Knockdown of Amyloid Precursor Protein Normalizes Cholinergic Function in a Cell Line Derived From the Cerebral Cortex of a Trisomy 16 Mouse: An Animal Model of Down Syndrome

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We have generated immortal neuronal cell lines from normal and trisomy 16 (Ts16) mice, a model for Down syndrome (DS). Ts16 lines overexpress DS-related genes (*App*, amyloid precursor protein; *Sod1*, Cu/Zn superoxide dismutase) and show altered cholinergic function (reduced choline uptake, ChAT expression and fractional choline release after stimulation). As previous evidence has related amyloid to cholinergic dysfunction, we reduced APP expression using specific mRNA antisense sequences in our neuronal cell line named CTb, derived from Ts16 cerebral cortex, compared to a cell line derived from a normal animal, named CNh. After transfection, Western blot studies showed APP expression knockdown in CTb cells of 36% (24 hr), 40.4% (48 hr), and 50.2% (72 hr) compared to CNh. Under these reduced APP levels, we studied ^{3}H-choline uptake in CTb and CNh cells. CTb, as reported previously, expressed reduced choline uptake compared to CNh cells (75%, 90%, and 69% reduction at 1, 2, and 5 min incubation, respectively). At 72 hr of APP knockdown, choline uptake levels were essentially similar in both cell types. Further, fractional release of ^{3}H-choline in response to glutamate, nicotine, and depolarization with KCl showed a progressive increase after APP knockdown, reaching values similar to those of CNh after 72 hr of transfection. The results suggest that APP overexpression in CTb cells contributes to impaired cholinergic function, and that gene knockdown in CTb cells is a relevant tool to study DS-related dysfunction.

Key words: acetylcholine; cell line; amyloid; Down syndrome; antisense

Down syndrome (DS) in humans, or trisomy of autosome 21, represents the hyperdiploidy that most frequently survives gestation (Reeves et al., 1986), and it implies a gene overdose of 127 known and 98 unknown genes (Hattori et al., 2000). The condition presents multisystemic anomalies, mental retardation, marked alterations of action potential. and ionic current kinetics in human neurons (Nieminen et al., 1988; Caviedes et al., 1990) similar to those derived from the trisomy 16 mouse (Ts16) (Orozco et al., 1987; Ault et al., 1989), an animal model of DS, due to the synteny of murine chromosome 16 with human autosome 21, particularly in the distal portions of the long arms (Oster-Granite, 1986). Cholinergic deficits in the brain are a hallmark in both human DS and TS16 fetal mice, which exhibit reduced activities of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) (Fiedler et al., 1994).

The Ts16 mouse is inviable (Ault et al., 1989). To overcome this problem, we have established immortalized neuronal cell lines from the cerebral cortex of a Ts16 fetal mouse, named CTb, and a control line, named CNh, derived from a normal littermate (Cárdenas et al., 1999). As Ts16 cortical neurons, CTb cells exhibit cholinergic dysfunction expressed in decreased high affinity choline

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uptake and fractional acetylcholine release (Fiedler et al., 1994; Allen et al., 2000). Several DS-related genes may affect such cell membrane-related functions when overexpressed in the trisomic condition: 1) *Sod1* (Cu/Zn superoxide dismutase); 2) *App* (amyloid precursor protein); 3) *Slc5a3* (Na^+/myo-inositol cotransporter); and 4) *Grik1* (kainate receptor subunit) (Galdzicki et al., 2001). *App*, the gene that encodes for the amyloid precursor protein APP, merits attention. APP is an integral membrane protein (Sisodia and Price, 1995), and is reportedly overexpressed in DS (Head and Lott, 2004). APP is overexpressed in both Ts16 mice and our CTb line (Fisher and Oster Granite, 1990; Arriagada et al., 2000), and the latter accumulates the peptide in intracellular compartments, where important metabolic pathways exist (Cole et al., 1989; Koo et al., 1996; Skovoronsky et al., 1998; Coughlan and Breen, 2000). The Aβ peptide is a product of APP metabolism, but its proteolytic cleavage by α-secretase yields soluble Aβ (Esch et al., 1990). Conversely, cleavage by β and γ-secretase produce insoluble, potentially toxic 1-40/42 forms (Yankner et al., 1990; Pike et al., 1991; Price et al., 1992). Excess APP could saturate the α-secretase pathway, shifting metabolism toward insoluble Aβ production (Fukuchi et al., 1992). Increased APP in neurons has been linked to: 1) lipoperoxidation (Begni et al., 2003); 2) Ca^{2+} channel formation that alter intracellular Ca^{2+} homeostasis (Arispe et al., 1994; Kawahara et al., 2000); 3) impaired mitochondrial function (Busciglio et al., 2002); and 4) cholinergic dysfunction (Kar et al., 1996, 1998), similar to that documented in Ts16 central neurons (Fiedler et al., 1994) and in our CTb cell line (Allen et al., 2000).

