Master's Thesis

Effects of the knockdown of the Cu/Zn superoxide dismutase on the uptake of choline and fractional release of acetylcholine in cortical cell lines derived from a trisomy 16 mouse

Jura Mikkola

University of Jyväskylä

Faculty of Mathematics and Science

Department of Biological and Environmental Science

Chemical Biology

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Preface

The work was carried out in the Program of Molecular and Clinical Pharmacology, ICBM, Faculty of Medicine, University of Chile, under the supervision of Dr. Pablo Caviedes, and in the Department of Biological and Environmental Science, Faculty of Mathematics and Science, University of Jyväskylä under the supervision of Dr. Tuula Jalonen.

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University of Jyväskylä
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Abstract of Master´s Thesis

Author: Jura Mikkola
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Abstract

Down syndrome (DS) is the phenotypic manifestation of the trisomy of chromosome 21 in humans. It is the most common known genetic disorder associated with mental retardation, with an average incidence of approximately 1:700 live births. DS affects all major organ systems, including the central nervous system, and the patients typically suffer from an early onset of Alzheimer's disease. The various symptoms of DS have been attributed to the altered expression levels of the genes present in the triplicated chromosome. Due to the high level of homology between the human chromosome 21 and the murine chromosome 16, murine trisomy 16 (Ts16) is considered to be a model of DS. However, Ts16 mice are not viable and die *in utero*. The immortal neuronal cell lines CTb, derived from the cortex of a Ts16 fetus, and CNh, derived from the cortex of a normal mouse fetus, have been previously generated to overcome this problem and allow the study of gene overexpression in a fully trisomic environment. Several genes related to DS, such as APP (amyloid precursor protein), Slc5a3 (myo-inositol transporter), and SOD1 (Cu/Zn superoxide dismutase) are overexpressed in the trisomic CTb cells, and they also show altered cholinergic function (a decrease in choline uptake and acetylcholine release, as well as a reduced expression of choline acetyltransferase) similar to DS neurons. SOD1 overexpression is associated with increased levels of oxygen radicals that lead to oxidative stress and lipid peroxidation, affecting membrane composition and function. These effects suggest a possible link between SOD1 overexpression and the cholinergic dysfunction seen in DS. We chose to reduce SOD1 expression in CTb cells to the levels seen in the normal CNh cells using a knockdown protocol based on specific mRNA antisense sequences. At approximately 72 hr post-transfection, Western blot analysis showed that transfection with SOD1 antisense oligonucleotides had reduced the expression of SOD1 in CTb cells to the levels seen in CNh cells. We carried out choline uptake and fractional acetylcholine release experiments, using ^{3}[H]-choline, in transfected CTb cells as well as non-transfected CTb and CNh cells. After one, two and five min of incubation with the isotope, choline uptake in non-transfected CTb cells was 41.5%, 36.1% and 41.5%, respectively, of that of CNh cells. The fractional release of acetylcholine, after stimulation with a depolarizing solution containing either K^{+}, glutamate or nicotine, was also reduced in CTb cells compared to CNh cells. At 24, 48 and 72 hr post-transfection with SOD1 antisense oligonucleotides, there were no significant changes in either choline uptake or acetylcholine release, at any of the incubation times tested. These results indicate that the normalization of SOD1 expression alone is not sufficient to correct the reduced choline uptake and acetylcholine release seen in CTb cell lines, and thus SOD1 overexpression alone is unlikely to be responsible for the cholinergic dysfunction in DS. The CTb cell line and gene knockdown remain valuable and relevant tools in the study of gene dosage effects in DS.

Keywords: Down syndrome, acetylcholine, cholinergic, superoxide dismutase

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Tiivistelmä

Downin syndrooma (DS) on kromosomi 21:n trisomian fenotyyppinen ilmentymä ihmisessä. Se on yleisin tunnettu vajaaälyisyyteen liittyvä geneettinen häiriötila, joka voidaan todeta keskimäärin noin 1:700 elävänä syntyneestä lapsesta. DS vaikuttaa kaikkiin merkittäviin elimiin, kuten keskushermostoon, ja potilaille kehittyy tyypillisesti Alzheimerin tauti varhaisella iällä. DS:n lukuisien oireiden on ajateltu johtuvan ylimääräisessä kromosomissa sijaitsevien geenien ilmentymisen ja säätelyn muutoksista. Hiiren kromosomi 16:n ja ihmisen kromosomi 21:n keskisen korkean homologisuusasteen vuoksi hiiren trisomia 16:a (Ts16) on pidetty Downin syndrooman eläinmallina. Ts16-hiiret eivät kuitenkaan ole elinkelpoisia, vaan kuolevat kohtuun ennen synnytystä. Kuolemattomat eli jatkuvasti jakautuvat solulinjat CTb (johdettu Ts16-sikiön aivokuoresta eristetyistä soluista) ja CNh (johdettu normaalin hiiren sikiön aivokuoren soluista) on kehitetty aiemmin juuri tämän ongelman ratkaisemiseksi. Näin on saatu aikaan malli, jolla voidaan tutkia geenien yli-ilmentymistä soluissa, joissa ilmenee täysi Ts16. CTb-soluissa yli-ilmennetään useita DS:aan liittyviä geenejä, kuten APP:a (beeta-amyloidin esiaste), Slc5a3:a (myoinositolin kuljettajaproteiini) ja SOD1:a (Cu/Zn superoksididismutaasi). Lisäksi niiden kolinergisessa toiminnassa on havaittavissa DS-tyyppisiä muutoksia (heikentynyt koliinin sisäänotto ja asetyylikoliinin vapautus, sekä koliini-asetyylitransferaasin heikentynyt ilmentyminen). SOD1:n yli-ilmentymisen on todettu aiheuttavan happiradikaalien määrän kohoamiseen solussa, mikä johtaa hapettavaan rasitustilaan sekä lipidien peroksidaatioon, mikä taas vaikuttaa solun kalvojen rakenteisiin ja toimintoihin. Nämä vaikutukset viittaavat mahdolliseen yhteyteen SOD1:n yli-ilmentymisen ja DS:ssa havaittujen kolinergisten häiriöiden välillä. Päätimme alentaa SOD1:n ilmentymistä CTb-soluissa normaaleissa CNh-soluissa havaitulle tasolle käyttäen geenin vaimennusmenetelmää, jossa spesifeillä antisense oligonukleotideilla estetään mRNA:n translaatio proteiiniksi. Western blot –analyysi paljasti, että noin 72 tunnin kuluttua transfektoimisesta antisense oligonukleotideilla SOD1:n määrä soluissa oli suurinpiirtein samalla tasolla kuin CNh-soluissa. Tämän jälkeen suoritimme ^{3}[H]-koliinilla kokeet koliinin sisäänotosta ja asetyylikoliinin vapautuksesta sekä transfektoiduissa CTb-soluissa että transfektoimattomissa CTb- ja CNh-soluissa. Yhden, kahden ja viiden minuutin inkubaation jälkeen koliinin sisäänotto transfektoimattomissa CTb-soluissa oli 41.5%, 36.1% ja 41.5%, em. järjestyksessä, CNh-solujen vastaavasta. Asetyylikoliinin vapautus depolarisoivalla liuoksella (K^{+}, glutamaatti tai nikotiini) oli myös heikompi transfektoimattomissa CTb-soluissa kuin CNh-soluissa. Koliinin sisäänotossa ja asetyylikoliinin vapautuksessa ei tapahtunut merkittäviä muutoksia 24, 48 eikä 72 tunnin kuluttua transfektion jälkeen missään testatuista inkubaatioajoista. Nämä tulokset viittaavat siihen, että SOD1:n ilmentymisen palauttaminen normaalille tasolle ei yksinään ole riittävä menetelmä korjaamaan CTb-soluissa havaittuja heikentyneitä koliinin sisäänottoa ja asetyylikoliinin vapautusta. Näinollen on luultavaa., että SOD1:n yli-ilmentyminen ei yksinään ole syynä DS:ssä havaittuihin kolinergisiin häiriöihin. CTb-solulinja ja geenin vaimennus ovat arvokkaita ja käyttökelpoisia työkaluja geenien yli-ilmentymisen tutkimisessa DS:ssa.

Avainsanat: Downin syndrooma, asetyylikoliini, kolinerginen, superoksididismutaasi

Table of Contents

Abbreviations

ACh	acetylcholine
AChE	acetylcholine esterase
AD	Alzheimer's disease
APP	amyloid precursor protein
AS	antisense sequence
ChAT	choline acetyltransferase
ChT	choline transporter
CoA	coenzyme A
dpm	disintegrations per minute
DS	Down syndrome
EC	extracellular fraction
HSA21	human chromosome 21
IC	intracellular fraction
mAChR	muscarinic acetylcholine receptor
nAChR	nicotinic acetylcholine receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate based saline
SCR	scrambled sequence
SDS	sodium dodecyl sulfate
SOD1	Cu/Zn superoxide dismutase
Ts16	murine trisomy 16
Ts21	human trisomy 21
VAChT	vesicular acetylcholine transporter

1 Introduction

The present study focuses on the effects of the overexpression of a single gene, coding for the enzyme Cu/Zn superoxide dismutase, on cholinergic function in a cell line derived from the cerebral cortex of a trisomy 16 mouse, a model of the human condition known as Down syndrome.

