becton-Dickinson, Mississauga, Ontario, Canada); 1:100 in PBST, 2 h), followed by anti-mouse Cy2-conjugated IgG (Jackson; 1:50 in PBST) or biotinylated goat anti-mouse IgG. Processing was as described above.

In situ Hybridization
Previously detailed probes and procedure (Meshorer et al., 2002) were modified as follows. Cy5-conjugated streptavidin and Cy3-conjugated anti-digoxigenin were employed for detection of biotin- and digoxigenin-labeled probes, respectively [1:200 in Tris-buffered saline with 0.1% Tween-20 (TBST; Jackson)]. In situ hybridization was combined with TUJ1-immunofluorescence as detailed above, or with BrdU-immuno-fluorescence applying fast-red reaction with alkaline-phosphatase (AP)-conjugated streptavidin (Zymed Laboratories, San Francisco, CA; 1:25 in TBST, 1 h), followed by BrdU-immuno-fluorescence with Cy2-conjugated anti-mouse IgG.

Confocal Microscopy
Images of 1-μm-thick sections were captured by excitation at 488, 543, 633 and 488 nm of Cy2, Cy3, Cy5 and Fast-Red, respectively. Emission was measured with band-passes of 505–545 or 560–615 nm or long-passes of 650 and 560 nm, respectively. The microscope's detector and amplifier were calibrated by referring to sections expected to have the optimal signal intensity. For subsequent sections, the focus was adjusted but the same amplifier and detector values were maintained to reach the narrow depth of maximal signal intensity.

Regions of Analyses
Sectors of analysis were 200 μm wide and distant 100 μm from the medial edge of the lateral ventricle, within the posterior-medial portion of the future somatosensory area. Digitized images were analyzed in a "blind" manner. BrdU-immunostaining was considered positive if nuclei were darkly stained or at least three puncta were discerned.

Image Analyses
At least three embryonic brains from at least three different litters were analyzed for each group. Averaged cell counts were obtained by averaging values from three or four non-consecutive sections from each brain, for all analyzed brains in each group. Confocal signal was converted to grayscale for intensity measurements of pixel values (Scion Image; Scion Corporation, Frederick, MD). Analysis of variance (ANOVA; Statistica software, StatSoft, Tulsa, OK) was used to compare multiple groups and a one-tailed t test (Microsoft Excel) was used to compare two groups.

Immunoblots
Cerebral homogenates yielding soluble AChE from E17 control and transgenic embryos were processed as described (Birikh et al., 2003). Immunodetection was with rabbit anti-ARP (1:250), goat anti-ASP (1:500) and goat anti-N-trm (1:500).

Catalytic Activity
Acetylthiocholine hydrolysis was measured spectrophotometrically as described (Kaufner et al., 1998). Iso-OMPA (tetraisopropyolphosphoramide) was used to block butyrylcholinesterase activity (5 × 10−5 M).

Results
Pre-AChE mRNA Splicing Shift at the Ventricular Zone
AChE gene expression was first studied by in situ hybridization in the VZ during the neurogenic interval (Fig. 1B). At the onset of neurogenesis (E11), a time of intense progenitor cell proliferation, cytoplasmic AChE-R and AChE-S mRNA (Fig. 1C) were co-localized in most VZ cells (Fig. 2A). Expression was most intense in the apical portion of the VZ, close to the ventricular lumen. With the advance of neurogenesis, e.g. at E13, cytoplasmic expression of the AChE isoforms became pronounced in clusters of adjoining cells in the basal portion of the VZ (Fig. 2A,C). These AChE-expressing clusters, which included from two to >20 cells, coalesced at various points along their common borders. By E15, AChE-expressing cell clusters were smaller, and by E17 they were limited to very small clusters or single cells (Fig. 2B). The subcellular distribution of AChE mRNA had also changed, so that intense signals were observed primarily in the basal pole of labeled cells (Fig. 2B). Densitometric measurements of AChE expression demonstrated a gradual reduction in labeling intensity throughout the VZ during neurogenesis. Expression areas of AChE splice variants exhibited a parallel reduction (Fig. 2C), implying decreasing numbers of expressing cells. Both AChE-R and AChE-S mRNA declined in the VZ. However, some of the cell clusters at E13 maintained the intense level of AChE-R expression (Fig. 2A, open arrow). A statistically significant reduction in AChE-S expression, as determined for both intensity and signal area, was observed from E11 to E13, while reduction of AChE-R was delayed until E15, apparently reflecting a transient dominance of AChE-R over AChE-S at E13 (Fig. 2C).