We propose that APP overexpression in CTb cells affects choline uptake and acetylcholine release, and therefore normalizing APP levels would revert these effects. We studied APP expression with blotting techniques, and subsequently decreased APP expression to levels comparable to those of the normal CNh line, using specific RNA antisense sequences. Once this APP knockdown was achieved, we evaluated membrane-related cholinergic function with labeled precursors. Part of this work has been published in abstract form (Opazo et al., 2005).

MATERIALS AND METHODS

Establishment and Culture of Cell Lines

The establishment of both CNh and CTb cell lines is reported elsewhere (Cárdenas et al., 1999; Allen et al., 2000). Briefly, trisomy 16 and normal mice fetuses were obtained by breeding double heterozygous (Rb 2H/RB 32 Lub) males with normal C57BL females. The pregnant females were anesthetized with CO_2 and killed by cervical dislocation after 12–16 days of gestation. Trisomic fetuses were identified by their characteristic massive edema. Normal littermates were used as contemporaneous controls. Whole brains from trisomic and littermate control fetuses were removed and the cerebral cortex was carefully dissected, sliced and digested in 3 ml of PBS containing 0.12% (w/v) of trypsin (Gibco Invitrogen, Carlsbad, CA) and incubated for 30 min at 37°C. Trypsin reaction was stopped by adding an equal volume of plating medium, consisting of DMEM/ Ham F_{12} nutrient mixture (1:1) (Sigma, St. Louis, MO) modified to contain 6 g/l glucose, 10% bovine serum, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma). The suspension was centrifuged and the pellet resuspended in 2 ml of plating medium. The tissue was dissociated by passages through a fire-polished Pasteur pipette, and the cells were then plated in a collagen (Calbiochem, La Jolla, CA)-coated 60-mm culture dish at a density of 40,000 cells/cm^2. At the time of seeding, the plating medium was supplemented with 10% (v/v) of medium conditioned by a rat thyroid cell line, UCHT1, which reportedly induces transformation in vitro (Cárdenas et al., 1999; Allen et al., 2000). The onset of transformation foci became evident after 7 months and signaled the establishment of cell lines CNh (derived from normal cortex) and CTb (derived from trisomic cortex).

For standard growth conditions, the cells were plated in glass Petri dishes and maintained in feeding medium, composed of DMEM/ Ham F_{12} nutrient mixture (1:1) modified to contain 6 g/l glucose, 10% bovine serum, 2.5% FBS, and 40 μg/ml gentamycin. Passages were carried out by detaching the cells with 0.1% trypsin (Gibco Invitrogen). For ^{3}H-choline uptake and release experiments, the cells were scraped instead of trypsinization and then seeded onto 35-mm plastic Petri dishes.

APP Knockdown

APP knockdown was carried out using a specific phosphorothioate mRNA antisense sequence as previously described (Kumar et al., 2000). The anti-APP sequence utilized, called As-APP, was: GGCGCCTTTGTTCGAACCCACATCTTCAG-CAAAGAACACCAG. For control, we used a scrambled sequence, Sc-APP, composed of the same amino acids in As-APP but rearranged randomly. This sequence was: ATATCTA-CACGCCTTGCCAAAGATCACCGGGCATGCCAGCAT.

CTb and CNh were cultured in 3.5 cm diameter culture wells, until reaching 80% confluence. Antisense sequences were incorporated into CTb cells with cationic liposomes (Lipofectamine; Invitrogen, Inc., Fredericksburg, MD) according to recommendations by the manufacturer with modifications. Briefly, 5 μL antisense at 50 μM concentration were mixed with 45 μL DME/F12 media without serum or antibiotics. This mixture was incubated for 15 min, and later mixed with 2.5 μL Lipofectamine and 47.5 μL DME/F12 media for another 15 min. This mixture was added to each 3.5 cm well, and completed to 500 μL with DME/F12 media. For non-transfected cells (CNh and CTb), the cultures were kept in 500 μL DME/F12 media. The cultures were incubated at 37°C and 5% CO_2 for 5 hr. Transfections were stopped by addition of 1 ml DMEM/F12 supplemented with adult bovine serum (10%, v/v), fetal bovine serum (2.5%, v/v), and gentamicin 40 mg/lt.