1.1 Down Syndrome

Down syndrome (DS) was first described by the English physician John Langdon Down in 1866 and was in 1959 shown to be caused by trisomy 21 (fig. 1), a form of aneuploidy in which an extra copy of chromosome 21 (HSA21) is present in the genome (Jacobs et al., 1959; Lejeune et al., 1959).

HSA21 is an acrocentric chromosome. It is the smallest of human chromosomes, the long q arm representing only about 1% of the total sequences obtained from the human genome. The total number of genes (protein-coding and RNA-coding DNA) on 21q has not been conclusively determined, but the estimates range between 225 and 382 (Hattori et al., 2000).

DS is frequently lethal in early development, and it has been estimated that 75-90% of conceptuses with Ts21 die *in utero*. DS has an average incidence of approximately 1:700 live births (Reeves et al., 1986; Morris et al., 2002). The probability of a maternal reproductive cell having an extra HSA21, or a part thereof, increases with the age of the mother. For women who become pregnant at under 30 years of age the probability of having a baby with DS is less than 1:1000. This increases to 1:350 for women who become pregnant at the age of 35 and to approximately 1:100 at the age of 40 (Morris et al., 2002). However, since only about nine percent of all pregnancies occur in the age group of 35 or older, in 75% of all cases of DS the mother is younger than 35 years.

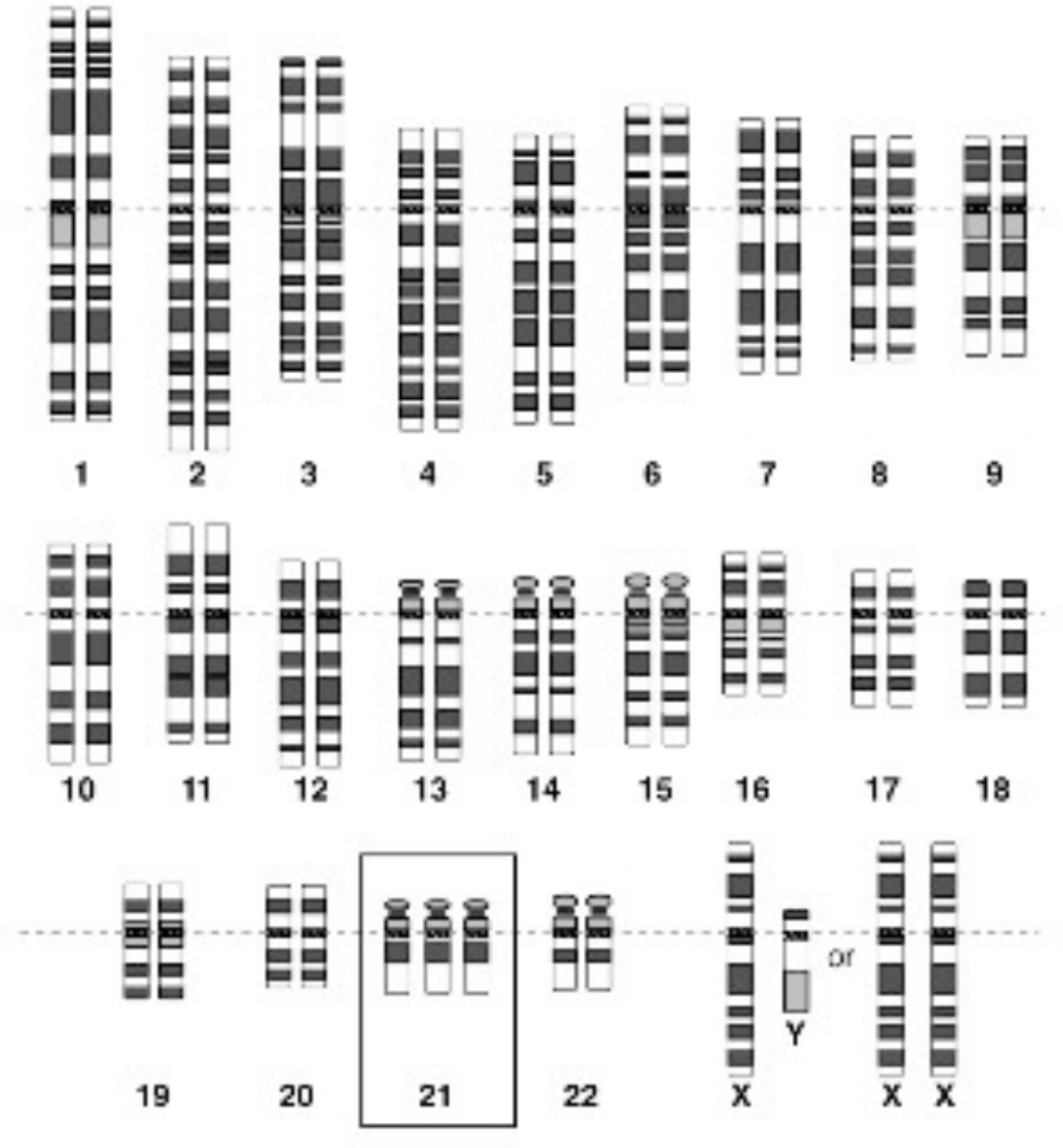

Figure 1.

The Down syndrome karyotype with three copies of chromosome 21. Picture from National Human Genome Research Institute (http://www.genome.gov/Pages/Hyperion/DIR/VIP/Glossary/Illustration/Pdf/trisomy.pdf)

By far the most prevalent type of DS, accounting for approximately 92% of all cases, is caused by the presence of a third whole HSA21, usually of maternal origin (Hassold et al., 1984), and is termed full trisomy 21 (Ts21). Most commonly the extra maternal chromosome is the result of a non-disjunction during the first meiotic division of the oocyte. A second type of DS is caused by a Robertsonian translocation of HSA21 in

which the long arm of the chromosome becomes attached to another chromosome, usually 14, 21 or 22. Very rarely, DS can be caused by mosaicism, in which case the individual has 46 chromosomes in some cells and 47 in others. The severity and symptoms of the syndrome in such individuals may vary, depending on the type and proportion of cells that carry the extra HSA21.

DS affects all major organ systems, including the central nervous system, immune, bone and cardiovascular systems (Korenberg et al., 1994). It is the most common known genetic disorder associated with mental retardation (Hassold and Jacobs, 1984). In addition to these mental deficiencies (Rachidi and Lopes, 2007) DS patients suffer from early onset of Alzheimer's disease (AD) type neuropathology (Thase et al., 1984; Masters et al., 1985). It also includes a 20-fold increased incidence of childhood leukemias and several other types of cancers, although some types of tumors, particularly neuroblastomas and nephroblastomas, are underrepresented (Satge et al., 1998; Hasle et al., 2000). The number and severity of symptoms vary widely even among Ts21 patients, which can be at least partially attributed to the genomic variability of HSA21.

The effects of DS have been associated with excess products of several genes located in HSA21. At least parts of the region from 21q22.1 to 21qter of the chromosome, containing an estimated 50-100 genes, is required for the manifestation of DS (Hattori et al., 2000). Theoretically, the genes that are present in three copies are overexpressed 1.5-fold relative to the euploid state. Studies using a mouse model named Ts65Dn indicate that the presence of extra copies of genes also affects the regulation of gene expression, and in Ts21 many genes of HSA21 are expressed at levels significantly lower or higher than the theoretical value of 1.5-fold (Kahlem et al., 2004; Lyle et al., 2004).

1.2 Models of Down Syndrome

Studying DS in humans is difficult because of the length of time involved in the development of the various symptoms, and also gives rise to ethical dilemmas, for example, in studies requiring tissue samples. Because of these reasons several models of the condition have been developed.

1.2.1 Animal Models

Two important animal models have been designed for use in *in vivo* study of the physiopathological changes observed in DS: (1) transgenic mice, with either single or multiple DS related genes added to their genome, and (2) mice with additional fractions of the murine chromosome 16, which is highly homologous to HSA21. These models can only be used to study the role of a few genes or gene products at a time, since none of them present all the characteristics seen in the full trisomy of HSA21, but they have been highly useful in the study of the effects of overexpression of individual genes.

The first transgenic animal models for the study of a single gene suspected to play a role in DS were several strains of mice carrying several copies of the human Cu/Zn superoxide dismutase gene (Epstein et al., 1987). In four of these strains the Cu/Zn superoxide dismutase activity in the brain was increased 1.6- to 6.0-fold. Such models provide tools for the study of gene dosage effects in DS.