Proliferating Cells but not Terminally Differentiated Neurons Display Splicing Shift at the Ventricular Zone
A 2 h pulse of BrdU was used to distinguish between S+G2+M versus G1 or post-mitotic nuclei in order to determine the proliferative profile of AChE expressing cells in the VZ. During such a short pulse, BrdU is continuously available for incorporation into nuclei in the S-phase, while the earliest of these nuclei advance through G2 and initiate mitosis (Takahashi et al., 1992).
Combined with in situ hybridization, anti-BrdU immunofluorescence demonstrated that the AChE mRNA-labeled clusters included both BrdU positive and negative nuclei, suggesting that the clusters comprise both S+G2+M and G1 or post-mitotic cells (Fig. 3A). The majority of intensely AChE-expressing cells were located at the basal portion of the VZ, i.e. in the S-phase zone. Adjacent to the ventricular lumen, i.e. in the G2+M zone, AChE expression was relatively sparse, with cells in mitosis expressing the transcripts at their basal pole (Fig. 3A, arrows and insets).

BrdU-negative cells in the VZ represent either proliferative cells re-entering the cell cycle through G1 or post-mitotic cells in the process of migration out of the VZ. To differentiate between these two possibilities, in situ hybridization for AChE-R mRNA was combined with immunofluorescence for beta-III tubulin (TUJ1), an early marker of neurons (Geisert and Frankfurter, 1989) (Fig. 3B). The AChE-R mRNA positive clusters were TUJ1 negative, suggesting that AChE-expressing cells that were refractory to the 2 h BrdU pulse were proliferative cells, i.e. cells at G1 phase. Nevertheless, not all of the non-AChE-expressing cells were labeled by TUJ1, indicating the existence of another or intermediate cell population.

**AChE Gene Expression in Migrating Neurons**

AChE expression patterns in the IZ were examined to assess the potential involvement of the protein and its splice variants in neuronal migration from the VZ to the cortical plate. Both AChE-R and AChE-S mRNA were observed either as individual IZ cells or as clusters, with reduced labeling compared with the CP or VZ (Fig. 4A,B). These cells were radially oriented, suggesting that they were migrating from the VZ to the CP. Immunofluorescence
with the TUJ1 antibody intensely labeled horizontally oriented cells in the IZ that were apparently involved in tangential migration (Fig. 4B,C). Indeed, combined AChE-R mRNA/TUJ1 labeling demonstrated that the AChE-R expressing cells in the IZ were TUJ1-negative (Fig. 4B,C). With further development, AChE expression in the CP appeared in clusters of intensely labeled cells, surrounded by moderately expressing cells (Fig. 4A,B). The intensely labeled cells were more prominent in the superficial portion compared with the deep CP. This suggested that the younger, newly arriving cells in the CP expressed more AChE transcripts than earlier arriving cells that had already undergone some differentiation. Subsequently, at E17, the intense signals in the superficial cell layer of the CP became significantly higher than those of the deep CP portion. Combined AChE-R mRNA/TUJ1 immunofluorescence demonstrated a complementary pattern, similar to that observed in the VZ (Fig. 4B,C). The distribution of TUJ1 exhibited increasing density of labeled cells from the superficial to deeper portions of the CP, implying again that AChE was intensely expressed by the relatively undifferentiated cells, and declined as the number of differentiated cells increased. Although TUJ1 expression was essentially detected in the deeper portion of the CP, a few of these cells were seen at the superficial portion adjacent to the MZ (Fig. 4C). We assume these cells to be young neurons that were about to be displaced by incoming newly arriving migrating neurons or possibly cells in transition from AChE to TUJ1-expressing cells. In addition to this, some horizontally oriented TUJ1 cells were detected in the MZ, possibly Cajal-Retzius cells.