Determination of APP Protein Levels

APP protein contents were determined 24, 48, and 72 hr post-transfection with the respective antisense sequences using Western blot. Cells were washed twice in PBS composed of

130 mM NaCl, 5.4 Mm KCl, 0.14 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 7.4 pH, and detached with Trypsin-EDTA 0.1% solution for 3–5 min at 37°C. The trypsin solution was neutralized by addition of DMEM/F12 media, supplemented with 10% adult bovine serum and 2.5% fetal bovine serum. The cells were resuspended by pipetting, placed in Eppendorf tubes and centrifuged at 6,000 rpm for 5 min at 4°C. The resulting pellets were washed in chilled PBS, and lysed with a RIPA lyses buffer composed of 150 mM NaCl, 24 mM sodium deoxycholate; 0.1% (v/v) SDS; 1% (v/v) Nonidet; 250 mM EDTA; 100 mM TRIS base, supplemented with proteases inhibitors (PMSF, leupeptin). Samples were incubated at 4°C for 45 min, and later sonicated for 30 sec. The samples were then centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatant was collected and stored at a −20°C for later study. Total protein content was determined using the Bradford method.

For Western blot analysis, 12% SDS-PAGE was carried out to evaluate total APP content. β-Tubulin was used as a loading control. Proteins were transferred to a nitrocellulose membrane for 1.5 hr at 4°C at 400 mA current. Blots were preincubated with skim milk 3%, Tween-20 1% for 1 hr at room temperature. Membranes were incubated for overnight at 4°C with monoclonal primary antibody 22C11, directed against amino acids 66–81 of the N-terminus portion of APP (1:1,000, Chemicon Corp., Temecula, CA), and monoclonal antibody directed against β-tubulin (1:2,000, Boehringer-Mannheim), which was used as loading control. After primary antibody incubation and washing, incubation with secondary antibodies (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) was carried out and detection was carried out using a chemiluminescence kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), according to instructions by the manufacturer. The resulting membranes were scanned at 900 dpi resolution, and saved in a PC computer for later analysis. Pixel density for the resulting bands was determined by the image analysis software ImageJ (NIH, Bethesda, MD), using a Gel plot macro included with the program, and APP amounts in each condition were estimated by dividing the values of these bands with those of the corresponding β-tubulin values.

[^{3}H]-Choline Uptake

[^{3}H]-Choline uptake into cells was measured at room temperature using methods described by Allen et al. (1997). At the time of experiment, 1 μCi [^{3}H]-choline (Sp. Activity = 50 mCi/mmol; Dupont NEN, Wilmington, DE; total volume = 1 μl) was added to each dish and uptake was assessed at 1, 2, and 5 min incubation periods (final choline concentration ~0.013 μM). At the end of the uptake period, 50 μL of extracellular solution medium were removed and kept to determine the extracellular fraction. The cells were then rapidly washed 4× with 1 ml cold (4°C) normal Krebs solution, composed of 120 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM Na_2HPO_4, 20 mM HEPES, 10 mM dextrose, 7.4 pH to remove nonselective bound [^{3}H]-choline. Cell membranes were disrupted with 1 ml of 0.1% SDS, and 900 μl of the cell/SDS extract was removed and analyzed for [^{3}H] content by liquid scintillation counting. The remaining 100 μl was used for protein determination. The amount of [^{3}H]-choline taken up into cells (dpm/mg cell protein) was determined at each time point for each well and normalized to the extracellular [^{3}H]-choline concentration (dpm/ml). [^{3}H]-Choline uptake (nmol/mg protein/min) was calculated as:

$$U[^3H] - C = [IC(dpm)/0.9]/[EC(dpm/0.05)]/mgProt. \quad (1)$$

where U ^{3}H-C is the [^{3}H]-choline uptake (or distribution volume), IC is the intracellular fraction, EC is the extracellular fraction, 0.9 and 0.05 are the aliquot factor for the intracellular and extracellular fraction, respectively. mg Prot. is the protein content per dish.

Acetylcholine Release Experiments

For fractional acetylcholine release experiments, cells were incubated for 30 min with 1 μCi [^{3}H]-choline, followed by 4× washes in Krebs solution at 37°C (Fiedler et al., 1994). To induce release, the cells were stimulated with 50 mM K^+, 200 μM glutamate, and 100 μM nicotine, as described previously (Fiedler et al., 1994; Cárdenas et al., 1999; Allen et al., 2000). To account for the basal efflux of fractional acetylcholine, determined as tritium release, samples of the extracellular fluid were taken at 1, 3, 6, and 9 min, and their radioactivity was determined. Stimulation was carried out at 10 min, and extracellular fluid samples were taken at 11 min. The basal efflux was extrapolated from the curve ands subtracted from the total values of radioactivity to determine the specific, active release (Fiedler et al., 1994; Allen et al., 2000). Later, the cells were lysed with 1% (v/v) Triton X-100 to determine the intracellular fraction of acetylcholine.

Statistics

Statistical significance was assessed with the aid of a statistical software Graphpad Instat (Graphpad Software, San Diego, CA), using one-way ANOVA, followed by Dunnett's test as multiple comparison post-test, where all values were compared to those of CTb cells. Significance was accepted at the $P < 0.05$ level.