The animal condition with most similarities to the DS seen in humans is the murine trisomy 16 (Ts16). As mentioned above, the murine chromosome 16 is highly homologous to HSA21, which is why Ts16 presents an animal model for the study of Ts21. However, mice with full Ts16 are not viable and die *in utero* between days 14 and 16 of gestation. To overcome this obstacle, models of partial trisomy have been created where only the distal portion of chromosome 16, the region most homologous to HSA21, has been triplicated (Ts65Dn) (Davisson et al., 1990). This region corresponds to the homologous region 21q21-22.3 of HSA21, known as the DS critical region, which was thought necessary to be completely triplicated for the manifestation of full DS. Recently it has been shown in mice that this region is not sufficient and for the most part not necessary to produce certain aspects of the DS phenotype (Olson et al., 2004).

Another model of partial trisomy is the Ts1Cje, which has a smaller portion of the critical region triplicated (Sago et al., 1998). These partially trisomic mice are viable, and thus provide useful animal models for the *in vivo* study of DS. The most used animal model is the Ts65Dn, in which are present many of the phenotypical changes seen in DS patients, including neurochemical alterations (Dierssen et al., 1996; Dierssen et al., 1997; Ruiz de Azua et al., 2001).

1.2.2 Cellular Models

In some studies, primary cultures derived from human trisomy 21 patients have been used, but the ethical and practical problems associated with the use of human tissues have limited their use. The cellular models used in the study of the physiopathologies associated with DS consist mostly of primary cultures derived from the animal models discussed above, as well as cell lines derived from such primary cultures. The usefulness of primary cultures is limited by their short lifespans and the small amount of cells acquired compared to the virtually unlimited amount of cells produced by a continuous cell line.

Studies using primary cultures have shown electrophysiological alterations, such as an abnormal action potential, in trisomic cells derived from the dorsal root ganglion and the hippocampus (Orozco et al., 1987; Orozco et al., 1988; Caviedes et al., 1990; Galdzicki et al., 1993). In addition to these alterations, levels of oxygen radicals sufficiently high to be cytotoxic have been found in neurons derived from trisomy 16 mice, leading to cellular damage and a shortened cellular lifespan (Busciglio and Yankner, 1995).

As mentioned above, Ts16 mice are not viable, and thus are not a suitable model for studies *in vivo*. Cellular models have nontheless been derived from Ts16 fetuses, and some immortalized cell lines have been successfully produced from such cells, providing an unlimited amount of readily available trisomic cells for studies *in vitro*. The group of Dr. Pablo Caviedes has established several neuronal cell lines (expressing neuronal markers) derived from various neuronal tissues from both normal and trisomic mice fetuses (Allen et al., 2000; Allen et al., 2002; Cardenas et al., 2002a; Cardenas et al., 2002b). The cell lines have been immortalized using a protocol involving the use of growth medium conditioned with the rat thyroid cell line UCHT1 (Caviedes and Stanbury, 1976), which reportedly induces a stable transformation *in vitro* while conserving the differentiation characteristic of the tissue of origin (Caviedes et al., 1993; Caviedes et al., 1994; Liberona et al., 1997; Cardenas et al., 1999; Allen et al., 2000).

Changes in basal cytosolic calcium levels, altered glutamatergic function and responses to the stimulation of glutamate reseptors, alterations in cholinergic functions, as well as reduced levels of acetylcholine precursors and diminished acetylcholine release resulting from reduced levels of choline acetyltransferase (ChAT) have been observed in

the trisomic cell line CTb, derived from the cerebral cortex of a Ts16 fetus (Cardenas et al., 1999; Allen et al., 2000).

1.3 Acetylcholine and cholinergic function

Acetylcholine (ACh) is a neurotransmitter synthesized in the neuronal cytoplasm from choline and acetic acid via acetyl coenzyme A (acetyl CoA). It is loaded into synaptic vesicles by the vesicular acetylcholine transporter (VAChT) and stored there until it is released at the nerve terminals into the synaptic cleft in response to a presynaptic action potential. The release of ACh in response to nerve impulses is dependent on the intracellular Ca^{2+} concentration and its gradient. After it is released, ACh diffuses and binds to receptor sites on the postsynaptic cell. (fig. 2)

Two classes of acetylcholine receptors exist, nicotinic (nAChR) and muscarinic (mAChR). In the context of this study the focus will be on nAChRs as they function directly as ion channels, whereas mAChRs are coupled with intracellular G proteins that function as secondary messengers to activate ion channels on the cell membrane. The binding of two molecules of ACh to nAChR causes a conformational change in nAChR, opening a channel through the receptor and allowing positively charged ions, particularly sodium and potassium, to pass through according to their electrochemical gradients, thereby altering the membrane conductance and potential of the postsynaptic cell.

The enzyme acetylcholine esterase (AChE), present in the synaptic cleft on the surface of the postsynaptic membrane, quickly degrades acetylcholine into acetate and choline, ensuring that the signal does not overstimulate the postsynaptic cell. Acetate and choline are then reabsorbed by the presynaptic cell, the acetate is used in the synthesis of acetyl CoA, and acetyl CoA and choline are then used to replenish ACh reserves for subsequent chemical transmission (Rand, 2007). Choline uptake is mediated by the high-affinity choline transporter (ChT), which is also the rate-limiting step at the *de novo* synthesis of ACh (Ferguson and Blakely, 2004).

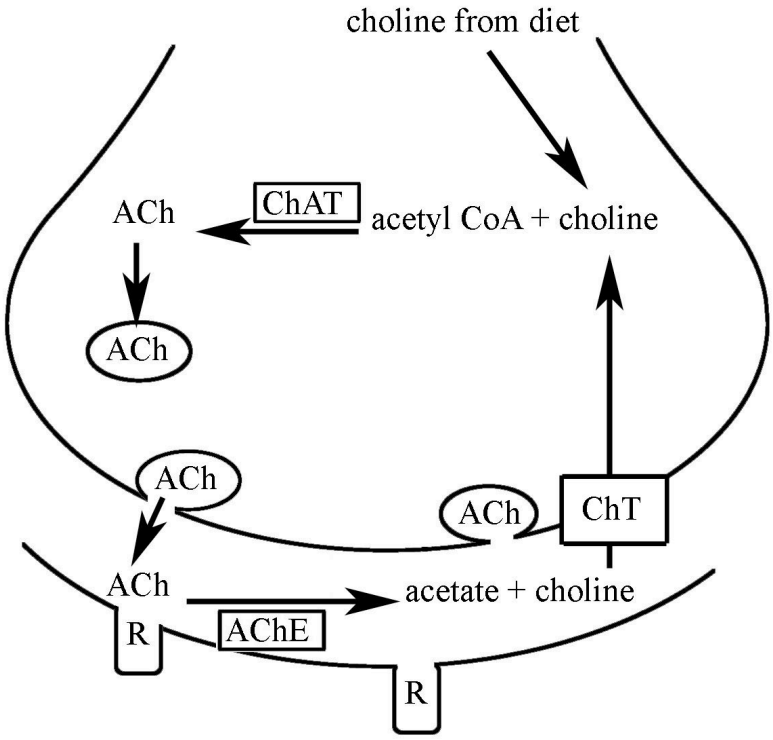

Figure 2.

Acetylcholine (ACh) pathways. ACh is synthesized from acetyl CoA and choline by choline acetyltransferase (ChAT) and then stored in synaptic vesicles from where it is then released into the synaptic cleft. There it binds into the acetylcholine receptors (R) on the postsynaptic membrane. It is then quickly degraded into acetate and choline by acetylcholine esterase (AChE) which is also located on the postsynaptic membrane. Choline is recycled back into the presynaptic cell through choline transporters (ChT) where it is used again in the synthesis of ACh.

ACh is a unique transmitter substance in that it is not an amino acid nor derived directly from one (fig. 3). The biosynthetic pathway for ACh has only one enzymatic reaction, that catalyzed by choline acetyltransferase (ChAT):

$$acetyl\ \ CoA + choline \xrightarrow{ChAT} CoA + ACh$$

The biosynthesis of the cosubstrate acetyl CoA is not specific to cholinergic neurons, because this substance participates in many metabolic pathways. Nervous tissue cannot synthesize choline, which is ultimately derived from the diet and delivered to neurons through the blood stream. As mentioned above, choline is also carefully recycled

after ACh has been hydrolyzed in the synaptic cleft. At normal brain concentrations ChAT is unsaturated with choline, and thus an increase in the intracellular choline concentration leads directly to an increase in the amount of ACh being synthesized (Fernstrom, 1977).

In the autonomic nervous system ACh is the transmitter for all preganglionic neurons and for the parasympathetic postganglionic neurons as well. It is used in many synapses throughout the brain. In particular, in the nucleus basalis there are many cell bodies that synthesize ACh and these neurons have widespread projections to the cerebral cortex. In addition, ACh is the transmitter used at all nerve-skeletal muscle junctions in vertebrates. ACh is also synthesized in various non-neuronal cells and tissues, such as T lymphocytes, oligodendrocytes, airway bronchial epithelial cells and the placenta, where it may function as an autocrine or paracrine signaling molecule (Fujii et al., 1996; Lan et al., 1996; Wessler et al., 2001; Proskocil et al., 2004).