Distinct Localization Patterns of AChE Splice Variants during Cortical Development

AChE splice variants are identical in most of their sequence, differing, primarily in their C-termini (30 residues of AChE-R peptide, ARP and 39 residues of AChE-S peptide, ASP; Fig. 1D). ARP, ASP and the common N-terminus all demonstrated cytoplasmic immunostaining patterns in VZ cells, similar to that of AChE mRNA, with a gradual decrease in intensity and in the number of expressing cells, as well as reduced clustering of intensely labeled cells (Fig. 5). Moreover, at E15, ARPs immunoreactive cell processes were observed ascending from the VZ (Fig. 5, arrows) and extending radially through the total thickness of the cortical wall to terminate at the pial surface. This pattern, which is characteristic of radial glia cells (Gadisseux et al., 1989), was most readily observed in the medial neocortex. There, fibers could be clearly traced into the marginal zone (MZ), where they arborized before terminating at the pial surface (Fig. 6A).

**Developing Brain AChE-R is C-terminally Cleaved**

The immunoreactivity of radial glia to antibodies targeted at the C-terminus of AChE-R, but not to its N-terminus (Fig. 5), suggested cleavage of AChE-R to separate the C-terminal domain that includes ARP, from the core AChE-R protein (Fig. 1D). This is consistent with AChE found in blood (Grisaru et al., 2001). Consistent with this observation, soluble proteins extracted from E17 cerebral cortex demonstrated an intense ARP immunoreactive band of 18 kDa in addition to a 65 kDa band that appears to be intact AChE (Fig. 6B). In contrast to this, antibodies directed against the N-terminal domain (N-trm) common to all AChE variants (Fig. 1D) revealed several slowly migrating bands (Fig. 6B). These included a lightly labeled band that paralleled the 65kDa band shown with anti-ARP and representing the non-cleaved AChE-R, while the most intense band was of ~55 kDa, reflecting the core AChE-R domain following removal of the C-terminus. Negligible immunoreactivity was observed to antibodies directed against ASP.
suggesting that ASP remained attached to AChE-S, rendering it insoluble and therefore not extractable by this procedure. These results indicate that AChE-R, but not AChE-S, is subject to cleavage of its C-terminal domain in the brain and that the vast majority of AChE-R in the developing cortex undergoes C-terminal cleavage.

Anti-ARP, which was originally raised against a glutathione S-transferase–human ARP fusion protein, was not immunoreactive against synthetic ASP (not shown), attesting to its specificity, yet displayed clear immunoreactivity to synthetic murine ARP (Fig. 6B). The immunoreactivity of these two distinct amino acid sequences to the same antiserum suggested evolutionary conservation of ARP structural epitopes, despite the disparity in sequence.

Anti-ARP Labels Migration-associated Glial Processes

With the thickening of the cortical wall, ARP-labeled fibers in the IZ became arched (from medial to lateral), and resumed a radial alignment, orthogonal to the pial surface as they entered the CP (Fig. 6D-2,6). Immunohistochemistry for nestin, a marker of radial glia (Lendahl et al., 1990), exhibited a similar pattern of fibers in adjacent sections (Fig. 6D-1,5). This alignment is typical of the morphology of radial glia (Gadisseux et al., 1989; Misson et al., 1988). In contrast to the pronounced staining by anti-ASP and anti-N-trm antisera, ARP labeling was faint in the cytoplasm of migrating cells in the IZ (Fig. 6D-2,6).
Within the CP, intense immunoreactivity of ASP and the N-terminus was observed in the cells at the superficial cell layer, i.e. newly arriving CP cells, similar to the pattern of AChE gene expression (Fig. 6D-3,4). In contrast, ARP immunoreactivity in this cell layer was sparse, compared with its intensity in the VZ (Fig. 6D-2).