RESULTS

APP Is Overexpressed in CTb Cells, and Its Levels Can Be Normalized With a Specific Antisense Sequence

The cationic liposomes method used proved to be a highly efficient transfection method in CTb cells, as shown in control experiments where CTb cells were transfected with eGFP (Green fluorescent protein), which yielded up to 90% fluorescent at the times tested (data not shown). Figure 1 shows a representative Western blot of the effect of the As-APP in the expression of APP. Up to three bands were evident in the 110 kD range, corresponding to those expected for all APP isoforms detected by the antibody. After correction with β-tubulin, APP was found to be overexpressed in CTb cells by approximately two-fold, compared to the normal CNh line. As-APP induced a rapid and significant reduction of APP expression in CTb cells, estimated at 36% 24 hr post-transfection, and

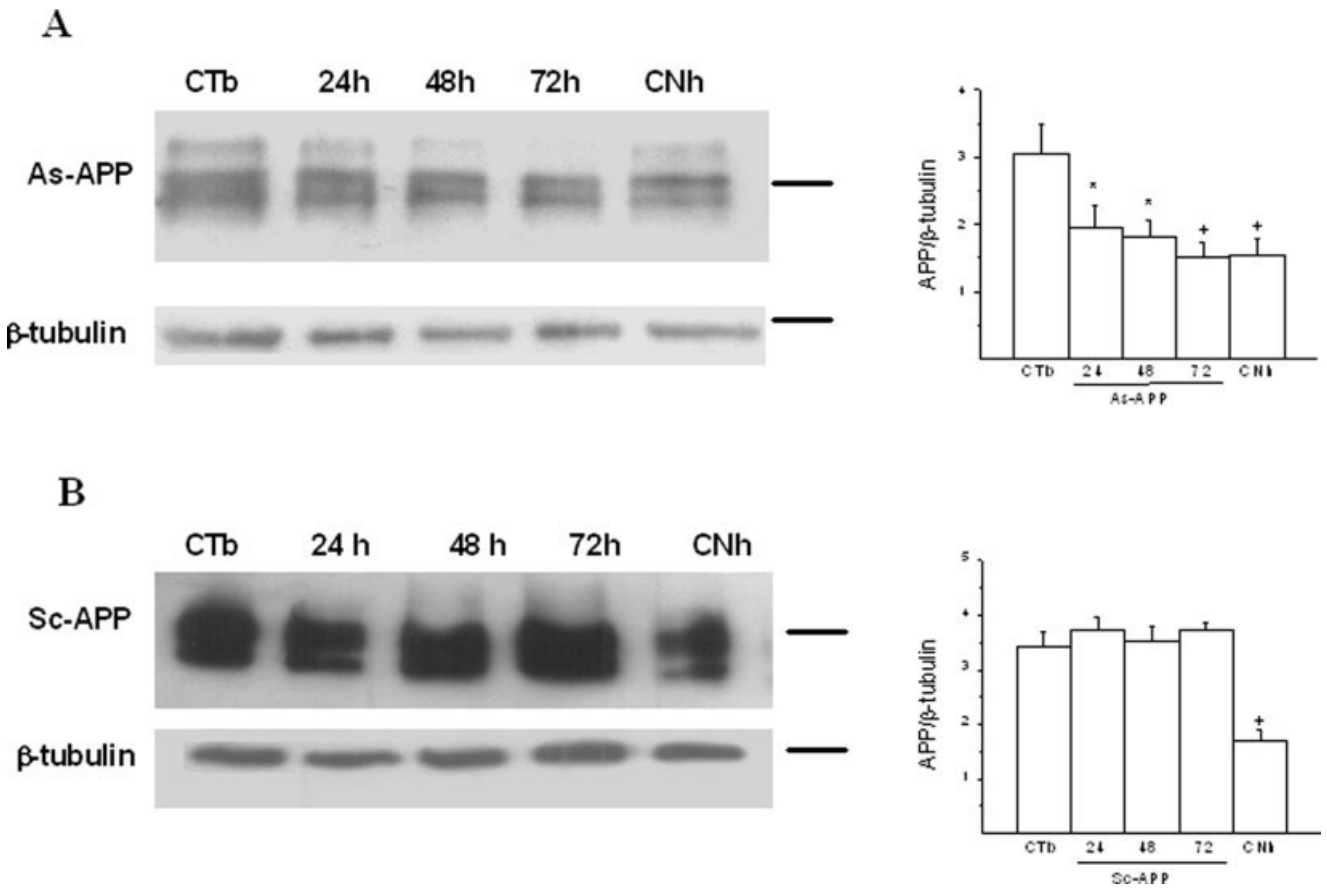


Fig. 1. APP antisense sequences were incorporated into CTb cells with cationic liposomes in a concentration of 8 mg/ml for 5 hr. Subsequently, APP expression was determined by Western blot at the noted times. **A:** APP expression was reduced by 36% (24 hr), 40.4% (48 hr), and 50.2% (72 hr) compared to non-transfected CTb cells. **B:** Control gel showing untreated cells (CTb) vs. cells transfected with a scrambled sequence (Sc). The graphs on the right show relative pixel density values of As-APP and Sc-APP antisense treated cells, respectively, corrected by β-tubulin (n = 6) $\star P < 0.05$; $+P < 0.01$ compared to CTb. ANOVA, $P = 0.0108$ (As-APP); $P = 2.1 \times 10^{-06}$ (Sc-APP). Values represent mean ± SEM. Bars = 110 kD for APP; 55 kD for β tubulin.