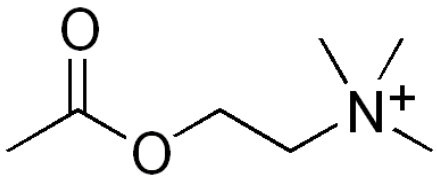

Figure 3.

Acetylcholine is an ester of acetic acid and choline. Its chemical formula is $CH_3COOCH_2CH_2N^+(CH_3)_3$ and systematic name *2-acetoxy-N,N,N-trimethylethanaminium*.

In DS, a reduction in cholinergic function has been observed (Epstein, 1986; Coyle et al., 1988), which is presumbaly one of the mechanisms underlying the cognitive deficits presented by the condition. Ts16 mice reportedly exhibit decreased activities of ChAT and AChE (Ozand et al., 1984; Sweeney et al., 1989), as well as reduced ACh synthesis and choline uptake in the brain (Fiedler et al., 1994). The trisomic murine cell line CTb, used in the present study, has been shown to exhibit reduced choline uptake and acetylcholine release as well as reduced ChAT expression (Allen et al., 2000). An increase in basal Ca^{2+} levels has also been observed in CTb cells (Cardenas et al., 1999), which could play a role in the altered cholinergic function.

In the present study, acetylcholine release from CTb and CNh cells was induced using three different depolarizing solutions: 50mM K^+, 200μM glutamate and 100μM nicotine. Potassium generates depolarization by causing a change in the membrane potential, which causes opening of voltage gated Ca^{2+} channels, followed by an influx of Ca^{2+} ions into the cellular body, eventually resulting in ACh release. Glutamate, which itself functions as a neurotransmitter, binds to NMDA receptors (named after the specific agonist *N*-methyl *D*-aspartate) as well as kainate and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, all of which undergo conformational changes to open cationic channels, allowing the influx of Ca^{2+} and Na^+ and the efflux of K^+. The resulting change in membrane potential triggers voltage gated Ca^{2+} channels, further increasing the influx of Ca^{2+}, which eventually causes the release of ACh. Nicotine is the agonist after which the nicotinic acetylcholine receptors are named. Its binding to a nAChR causes a conformational change which opens a channel through the receptor, allowing the influx of Na^+ and efflux of K^+ (according to their individual electrochemical gradients). The resulting depolarization of the postsynaptic membrane leads to the opening of voltage gated Na^+ channels and an increased influx of Na^+. As the depolarization increases, more Na^+ channels are opened, and eventually this results in an action potential that triggers the release of ACh.

1.4 Cu/Zn Superoxide Dismutase

Cu/Zn superoxide dismutase (SOD1) is a key enzyme in the metabolism of free radicals. It converts superoxide molecules into molecular oxygen and hydrogen peroxide through the two-step reaction:

$$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2$$
$$Cu^+ + O_2^- + 2H^+ \rightarrow Cu^{2+} + H_2O_2$$

SOD1 is a 32kDa homodimeric enzyme found predominantly in the cytosol (McCord and Fridovich, 1969a, b), and is one of the most thermally stable enzymes found in mesophilic organisms. Its dismutase activity declines at 80^oC with a corresponding

melting temperature at 90°C (Roe et al., 1988). The protein is stable in the presence of strong denaturants, and the activity is observed in 4% sodium dodecyl sulfate (SDS) or 10M urea (Forman and Fridovich, 1973). Structural properties of SOD1 that contribute to this remarkable thermochemical stability are thought to include an eight-stranded β-barrel motif, hydrophobic interactions associated with dimerization, coordinate covalent bonds, and an intrasubunit disulfide bond between highly conserved pair of cysteins (Cys^{57} and Cys^{146} in the human form). To attain the correctly folded and enzymatically active quaternary structure, SOD1 requires several post-translational modifications. These include the acquisition of copper and zinc ions, formation of the disulfide bond mentioned above, and dimerization. It has been shown that only the most immature form, before any post-translational modifications, favors the monomeric state under physiological conditions (Arnesano et al., 2004).

Each subunit of SOD1 is formed by a β-barrel, made of eight antiparallel β-strands, connected by seven turns and loops (fig. 4). Three of the loops (IV, VI and VII) are long and have nonorganized secondary structure. The active site of each monomer contains one copper ion and one zinc ion. They are located between loops IV and VII, outside the β-barrel. In the oxidized enzyme, copper(II) is coordinated by four histidine residues, and the zinc is bound to three histidine residues and one aspartate residue. The orientation of the metal ligands in determined by a complex network of hydrogen bonds (Ogihara et al., 1996).

Mutations in SOD1 are associated with familial amyotrophic lateral sclerosis (Rosen, 1993). Oxygen free radicals and oxidative stress have also been implicated by several studies in other neurogenerative diseases, such as Alzheimer's and Parkinson's diseases. The gene encoding SOD1 is over 9200 base pairs long and is located in HSA21 band 21q22.11. It is overexpressed in both Ts21 and Ts16, and the levels of its products, both mRNA and protein, are elevated in the trisomic tissues (Epstein et al., 1987).

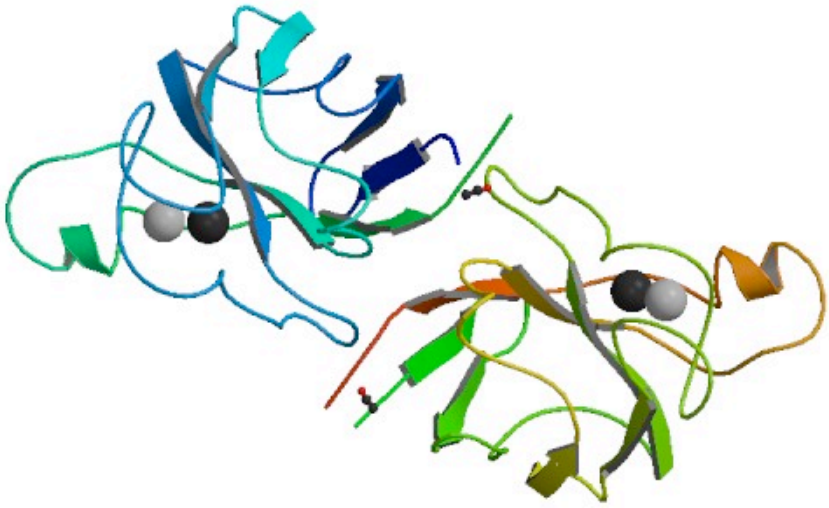

Figure 4.

A ribbon model of SOD1 (Protein Data Bank (PDB) code 1SPD) with the Cu and Zn ions visible. Image from RCSB PDB (http://www.pdb.org).

The increase in SOD1 enzymatic activity is associated with elevated levels of H_2O_2 and hydroxyl radicals, reactive oxygen species (Halliwell and Gutteridge, 1984) which can cause mutations in DNA, changes in the structure and function of enzymes and other proteins, and initiate the autocatalytic process of lipid peroxidation in the cell membrane (Halliwell and Gutteridge, 1985). In DS patients the elevated levels of hydrogen peroxide and hydroxyl radicals lead to lipid peroxidation and neuronal damage (Avraham et al., 1988; Bar-Peled et al., 1996; Peled-Kamar et al., 1997). In cortical neurons of DS patients this results in neuronal death, which could be prevented by antioxidants (Busciglio and Yankner, 1995). An increase in lipid peroxidation has been recently confirmed in patients with DS (Casado et al., 2007).

Transgenic mice overexpressing human SOD1 exhibit deficiency in spatial memory and long-term potentiation (Harris-Cerruti et al., 2004). In partial trisomy 16 mice it has

been observed that learning impairment is much less severe when SOD1 is not functional (Sago et al., 1998). In DS, the overexpression of another protein located in HSA21, amyloid precursor protein (APP), further contributes to the oxidative damage resulting from the overexpression of SOD1 by reducing antioxidant formation (Takahashi et al., 2000). Several SOD1 mutants are also vulnerable to oxidative damage, as the oxidation of select histidine residues that bind metals in the active site can mediate SOD1 aggregation (Rakhit et al., 2002).

Groner et al. (1994) have shown that rat PC12 cells expressing elevated levels of transfected human SOD1 show impaired neurotransmitter uptake, suggesting a possible effect on choline uptake in cholinergic cells. They also discovered that several strains of mice carrying extra copies of human SOD1 expressed similar morphological changes in their neuromuscular junctions as aging mice and rats as well as DS patients. These changes include withdrawal and destruction of some terminal axons and the development of multiple small terminals, and a decrease in the ratio of terminal axon area to postsynaptic membranes. DS patients diagnosed with partial or mosaic Ts21 have shown normal SOD1 activity, suggesting that overexpression of SOD1 is not critical to the development of the DS phenotype (De La Torre et al., 1996).