To examine whether ARP immunoreactivity is apparent in other migration-associated glial processes, immunoreactivity was examined in the lateral cortical stream (LCS) and in the striatum (Fig. 6C). The dense glial bundles of the LCS were strongly positive for both nestin and ARP (Fig. 6D-9,13 and 10,14, respectively), extending ventrolaterally from the lateral edge of the VZ between the neocortex and the striatum. Both nestin and ARP demonstrated ramification of this bundle into fibers that assume an orthogonal orientation to the pial surface as they penetrate the neocortex.

Antisense Suppression of AChE-R mRNA Attenuates Neuronal Migration

The distinct patterns of ARP and ASP immunostaining we observed suggested that these two peptides and/or their corresponding proteins may play distinct roles during neuronal migration. To challenge this hypothesis, we labeled a cohort of migrating cells with BrdU prior to their terminal mitosis in the VZ (Fig. 7A). To reduce AChE-R during neuronal migration, we employed mouse EN101, an antisense oligonucleotide capable of inducing selective destruction of mouse neuronal AChE-R mRNA (Cohen et al., 2002). Fluorescent double-labeling in situ hybridization was performed to quantify AChE mRNA variants in the cortical wall following EN101 injection during neuronal migration. Cell density within the VZ, as well as its thickness were similar in control and EN101-treated brains. A reduction in labeling was observed, however, which could not be attributed to reduction in the number of AChE expressing cells. Signal intensity for AChE-R mRNA, measured and compared in uniform 100 x 50 µm square samples in the apical portion of the VZ (121 ± 12 cells; Fig. 7B) exhibited a 34% reduction following EN101 compared with control treatment with the inversely oriented oligonucleotide sequence, INV101, both at 100 µg/kg (n = 10, P < 0.05). In contrast, AChE-S mRNA labeling was reduced by only 7% (n = 10), which was not statistically significant (Fig. 7B).

The effect of AChE-R mRNA reduction on cell migration was evaluated by counting the number of BrdU immunoreactive nuclei present in the dorsomedial CP 48 h after BrdU injection (Fig. 7C). EN101 treatment significantly reduced the number of BrdU immunoreactive cells in the CP by 26% compared with the INV101 treatment (n = 13; ANOVA, P < 0.01; Fig. 7D). Lower concentrations of EN101 or INV101 (40 µg/kg) failed to elicit...
discernible effects on cell migration to the CP (Fig. 7D), indicating dose-dependence. Consistent with this, significantly more BrdU-immunoreactive cells were detected in the IZ under EN101 treatment (at 100 μg/kg) compared with INV101 or low dose EN101 \((n = 13)\) (ANOVA, \(P < 0.05\)) (Fig. 7C, D). The total number of BrdU-immunoreactive cells in the CP and IZ was similar between all treated groups, demonstrating that postmitotic cell survival was unchanged (Fig. 7D). Therefore, the EN101-mediated reduction of neuronal migration reflected attenuated progression of cells from the IZ to the CP.

**AChE-R mRNA Destruction Increases Proliferation in the Ventricular Zone**

The gradual reduction of AChE gene expression during neurogenesis, in parallel with the restriction of proliferation in the VZ, suggested an involvement of AChE and the AChE-associated migration process with proliferation of progenitor cells (Fig. 8A). The effect of AChE-R on proliferation in the VZ was examined by EN101 treatment in animals treated with BrdU 48 h prior to sacrifice. Immunocytochemical labeling of the Ki67 nuclear antigen appeared in most of the cells at the M-phase region in the VZ of the mouse developing neocortex at E16. Few cells in the basal portion of the VZ were intensely stained, though, the majority of labeled cells in that region exhibited light, punctate nuclear staining (Fig. 8B). Compared with INV101, EN101 treatment significantly increased Ki67 expression in the VZ (Fig. 8C, D), suggesting increased re-entry into the cell cycle vs exiting the cell cycle following reduction of AChE-R expression by EN101.