reaching levels essentially similar to those of CNh at 72 hr post-transfection. This agrees with results by Kumar et al. (2000), who estimated that the As-APP sequence reduced expression or 'knocked down' APP protein levels by 30–40% within this time frame, and remained in those levels in subsequent days. When analyzing the pixel density of bands individually and correcting with β-tubulin expression, the time course of the reduction for the two most prominent bands was essentially similar. Indeed, the band with higher molecular weight (Fig. 1) in CTb cell was reduced from a value of 1.46–0.78 at 72 hr post-transfection, whereas the band with the lower molecular weight was reduced from 1.32 to 0.75 in the same conditions. This suggests that As-APP primarily affects the production of the precursor, and not a given isoform. Conversely, transfection of CTb cells with the Sc-APP caused no reduction in APP expression, thus confirming a specific effect of As-APP. The latter also suggests that contamination of these results due to possible cross reactivity of the 22C11 APP antibody with the amyloid precursor like protein-2, APLP2 (Slunt et al., 1994) is unlikely. Further, analysis of the individual bands in CTb cells showed no preferential effect of Sc-APP, where the high molecular weight band exhibited β-tubulin corrected values of 3.21 in control conditions vs. 3.45 at 72 hr post-transfection, whereas the band with the lower molecular weight showed control values of 3.02 vs. 3.25 at 72 hr post-transfection.

Reduced Uptake of Choline in CTb Cells Is Normalized by APP Knockdown

Figure 2 represents high affinity choline uptake in CTb and CNh cells after 1, 2, and 5 min incubation with [^{3}H]-choline. This uptake was largely carried out by hemicholinium-3 sensitive transporters in both cell types, where 10 μM concentration of the blocker resulted in a 79.9% and 79.5% reduction of choline uptake for CTb and CNh cells, respectively. CTb cells exhibited a reduction in choline uptake of 75%, 90%, and 69% after 1, 2,

and 5 min of incubation with 1 μCi of [^{3}H]-choline, respectively, compared to CNh. APP knockdown induced a marked and sustained increase in [^{3}H]-choline uptake in CTb cells in relation to the onset of the knockdown effect, in particular after 2 and 5 min incubation with the labeled choline, and reaching levels comparable to those of CNh cells, particularly at 72 hr post-transfection, where the differences in APP expression between the two conditions were negligible. Interestingly, no such effect could be observed in Sc-APP-transfected CTb cells, suggesting that the increase and subsequent normalization of [^{3}H]-choline uptake is related specifically to APP knockdown.