The purpose of the present study is to establish the effects of SOD1 overexpression on cholinergic function in Ts16 neuronal cells, specifically in context of the full trisomy and not just as a single gene. Since the expression levels of a large number of gene products are altered in DS as a result of the extra HSA21 (Kahlem et al., 2004; Lyle et al., 2004), the possibility that they are affected by one another should be taken into account when attempting to establish the effect of a knockdown of a single gene. For this reason we chose to use the CTb cell line, which expresses the full Ts16, rather than a cell line transgenic for SOD1 alone. The knockdown protocol, rather than a simple knockout of the SOD1 gene, which would completely stop its expression, was chosen so that the effect of returning the expression levels of SOD1 to normal could be observed.

There are several ways the overexpression of SOD1 could potentially play a role in the cholinergic dysfunctions (a reduced expression of ChAT and a decrease in both choline uptake and acetylcholine release) reported in the murine trisomic CTb cells (Allen et al., 2000). The decreased uptake of choline could be the result of damage to the cell membrane (lipid peroxidation), or some other direct or indirect effect of SOD1 overexpression on the

function or regulation of ChT. Lipid peroxidation could also cause the decrease in acetylcholine release by damaging either the cell membrane or the synaptic vesicles stored in the cytosol. Other potential targets for disrupting cholinergic function through oxidative stress would include ChAT and VAChT as well as nAChR [a decrease in acetylcholine release in response to nicotine stimulation has been observed in CTb cells (Allen et al., 2000; Opazo et al., 2006) compared to CNh cells]. The increase in basal intracellular Ca^{2+} levels, seen in CTb cells (Cardenas et al., 1999), could also be a result of changes to membrane composition due to lipid peroxidation, or damage to ion channels from oxidative stress.

To study the effects of SOD1 overexpression on cholinergic function in the CTb cell line we used a knockdown protocol described by Kumar et al. (2000) to bring the expression of SOD1 down to a normal level. For the normal level of expression, we used as a reference the CNh cell line, derived from the cerebral cortex of a normal mouse fetus. We then carried out experiments on choline uptake [methods described by Melega and Howard, (1981), and Allen et al., (1997)] and acetylcholine release [methods described by Fiedler et al., (1994), Cardenas et al., (1999), and Allen et al., (2000)] to see if the reduction in SOD1 expression caused any changes in these cholinergic functions. A positive result from either or both of these experiments would present a reason to carry out more thorough experiments on the specific interactions through which SOD1 overexpression affected these functions. A negative result would indicate that SOD1 overexpression by itself does not affect cholinergic function in Ts16, and is therefore unlikely to do so in Ts21.

2 Aim of the Study

The aim of the present study is to improve the understanding of the cellular conditions and mechanisms underlying the phenotypic manifestation of DS. A more detailed summary of the objectives of the study is described below.

2.1 General Objectives

The general objective of the present study is to normalize the overexpression of the Cu/Zn superoxide dismutase SOD1 in a trisomic murine cortical cell line (CTb) using a knockdown protocol, and to study whether the change in the intracellular level of SOD1 would have any effect on the uptake of choline or the fractional release of acetylcholine, both of which are impaired in CTb cells (Allen et al., 2000; Opazo et al., 2006). A non-trisomic murine cortical cell line (CNh) is used as a model of normal cells for comparison. The results will then present clues to the role of the overexpression of SOD1 in the cholinergic dysfunction exhibited by Ts16 and Ts21 neuronal cells.

2.2 Specific Objectives

The specific objectives of the study were:

1. Transfection of CTb cells with oligonucleotides specific for SOD1.
2. Documentation of the normalization of SOD1 levels in transfected CTb cells.
3. Comparision of the uptake of ^{3}[H]-choline from extracellular medium in transfected CTb cells and CNh cells.
4. Comparision of the fractional release of acetylcholine in transfected CTb cells and CNh cells.
5. Correlation of the effect of the knockdown with the uptake of ^{3}[H]-choline and fractional release of acetylcholine in CTb cells.

3 Materials and Methods

3.1 Cell Lines

The cell lines used in the study were previously generated from primary cultures derived from the cerebral cortex of both normal and Ts16 mice. Ts16 mice were generated by crossing mice with Robertsonian translocations Rb (14:16) and Rb (9:16). The F1 generation is heterozygous, and crossing them with normal parents results in an F2 generation with 25% of each of the following genotypes: Rb (14:16), Rb (9:16), monosomy 16 and trisomy 16. Of these genotypes only the first two are viable. The monosomic zygotes fail to implant themselves in the uterus and the trisomic fetuses die *in utero* between days 14 and 16 of gestation. For the generation of trisomic cortical cell lines it was necessary to obtain the tissues for the primary cultures from fetuses between 14 and 16 days old just before they would naturally die. The phenotype of a trisomic fetus differs from a normal one's, and they can be identified from morphological features during the dissection of the cortical tissue. (Allen et al., 2000)

The murine cortical cell lines CNh (normal) and CTb (trisomic) were generated by the laboratory of Dr. Pablo Caviedes in the University of Chile. These cell lines were immortalized from the primary cultures using a growth medium conditioned with the rat thyroid cell line UCTH 1. The cell lines immortalized using this protocol preserve the differentiation characteristic of the tissue of origin (Caviedes et al., 1993).

3.2 Cell Cultures

For standard growth conditions, the CTb and CNh cell lines were cultured on separate glass Petri dishes and maintained in DMEM/F-12 (1:1) (Invitrogen, Grand Island, NY, USA) growth medium modified to contain 6 g/l glucose, 5% (v/v) adult bovine serum, 5% (v/v) fetal bovine serum, and 40 mg/l gentamycin. The medium on the culture dishes was changed to a fresh one every two or three days. The cells were kept in a humidified incubator at 37^{o}C and 5% CO_2. At 80-90% confluence new subcultures were prepared, using a modified phosphate based saline (PBS) pH 7.2, termed solution D (appendix 7.1),

for washing the cells and 0.1% trypsin (Invitrogen) to detach the cells from the culture plates.

3.3 Knockdown of SOD1

Generally, a transient knockdown can be induced by transfecting the target cells with oligonucleotides specific to a certain gene or its transcripts. The binding of the oligonucleotides to the active gene or its transcripts causes decreased expression through blocking of transcription, degradation of the mRNA transcript or blocking of translation. In the present study, the knockdown protocol was used to normalize the expression of SOD1 in the trisomic CTb cells, bringing it down from the elevated level (caused by the overexpression resulting from the trisomy) to the level observed in the non-trisomic CNh cells. The knockdown was carried out using a specific phosphorothioate mRNA antisense sequence (AS) as described by Kumar et al. (2000). Briefly, the AS oligonucleotides bind to the mRNA transcripts of SOD1, blocking them and preventing their translation, thus preventing the synthesis of new SOD1 molecules and causing a gradual decrease in the amount of SOD1 present in the cells. The sequence used was based on the rat SOD1 antisense sequence used by Troy and Shelanski (1994), modified to be specific against mouse SOD1. A scrambled sequence (SCR), composed of the same amino acid codons as AS but in a randomly rearranged order, was used as a control.

The transfection (see chapter 3.3.1) was carried out using cationic liposomes (Lipofectamine reagent; Invitrogen). This method has been previously shown to be effective in inducing the endocytosis of oligonucleotide sequences in CTb cells (Opazo et al., 2006).

3.3.1 Transfection With Oligonucleotides

The oligonucleotides used to induce the knockdown of SOD1 were commercially produced by Sigma-Proligo (The Woodlands, TX, USA). The antisense oligonucleotide (AS)

sequence used, based on rat SOD1 antisense sequence used by Troy and Shelanski (1994), was:

5'CCGCTTTCATCGCCATGCTTC,

and the scrambled oligonucleotide (SCR) sequence used as a control was:

5'AAGCCAAATGGTGACGGGGAG.

Stock solutions of 50µM of each AS and SCR were prepared.

CTb and CNh cells were seeded separately on 6-well plates (Ø 3.5 cm) and cultured until 80% confluent, usually between 2-4 days. The following cultures were prepared: CTb cells for transfection with AS, CTb cells for transfection with SCR (positive control), CTb cells not to be transfected (negative control) and CNh cells not to be transfected (to compare trisomic cells with normal cells), each to be observed at three time points (24, 48 and 72 hr post-transfection) to establish the time needed to normalize SOD1 expression.

For each well containing cells to be transfected with AS, 5 µl of 50 µM AS was mixed with 45 µl of DMEM/F-12 without sera or antibiotics for a final volume of 50 µl per well. The mixture was incubated for 15 min in room temperature. A similar solution was prepared using 50 µM SCR. For each well containing cells to be transfected, 2.5 µl of Lipofectamine reagent (Invitrogen) was mixed with 47.5 µl of DMEM/F-12 without sera or antibiotics for a final volume of 50 µl per well. Each AS and SCR solution was mixed 1:1 with the Lipofectamine solution for a final volume of 100 µl per well. The mixtures were incubated for 15 min in room temperature.