**Both Catalytic and Non-catalytic AChE Activities Affect Proliferation and Migration**

VZ neuronal progenitors express both AChE-S and AChE-R. The observed effect of EN101 described above, therefore, implied one of two possibilities: (i) AChE-R alone reduces proliferation; or (ii) AChE hydrolysis, common to AChE-R and AChE-S is responsible. To distinguish between these possibilities, we injected BrdU into E14 transgenic mice overexpressing (i) the membrane adhering AChE-S (TgS, Beeri et al., 1995); (ii) soluble AChE-R (TgR, Sternfeld et al., 2000); or (iii) an enzymatically inactive form of AChE-S (TgSin, Sternfeld et al., 1998) (Fig. 9A). The animals were sacrificed 48 h later. Reverse transcriptase-polymerase chain reaction was employed to confirm the expression of each of the AChE variants, and acetylthiocholine hydrolysis measurements confirmed increased catalytic activity in brain homogenates from the TgR and TgS but not TgSin strains (Fig. 9B). ARP expression in embryonic brains from the three transgenic and control groups, appeared similar by immunoblot analysis (not shown), suggesting that high AChE-R levels are maintained during cortical development. Arrival of cells in the CP from both TgR or TgS overexpressing embryos was unchanged relative to parent strain controls. However, in TgSin embryos, the average number of BrdU-immunoreactive cells in the CP was significantly reduced compared with control mice as well as those of TgS or TgR mice (Fig. 9B), suggesting a role for ACh in neuronal migration.

The effect of overexpressing AChE splice variants on progenitor cells proliferation was examined in the transgenic mouse strains by Ki67 labeling and density quantification in the VZ. Ki67 expression was reduced in both TgS and TgSin, but not in TgR embryos compared with controls (Fig. 9B). This suggests that the non-hydrolytic activity of AChE-S acts to reduce proliferation in the VZ, whereas its hydrolytic activity has a role in promoting neuronal migration to the CP.

**Discussion**

Using the regulation of AChE pre-mRNA processing as a case study, we explored the involvement of alternative splicing modulations in shaping the developing brain. We found transient changes in AChE pre-mRNA processing during murine cortical development. Using transgenic and antisense manipulations of these variants, we have further demonstrated causal involvement of such changes in progenitor proliferation and the shift toward neuronal migration and differentiation which together shape the mammalian cortical plate.

While many neuronal mRNAs are subject to alternative splicing modulations during brain development (e.g. the clathrin assembly protein 3 (AP-3) (Ishihara-Sugano and Nakae, 1997), the protein tyrosine phosphatases PTP- SL and PTPBR7

---

**Figure 8.** Antisense treatment increases proliferation in the ventricular zone and reduces cell arrival to the cortical plate. (A) Proliferating cells in the ventricular zone (VZ) can continue proliferating or migrate to the cortical plate (CP), possibly sending signals (broken arrow) that modulate proliferation. (B) Ki67-immunostaining (E16 coronal section counterstained with hematoxylin). Adjacent to the ventricular border, where mitosis occurs, note the intense nuclear labeling of cells in metaphase (large arrowhead), anaphase (small arrowhead) and G2 or beginning of G1 (long arrow). Some basal VZ cells were intensely stained (open arrows), but most were lightly labeled with punctated nuclear staining (small arrow). (C) Ki67-immunostaining following INV101 or EN101 treatments. 100 × 50 μm sectors at the inner VZ (box) served for density measurements. (D) Ki67-immunoreactivity density in the VZ following EN101 treatment. *P < 0.05. INV101 served as control.
AChE splice-variant manipulations show involvement with both proliferation and migration. (A) Schematic representation of cell and cell-membrane binding and interaction with acetylcholine (ACh) for AChE variants. Transgenic (Tg) strains over-expressing variant AChE are: TgS, overexpressing the membrane associated AChE hydrolysing AChE-S; TgR, overexpressing the soluble AChE hydrolysing AChE-R; and TgSin, overexpressing the membrane-associated AChE-S which has been mutated by sequence insertion encoding seven amino acids to the catalytic site to abolish its ACh hydrolytic activity. (B) Elman’s reaction, cell arrival to the cortical plate (CP) and Ki67-immunoreactivity in the ventricular zone (VZ) at E16 following BrdU injection at E14. *P < 0.05 when compared with control, TgR or TgS; **P < 0.01 when compared with control or TgR.