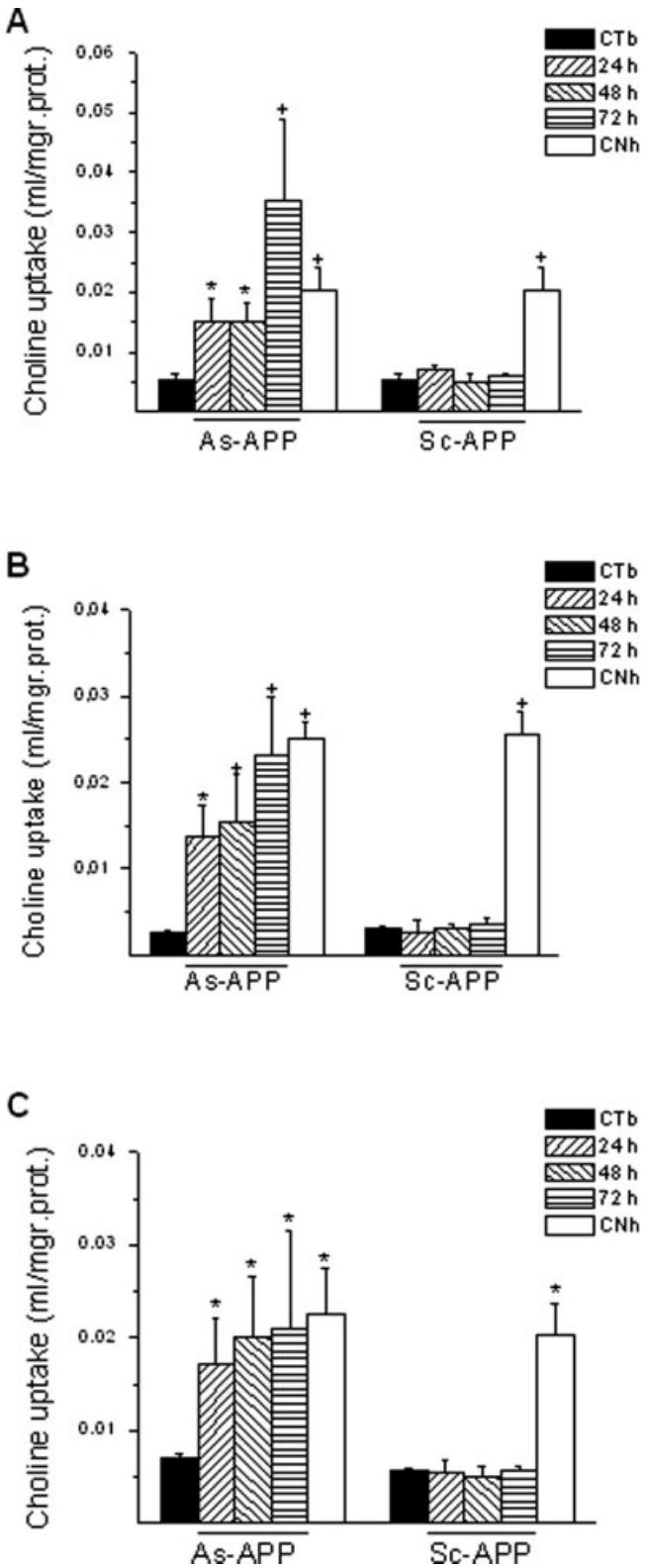


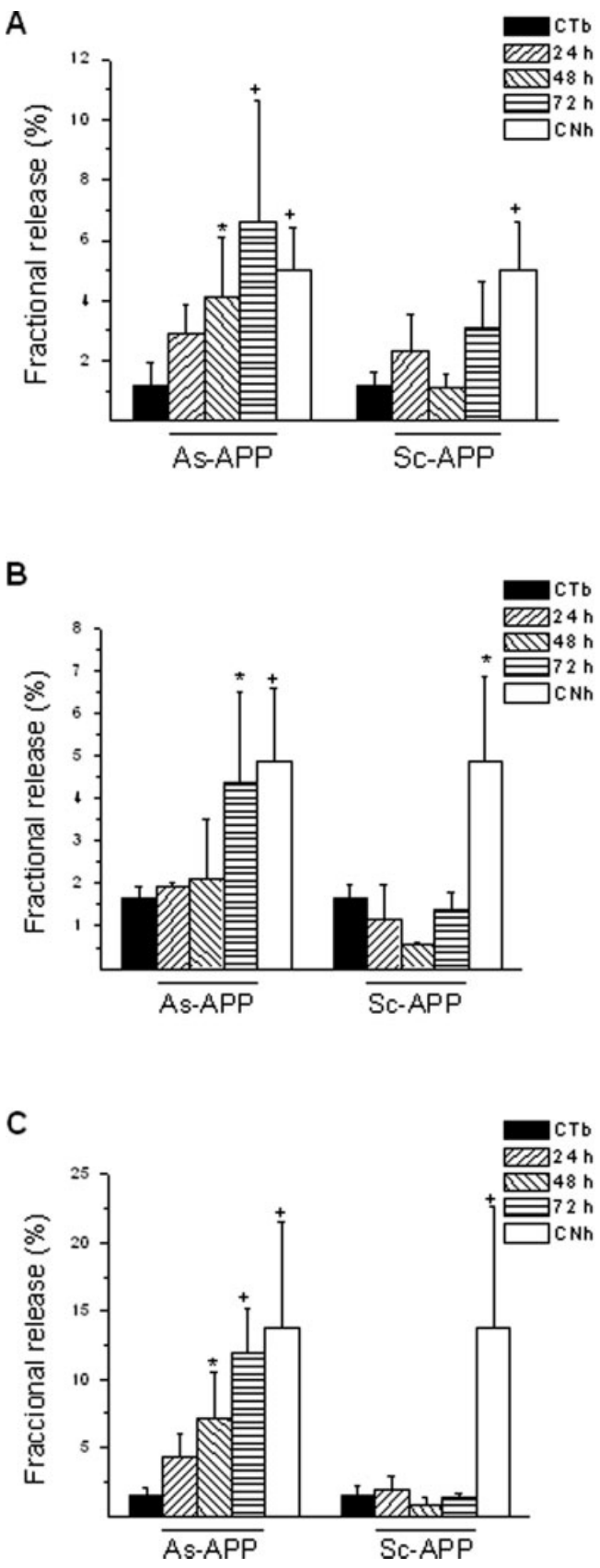
Fig. 2. [^{3}H]-Choline uptake in CNh and CTb cells, and trisomic cells transfected with antisense probes. Cells were incubated with 1 μCi [^{3}H]-choline for 1 min (**A**), 2 min (**B**), and 5 min (**C**). Note progressive normalization of choline transport with As-APP (left dashed columns), whereas no effect is seen with scrambled sequences (right dashed columns). Values represent mean ± SEM (n = 6–14 experiments, in triplicate). $\star P < 0.05$; + $P < 0.01$, with respect to CTb cortical cell line. ANOVA: $P = 0.0009$ (A), $P = 1.17 \times 10^{-16}$ (B), $P = 4.9 \times 10^{-11}$ (C).