Growth medium was removed from cells and replaced with 400 µl of DMEM/F-12 without sera or antibiotics. For controls not to be transfected, 500 µl was used. 100 µl of AS with Lipofectamine or SCR with Lipofectamine was added for cells to be transfected. The final concentration of AS or SCR in each well was 0.5 µM. All cells were incubated for 5 hr at 37^{o}C and 5% CO_2.

After the 5 hr incubation, medium was removed from cells, the cells were washed twice with solution D, and 1 ml of DMEM/F-12 with 5% adult bovine serum, 5% fetal bovine serum and 40 mg/l gentamycin was added. Normal growth conditions (37^{o}C and 5% CO_2) were resumed for 24, 48 and 72 hrs.

3.3.2 Cell Lysis

After 24, 48 and 72 hr incubation post-transfection, cell lysis was induced as follows:

RIPA lysis buffer (appendix 7.1) was prepared with protease inhibitors (PMSF, leupeptine). Medium was removed from cells and washed twice with PBS pH 7.4. After washing 400 µl of 0.1% trypsin-EDTA was added and the cells were incubated for 5-10 min in 37^{o}C to detach the cells.

After this 400 µl of DMEM/F-12 with 5% adult bovine serum, 5% fetal bovine serum and 40 mg/l gentamycin was added to neutralize the trypsin solution. Detached cells were transferred to an Eppendorf tube.

The cells were centrifuged for 5 min at 3000 rpm and 4^{o}C, after which the supernatant was removed and discarded. The cells were washed with 300 µl of chilled PBS, and centrifuged for 5 min at 3000 rpm and 4^{o}C. The supernatant was then removed and discarded, and 200 µl of lysis buffer was added on the pellet. The cells were incubated on ice for 45 min after which the lysate was sonicated for 30 sec and then centrifuged for 5 min at 14000 rpm and 4^{o}C.

The supernatant was collected and stored at -20^{o}C for later study. Aliquotes were taken from each sample and protein content was measured using the Bradford method at 595 nm (Bradford, 1976).

3.3.3 SDS-Electrophoresis

Once the protein content corresponding to each sample was determined (chapter 3.3.2), gels for 12% SDS-PAGE (table 1) and the running buffer (appendix 7.1) were prepared.

Aliquotes containing 20 µg of protein were prepared from each sample and loading buffer was added in a concentration of 1:5. The aliquotes were then incubated for 5min in 95^{o}C. For one lane per gel, 3 µl of a SeeBlue® molecular weight marker (Invitrogen) was used.

Table 1. Formulas used for preparing gels for SDS-PAGE.

	Stacking Gel (5%), 2 ml	**Resolving Gel (12%), 5 ml**
30% Acrylamide	335 µl	2000 µl
1.5M Tris-HCl pH 8.8	-	1250 µl
0.5M Tris-HCl pH 6.8	500 µl	-
ddH_2O	1125 µl	1645 µl
10% APS	20 µl	50 µl
TEMED	3 µl	5 µl
10% SDS	20 µl	50 µl

The samples were run in the running buffer at a constant voltage of 80 V through the upper gel and 120 V through the lower gel using a Bio-Rad (Hercules, CA, USA) Mini-PROTEAN® 3 electrophoresis cell.

3.3.4 Transfer of Proteins to a Nitrocellulose Membrane

The transference buffer (appendix 7.1) was prepared and proteins were transferred from the gel to a nitrocellulose membrane at a constant current of 300 mA for 1 hr 30 min at 4^{o}C using a Bio-Rad Mini-PROTEAN® 3 cell with a Bio-Rad tank blotting module.

3.3.5 Western Blot

The blocking solution (appendix 7.1) was prepared. The nitrocellulose membrane was blocked from nonspecific binding with the blocking solution for 1 hr at room temperature. After this, the membrane was incubated for 1 hr 30 min at room temperature with polyclonal primary antibody, directed against SOD1 (1:500), and monoclonal primary antibody directed against β-tubulin (1:2000; Boehringer Mannheim, Mannheim, Germany), which was used as a loading control.

The membrane was then washed 6 x 5 min with PBS and incubated with the secondary antibody (1:1000; conjugated with horseradish peroxidase; Santa Cruz

Biotechnology, Santa Cruz, CA, USA) for 1 hr in room temperature after which it was washed 6 x 5 min with PBS.

The antibody detection was carried out using a chemiluminescence kit (SuperSignal® West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA), and the blot was then exposed to a film.

The films were scanned and ImageJ software (NIH, Bethesda, USA) was used to quantify SOD1 and β-tubulin according to band size and intensity. To obtain relative quantity of SOD1, SOD1 values were divided with corresponding β-tubulin values.

3.4 ^{3}[H]-Choline Uptake

^{3}[H]-choline uptake into cells was measured using methods previously described by Melega and Howard (1981) and Allen et al. (1997). The cells were seeded and transfected as above (chapter 3.3.1) and the following procedure was repeated at 24, 48 and 72 hr post-transfection:

Scintillation vials were prepared by adding 10 ml of scintillation coctail to each one. 1 µl (1 µCi) ^{3}[H]-choline ([methyl-^{3}H]-choline chloride;specific activity range 60-90 Ci/mmol; Perkin-Elmer, Waltham, MA, USA) was added to each well of cells. After 1 min of incubation at room temperature a 50 µl sample was taken from each well, and these were placed in separate scintillation vials. This was repeated after 2 min and 5 min of incubation. These are the extracellular (EC) fractions.

The cells were then washed four times with 1 ml Krebs solution (Fiedler et al., 1994) pH 7.4 (appendix 7.1) to remove excess ^{3}[H]-choline. To obtain the intracellular (IC) fraction, 1 ml 0.1% SDS was added and the cells were incubated for 30 sec at room temperature to induce lysis. A 900 µl sample of the cell lysate was taken from each well and placed in separate scintillation vials. An additional 100 µl sample was then taken from each well, and the protein content was measured using the Bradford method at 595 nm.

A scintillation counter was used to measure the radioactivity of the samples as disintegrations per minute (dpm) of the ^{3}[H]-choline. The uptake of ^{3}[H]-choline was calculated from the following formula:

$$U^3[H]C = [(IC(dpm)/0.9)/(EC(dpm)/0.05)]/mg_{protein}$$

where $U^3[H]C$ is the uptake of ^{3}[H]-choline, IC(dpm) is the measured radioactivity of the intracellular fraction, EC(dpm) is the measured radioactivity of the extracellular fraction and $mg_{protein}$ is the protein content in mg. The protein content is assumed to be somewhat constant relative to the number of cells in a well, and can therefore be used to normalize the ratio of IC(dpm) vs. EC(dpm) in each sample, thus making the samples comparable to one another.

3.5 Fractional Release of Acetylcholine

The acetylcholine release experiments were carried out using methods previously described by Fiedler et al. (1994), Cardenas et al. (1999) and Allen et al. (2000).

The cells were seeded and transfected as above (chapter 3.3.1), and the following process was repeated at 24, 48 and 72 hr post-transfection with each of the three depolarizing solutions used (50 mM K^+, 200 µM glutamate or 100 µM nicotine).

Scintillation vials were prepared by adding 5 ml scintillation coctail (Optiphase Hisafe 2; Perkin-Elmer) to each one. The cells were incubated with 1 µCi ^{3}[H]-choline for 30 min after which they were washed four times with 2 ml of Krebs solution to remove excess ^{3}[H]-choline. Samples of 50 µl were taken at 1, 3, 6 and 9 min after the final wash and placed in separate scintillation vials. At 10 min after the final wash, acetylcholine release was induced by adding the depolarizing solution (50 mM K^+, 200 µM glutamate or 100 µM nicotine). 1 min after adding the depolarizing solution, another 50 µl sample was taken and placed in a scintillation vial. These are the extracellular fractions.

To obtain the intracellular fraction, the cells were lysed with 1 ml 1% Triton X-100. A scintillation counter was used to measure the radioactivity of the samples from the disintegrations per minute (dpm) of the ^{3}[H]-choline. The fractional release of acetylcholine was calculated from the following formula:

$$\%LF = \frac{EC_{total} - basal\ \ efflux}{EC_{total} + IC} * 100$$

where %LF is the fractional release of acetylcholine, EC_{total} is the total amount of radioactivity measured from the extracellular samples, IC is the amount of radioactivity measured from the intracellular sample and basal efflux is the basal efflux of acetylcholine at 1min after adding the depolarizing solution as extrapolated from a standard curve (time vs. dpm) based on the measured radioactivity of the EC samples from 1, 3, 6 and 9 min after the final wash. It is assumed that the radioactivity in the extracellular samples is mostly due to acetylcholine metabolized by the cells using ^{3}[H]-choline.

3.6 Statistical Analysis

The acquired data was processed using the statistical softwares inerSTAT-a v1.3 (Vargas M., Instituto Nacional de Enfermedades Respiratorias, Tlalpan, Mexico) and R (The R Foundation, Vienna, Austria).