Role in Glial Cell Differentiation
The dynamic changes that take place in the VZ during cortical development include increased cell cycle length (Takahashi et al., 1995), reduction of symmetric mitotic divisions (Chenn and McConnell, 1995), restriction in layer specification (McConnell and Kaznowski, 1991) and change in radial glia phenotype (Hartfuss et al., 2001). During brain development, AChE expression in the VZ decreased at the end of neurogenesis, when radial glia transform to astrocytes (Hartfuss et al., 2001). AChE involvement in cell proliferation was previously proposed in several brain and hematopoietic cell types (Karpel et al., 1992; Lev Lehman et al., 1997) and G protein isoforms (Morishita et al., 1999), the information accumulated on AChE’s splice variants and their putative roles in neuronal development and functioning provides added value to this particular example. First, the alternative splicing shift in AChE pre-mRNA processing occurred in proliferating progenitors prior to their neuronal commitment, marking a checkpoint between proliferation and migration. Secondly, our analyses pointed at four distinct functions for AChE in cortical development: (i) ACh hydrolysis, common to AChE-S and AChE-R; (ii) non-catalytic structural features of the core domain, also common to both variants; (iii) migration-supportive properties of ARP, the cleavable C-terminus of AChE-R; and (iv) adherent capacities of ASP, the corresponding uncleaved C-terminus of AChE-S, which joins AChE-S tetramers to a proline-rich membrane anchor (PRiMA) structural subunit (Perrier et al., 2002) but also drives AChE-S to the cell nucleus (Perry et al., 2002). In the following, we discuss the implications of each of these roles for cortical development.

Concerted Effects on Progenitor Migration and Proliferation
During murine brain development, alternative splicing modulation yields a relative dominance of AChE-R, which we found to be a pre-protein to its cleavable C-terminus ARP, compatible with its cleavage under stress in the mouse and human blood (Grisaru et al., 2001; Cohen et al., 2003; Pick et al., 2004). In the developing cortex, ARP interacts with migration-supportive radial glia, unlike the core AChE domain and the uncleaved variant AChE-S, which persist in migrating and differentiating neurons. Moreover, antisense suppression of AChE-R production attenuated neuronal migration to the CP, suggesting causal involvement of the splice shift in this process. In addition, the antisense treatment increased neuronal progenitor proliferation. This could have reflected a proliferation-inhibitory effect of AChE-R itself or of EN101-resistant AChE-S in the attenuated progenitors. To distinguish between these possibilities, progenitor proliferation and neuronal migration were compared in transgenic mice overexpressing distinct AChE variants. AChE-R excess had no effect, whereas both AChE-S and its genetically inactivated mutant AChE-Sin suppressed proliferation, and AChE-Sin further suppressed migration. These findings suggested a non-catalytic, proliferation-inhibiting effect for AChE-S, possibly acting through AChE-R or in an AChE-R-dependent manner. Thus, reduction of AChE-R following EN101 treatment abolished the capacity of AChE-S to attenuate proliferation. Additionally, suppression of neuronal migration by AChE-Sin is compatible with the assumption that ACh hydrolysis is pivotal for neuronal migration, supporting the view of ACh as a regulator of neuronal migration (Lauder and Schambra, 1999).
**Clustering of AChE-expressing Cells**