Acetylcholine Secretion Is Normalized in CTb Cells After APP Knockdown

Figure 3 shows the effect of APP knockdown on fractional release of labeled acetylcholine, as a function of the total choline incorporated, in response to stimuli noted. CTb cells exhibited a reduction in fractional release levels of 88% and 76%, in comparison with CNh. Release induced by 0.2 mM glutamate, was reduced by 66% in CTb cells compared to the normal cell line. Nicotine stimulation and K^+-induced depolarization in As-APP-transfected CTb cells resulted in a clear, significant increase in fractional choline release after 48 hr, and reached levels most similar to those of CNh cells after 72 hr post-transfection. Glutamate induced release also tended to progress toward those levels of CNh cells, in relation to APP knockdown. Again, transfection of CTb cells with Sc-APP had no effect on secretion, which remained at significantly lower levels, compared to CNh cells.

DISCUSSION

Western blot analysis showed a clear overexpression of APP in our Ts16 derived cell line, CTb, compared to the CNh line, established from a normal littermate. Interestingly, CTb cells exhibited a two-fold increase in APP expression with respect to CNh cells, which surpassed the amount expected by a 50% excess gene dosage effect inherent to the trisomic condition. A plausible explanation may be reduced APP metabolism present in CTb cells (Arriagada et al., 2000), particularly in the endosomal/lysosomal pathway, resulting in accumulation of APP in vacuole-like compartments in the cytoplasm. The latter has also been described in the brain of Ts65Dn partial trisomic mice (Cataldo et al., 2004). Interestingly, when the aforementioned mice were mated to APP knockout mice, thus yielding an offspring that is trisomic for most of the genes present in the Down syndrome critical region, but normosomic for the *App* gene, central neurons of this 'in vivo knockdown' model lack the intracellular deposits of amyloid. Hence, an overload of amyloidogenic pathways may result in accumulation of APP products both by



excess production and interference with metabolism. In our experiments, transfection of CTb cells with the specific As-APP antisense sequences resulted in a comparable situation: APP expression was significantly reduced to 64%, 59.6%, and 49.8% after 24, 48, and 72 hr of transfection, respectively, compared to APP levels in non-transfected CTb cells. Such a reduction cannot be explained by a general effect due to the transfection method, as the use of the Sc-APP sequence, that exhibits the same base composition as that of As-APP but rearranged in random order, had no effect whatsoever on APP expression. Our results then confirm those of Kumar et al. (2000) regarding the quantity and time course of APP knockdown produced by the As-APP oligo.

Once APP expression reached levels comparable to those of the CNh control cell line, we studied cholinergic function on both cell types. Our present results confirmed our previous findings in both Ts16 brain cells in primary culture (Fiedler et al., 1994) and in our cell lines (Allen et al., 2000). First, both CTb and CNh cells largely incorporate choline via the high affinity, Na^+-dependent, hemicholinium-3 sensitive transporter, and a significant reduction in high affinity choline uptake and fractional acetylcholine release in response to various stimuli was evident in CTb cells compared to CNh. After 24 and 48 hr of As-APP transfection, an increase in [^{3}H]-choline uptake in CTb cells is evident at all incubation times tested, compared to non transfected CTb (Fig. 2). After 72 hr of transfection, there was no significant difference in [^{3}H]-choline uptake between transfected CTb cells and CNh cells at all incubation times tested, which interestingly coincides with 'normalization' of APP expression in CTb cells compared to CNh (Fig. 1). This effect cannot be explained by a non-specific effect, as transfection with the scrambled Sc-APP oligo had no effect on choline uptake in CTb cells in all conditions tested, which remained in levels comparable to those of non-transfected CTb cells at all times. Our results suggest strongly that excess APP results in a specific reduction of high affinity uptake in the trisomic condition.