Statistical significance was assessed using analysis of variance (ANOVA) with Dunnett's test as *post hoc*. Significance was accepted at the $P < 0.05$ significance level.

4 Results

4.1 Knockdown of SOD1

4.1.1 Transfection with AS

The results (figures 5 and 6) show a clear overexpression of SOD1 in the trisomic CTb cells compared to the non-trisomic CNh cells. In CTb cells, SOD1 was expressed at levels 87.3% higher than in CNh cells.

Transfection with antisense oligonucleotides produced a knockdown effect, causing the expression of SOD1 to be normalized at approximately 72 hr post-transfection. The expression of SOD1 was reduced by 1%, 11.2% and 43.6% at 24, 48 and 72 hr post-transfection, respectively, compared to the non-transfected CTb cells. A one-way ANOVA on all the samples rejected the null hypothesis of equality of the means of the distributions (F_4=13.18;$p < 0.0001$;figure 6), indicating statistically significant differences between the samples. Dunnett's test showed a significant difference to the non-transfected CTb cells at 72 hr post-transfection as well as between CNh and CTb (Dunnett's test: $p < 0.01$;figure 6).

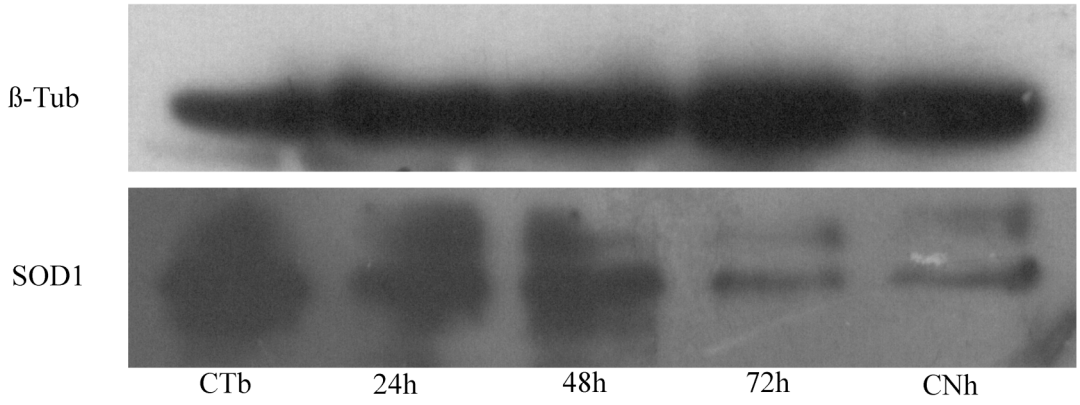

Figure 5.

Western blot of AS transfected samples. β-tubulin was used as a loading control. The overexpression of SOD1 in CTb cells is obvious when compared to CNh, and the effect of the knockdown can be seen particularly well at the 72 hr time point.

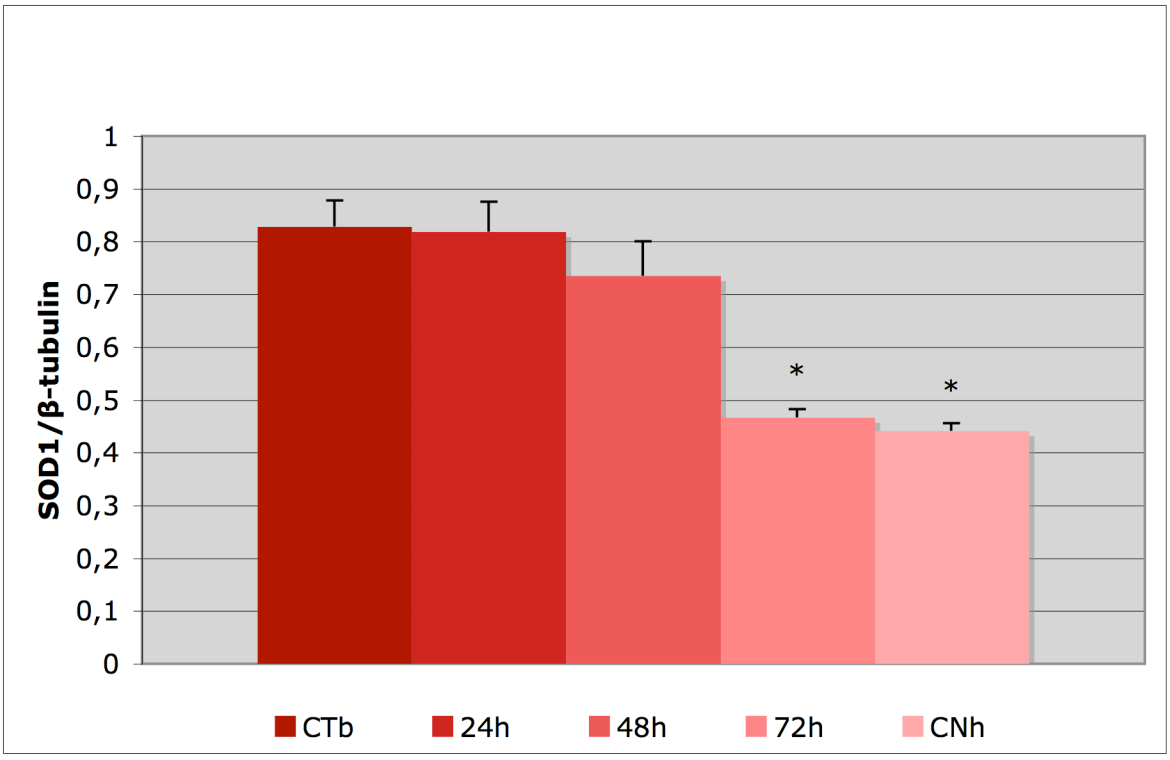

Figure 6.

Effect of transfection with AS on the expression of SOD1. β-tubulin was used as a loading control to normalize the results between samples. Values are mean ± SEM (n=4) SOD1/β-tubulin. Significant difference (Dunnett's test: $p < 0.01$) compared to non-transfected CTb is indicated by *.

4.1.2 Transfection with SCR

The results (figures 7 and 8) show that transfection with SCR, oligonucleotides not specifically targetted at SOD1, did not cause a knockdown effect on SOD1. There are no significant changes in the expression of SOD1 at 24, 48 or 72 hr post-transfection when compared to the non-transfected CTb cells (Dunnett's test: $p > 0.05$; figure 8). This confirms that the effect of transfection with AS, as shown above in figure 6, is specific.

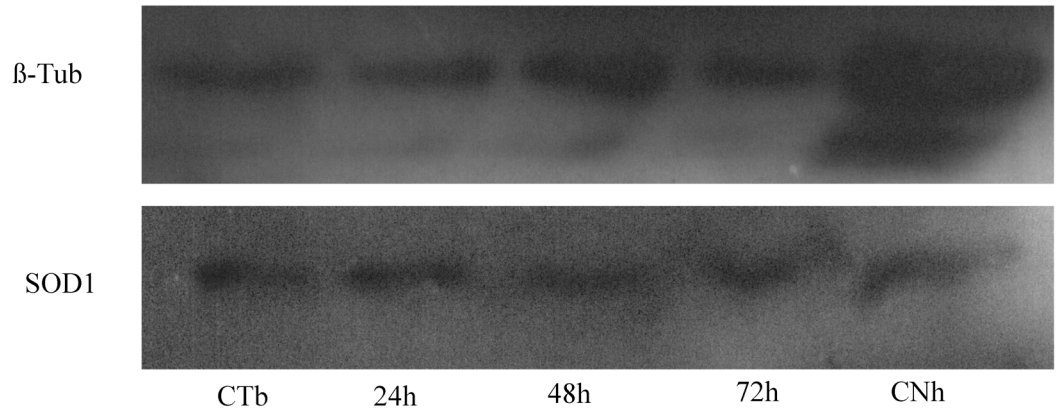

Figure 7.

The results from Western blot of SCR transfected samples. β-tubulin was used as a loading control.