Following the initiation of neurogenesis, AChE was detectable in clusters of Vz proliferating cells which included all phases of the cell cycle, though were sparsely detected during mitosis, and did not include post-mitotic neurons. This resembles previously shown cell clusters, thought to dynamically couple by gap junctions during all phases of the cell cycle except M, and contain radial glial cells but not migrating or post-mitotic neurons (Bittman et al., 1997). Cell clustering during cortical development likely reflects assembled clonally related dividing cells (Cai et al., 1997) and includes cell clusters expressing choline acetyltransferase (ChAT), the rate-limiting enzyme in ACh synthesis (Schambra et al., 1989). That ACh stimulates cortical precursor cell proliferation *in vitro* through muscarinic receptor activation (Ma et al., 2000) may suggest that AChE, expressed in such clusters, functions by hydrolyzing ACh and terminating its activity as a morphogenic cue. That TgSin embryos display reduced progenitors proliferation may suggest additional mechanisms that are not dependent on ACh hydrolysis. Alternatively, or in addition, AChE-Sin incorporation into progenitors’ membranes might have limited the incorporation of enzymatically active AChE to these sites, creating a cholinergic imbalance.

**ARP May Exert an Independent Migratory Effect**

By E13, the neuroepithelium exhibits the radial glial phenotype (MalATESTA et al., 2003; Tamamaki et al., 2001), stretching fibers to the pia matter. ARP was detected throughout the full length of these cells, whereas the perikaryons of migrating cells in the IZ and arriving cells in the CP were positive for both ASP and the N-terminal core of AChE. Cleaved ARP was detected in the mouse serum following forced swim stress, where its presence accompanies blood cell progenitor proliferation (GRISARU et al., 2003) and in humans following lipopolysaccharide (LPS) exposure, concomitant with the psychological impact of such exposure (COHEN et al., 2003). Migrating cells in the IZ express AChE-R mRNA but not ARP, suggesting secretion of this soluble peptide. Conversely, radial glial fibers are decorated for ARP but not the common N-terminus, and protein blot analysis demonstrated that the C-terminus of AChE-R, including ARP, is detached from the larger, N-terminal portion of AChE. Combined with the antisense and transgenic manipulations, these findings support the notion that ARP participates in the neural migration role of radial glia within the developing cortex.

**Radial Migration of Intermediate Zone Neurons**

AChE’s involvement in cell migration was proposed previously based on its expression in migrating sensory rat dorsal thalamic neurons (SCHLAGGER et al., 1993). Furthermore, an AChE-coated substrate induced migration and clustering of cultured spinal motoneurons (BATAILLE et al., 1998), suggesting an extracellular effect of AChE on cell migration and cell–cell interaction. In our study, transient *in vivo* antisense reduction of AChE-R reduced cell arrival to the CP, with cells remaining on their way, in the IZ. In contrast, constitutive overexpression of AChE-R in transgenic mice did not elicit an increase in cell arrival to the CP. Also, ARP levels were similar in control, TgR, TgS and TgSin embryos, suggesting robust control over ARP in brain development, with increased but limited production of ARP-R, in turn suggesting that its transgenic overexpression did not contribute to neuronal migration at that phase. Nevertheless, AChE-Sin overexpression exhibited a reduction in cell arrival to the CP, suggesting that the hydrolytic activity of AChE-S promotes neuronal migration. Nevertheless, ChAT is expressed primarily in tangentially oriented cells in the IZ (Schambra et al., 1989), suggesting that these cells do not migrate radially, and indicating that ACh hydrolysis may indirectly influence radial migration.