With respect to tritium efflux, the strongest reductions were observed in CTb cells after stimulation with 50 mM of K^+ and 0.1 mM of nicotine, whereas glutamate-induced release in the trisomic cells resulted in more modest reductions, compared to values of the control cell line. This lesser effect of glutamate is comparable to the results by Allen et al. (2000), and could be explained by a preferential effect of nicotine and K^+-induced depolariza-

Fig. 3. Fractional release of labeled acetylcholine in previously loaded cells, in response to depolarization and external application of 100 μM nicotine (**A**), 200 μM glutamate (**B**), and 50 mM KCl (**C**). Note the shift of fractional release levels in As-APP transfected CTb cells (left dashed columns), toward those of the normal CNh line, as the knockdown of the protein is progressively achieved. Values are mean ± SEM (n = 3–6 experiments, in triplicate). $\star P < 0.05$; $+ P < 0.01$, with respect to the CTb cortical cell line. ANOVA: $P = 0.00033$ (A), $P = 0.002$ (B), $P = 0{,}0008$ (C).

tion on neurites and the synapse (i.e., voltage gated Ca^{2+} channels), whereas glutamate has more complex effects that involve mechanisms residing in the soma and nuclei (Cárdenas et al., 1999; Allen et al., 2000). After 48 hr of As-APP transfection, fractional release progressively increased in CTb cells compared to CNh cells in all stimuli tested. Indeed, K^+-induced depolarization, nicotine, and glutamate stimulation resulted in progressive increase of fractional release in CTb cells, after the onset of the knockdown effect in a quite linear fashion. At 72 hr post-transfection, fractional release levels in CTb cells reach levels that are most comparable to CNh cells in all conditions tested. Interestingly, at 24 hr post-transfection with As-APP, choline release in CTb cells showed no significant differences with non-transfected CTb cells (Fig. 3) in all conditions tested. This coincides with a lesser reduction in APP expression at that time, and can be related to the APP knockdown effect on intracellular Ca^{2+} signals evoked by the same agonists used herein (Encina et al., 2004). Indeed, altered time dependent kinetics of such responses in CTb cells are reverted only 48 hr after As-APP transfection, suggesting a relationship between normalized Ca^{2+} responses with the improvement of a secretory function. Again, transfection of CTb cells with the scrambled Sc-APP oligo had no effect on fractional release, suggesting a specific role of overexpressed APP in the reduction of acetylcholine secretion.

The mechanisms underlying the aforementioned effects of excess APP on cholinergic dysfunction in the trisomic condition are unclear. Yet, several facts may shed light in this regard. Studies have shown that the injection of the Aβ peptide within septal nuclei induces a specific damage of cholinergic but not GABAergic neurons (Harkany et al., 1995). According to this, it is possible that a yet uncharacterized Aβ peptide receptor may exist in cholinergic neurons that may initiate or cause specific deleterious effects (Jhamandas et al., 2001). Neurotoxicity induced by exposure to high Aβ concentrations (5–50 μM) is associated with a reduction of glucose uptake (Mark et al., 1997). Hence, the Aβ peptide could reduce the availability of acetyl Co-A and acetylcholine synthesis by inhibiting the activity of the pyruvate dehydrogenase (PDH) (Hoshi et al., 1997). Aβ could reduce cholinergic transmission by affecting acetylcholine production. Interestingly, in Alzheimer's disease, a high affinity binding can occur between $A\beta_{1-42}$ and the nicotinic receptor α7nAchr, thus inhibiting acetylcholine release (Kar et al., 1998) and altering Ca^{2+} homeostasis (Seguela et al., 1993), which may eventually lead to cell stress and degeneration. Finally, at the membrane level, Nitsch et al. (1992) suggested after a series of experiments that phospholipid metabolism is abnormal in the cerebral cortex of Alzheimer's patients, evidenced by a 10–12% reduction in the levels of phosphatidylethanolamine and phosphatidylcholine in the parietal and frontal cortex, compared to normal subjects. This represents a reduction in total content in the order of 2 nmol/μg of total DNA. This abnormal phospholipid content could increase amyloid deposits by altering APP processing or exposing the transmembrane domain of APP. This could favor the formation of amyloidogenic fragments from APP, which could in turn affect membrane function.

Our data suggest that specific gene overdose in Ts16 could explain certain membrane related dysfunction associated with proteins such as receptors and ionic channels, particularly in cholinergic cells. The establishment of stable in vitro models of the murine Ts16 mouse could provide an important tool to effectively ascertain the relationship between gene expression and specific functional alterations in tissues that reproduce the disrupted cell homeostasis of DS-related excess gene dosage. In this regard, the CTb cell line seems an interesting and reproducible model, and could serve as an important tool in the study in potential therapies or altered cell function. The present study suggests that stably reducing APP overexpression to normal physiological levels, may be an important therapeutic target. For example, a drug that reduces the amount of intracellular in such tissues, such as certain phenolic derivatives, (Paula Lima et al., 2002) could constitute a strategy to revert cholinergic dysfunction in DS.

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