**Cortical Plate Differentiation**

Transient AChE expression was previously described in young post-mitotic neurons in a superficial layer of the chick neuroepithelium (Layer et al., 1988), which later comes to cover the entire surface of the embryonic chicken brain. Furthermore, shortly after chick neurons initiate AChE expression, they extend long projecting neurites (Layer, 1991) and establish distant connections (WEIKERT et al., 1990). Murine ChAT immunoreactivity was reported in the early arriving cells of the margin between the IZ and CP (Schambra et al., 1989), suggesting that ACh may possibly induce AChE expression. In our study, AChE-R and AChE-S mRNAs were both expressed in clusters of newly arriving, i.e. undifferentiated, neurons. ASP and the common N-terminus exhibited similar immunoreactivity to that of both AChE-S and AChE-R transcripts in the CP, whereas ARP was located along radial glia. AChE-R secreted from differentiating cells at the CP may hence regulate cell migration. Conversely, the effect of AChE-S on neurite extension was attributed to the adhesion properties of its neuroligin-like core domain (Andre et al., 1997; GRIFFMAN et al., 1998; STERNFELD et al., 1998), independently of its catalytic activity (for review see Soreq and Seidman, 2001). The neuroligin family of brain-specific mammalian AChE-homologues (ICHTECHENKO et al., 1996), is of particular importance to brain development, especially in excitatory synapses (ICHTECHENKO et al., 1996; SONG et al., 1999). In PC12 cells, antisense suppression of AChE-R restricted differentiation and neurite extension in a manner restorable by transfected neuroligin-1 (GRIFFMAN et al., 1998). This suggested redundant properties for AChE and neuroligins, possibly through binding to neurexin Iβ, and provided a possible mechanism for AChE’s involvement in neuronal differentiation and network formation in the cortical plate. Mutated neuroligin increases the risk of autism (JAMAIN et al., 2003), likely through impaired interaction with β-neurexins, neuronal surface proteins (ULLRICH et al., 1995) involved with neuronal differentiation, axogenesis and neural network formation (DEAN et al., 2003). That overexpressed AChE-S suppresses neurexin Iβ production in embryonic motoneurons of TgS mice (Andre et al., 1997), thus highlights the putative importance of the alternative splicing shift for brain development.

**Prenatal Stress and AChE Malexpression**

In the adult brain, stress and blockade of AChE enhance ACh release, with balance retrieved by AChE-R overproduction (KAUFER et al., 1998). Our findings suggest that both ACh release and AChE-R excess may interfere with cortical development. This provides a tentative explanation to the effects shown for acute, transient or chronic embryonic stress as well as anti-AChE intoxication in later forming years. Even defects that are morphologically non-apparent may result with aberrant microstructures, as may be the case with TgS mice which are subject to early neurodegeneration. No structural or cortical lamination abnormalities were observed in these mice; nevertheless, they display progressive accumulation of pathologic, curled neuronal

---

Dori et al. 428 'Readthrough' AChE Variant in Murine Neocortical Development

---
processes in the somatosensory cortex, whereas transgenic excess of AChE-R attenuates this appearance (Sternfeld et al., 2000). The developmental construction of the mammalian cortical plate thus reflects a well-concerted balance of alternative splicing shifts which may be perturbed under environmental exposure to anticholinesterases (e.g. common agricultural insecticides) or traumatic experiences.

Notes
The authors are grateful to Dr A. Frankfurter for anti-TUJ1 antibodies. This study was supported by US Army Medical Research and Material Command Grant DAMD 17-99-1-9547, Israel Science Fund Grant 618/02 (to H.S.). A.D. was a Post-Doctoral Fellow of the National Institute for Psychobiology in Israel (Fellowship in Memory of Mrs Leah M. Smith), at the Hebrew University.

Address correspondence to Hermona Soreq, Department of Biological Chemistry, Institute of Life Sciences, The Edmond J. Safra Campus, The Hebrew University of Jerusalem, Givat Ram, Jerusalem 91904, Israel. Email: soreq@cc.huji.ac.il.

References