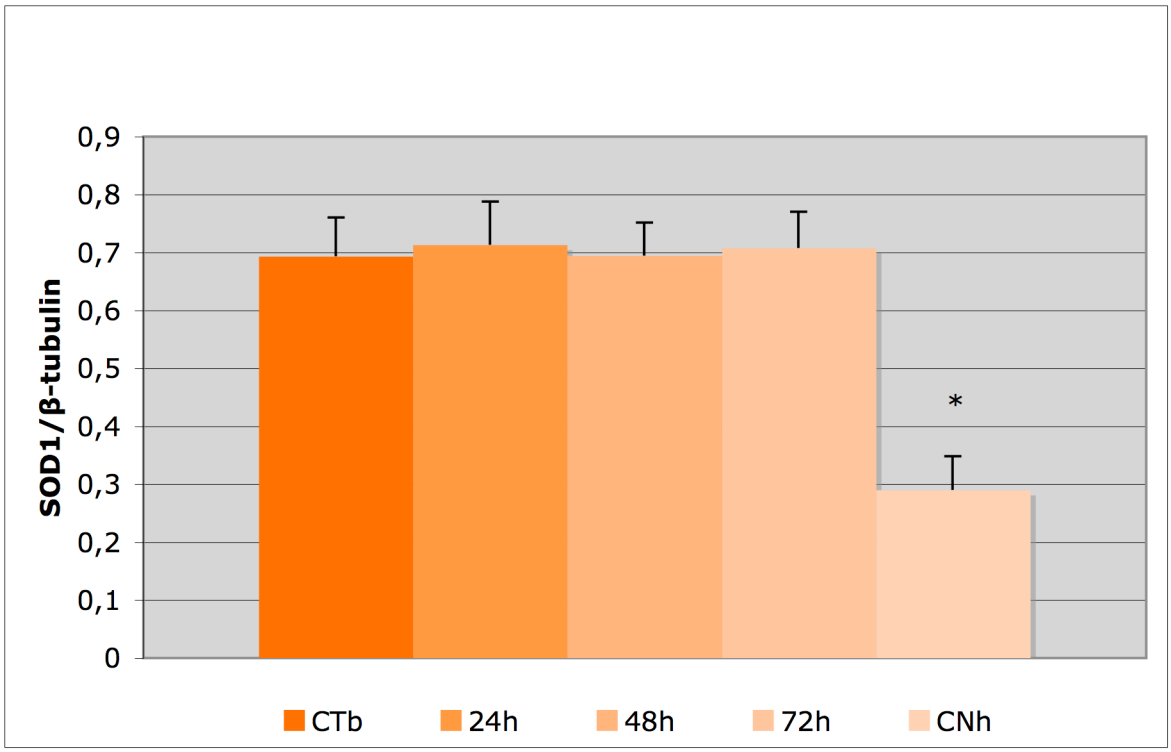

Figure 8.

Effect of transfection with SCR on the expression of SOD1. β-tubulin was used as a loading control to normalize the results between samples. Values are mean ± SEM (n=3) SOD1/β-tubulin. Significant difference (Dunnett's test: $p < 0.05$) compared to non-transfected CTb is indicated by *

4.2 ^{3}[H]-Choline Uptake

After one minute of incubation with ^{3}[H]-choline the choline uptake of non-transfected CTb cells was 41.5% of the level detected in the non-trisomic CNh cells. Statistically, there was no change at all at 24, 48 or 72 hr post-transfection with AS (one-way ANOVA: F_4=15.85; $p < 0.0001$; figure 9), and the difference between CNh and CTb cells in all conditions remained significant (Dunnett's test: $p < 0.01$; figure 9).

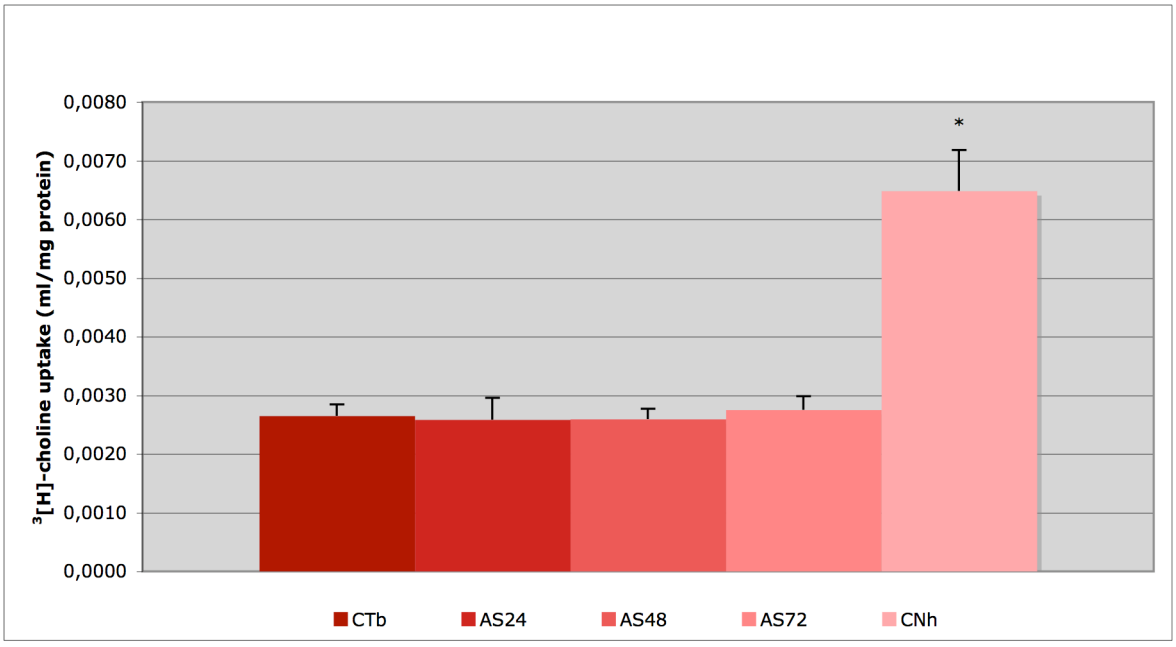

Figure 9.

Uptake of ^{3}[H]-choline in controls (CTb and CNh) and CTb cells transfected with AS after incubation with 1 µCi ^{3}[H]-choline for 1 min. Values are mean ± SEM (n=5 for AS48; n=6 for other samples). *Indicates a significant difference (Dunnett's test: $p < 0.01$) compared to non-transfected CTb cells.

After two minutes of incubation (figure 10) with ^{3}[H]-choline the choline uptake of non-transfected CTb cells was 36.1% of the level detected in the non-trisomic CNh cells. At 24 and 48 hr post-transfection with AS there was again no change, but at 72 hr post-transfection the choline uptake of the AS-transfected CTb cells increased to 44.6% of that of CNh cells. A one-way ANOVA rejected the equality of the means of the distributions

(F_4=67.05; p<0.0001; figure 10) and Dunnett's test showed that the difference between CNh and CTb in all conditions remained significant (p < 0.01; figure 10).

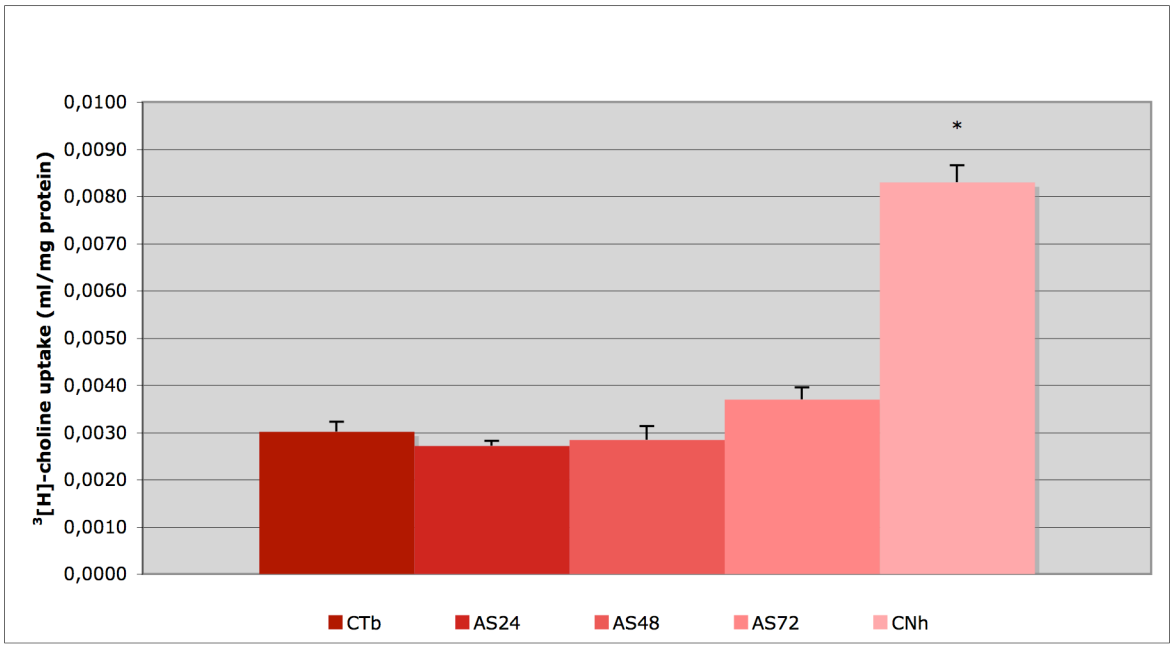

Figure 10.

Uptake of ^{3}[H]-choline in controls (CTb and CNh) and CTb cells transfected with AS after incubation with 1 µCi ^{3}[H]-choline for 2 min. Values are mean ± SEM (n=5 for CTb; n=6 for other samples). *Indicates a significant difference (Dunnett's test: p < 0.01) compared to non-transfected CTb cells.

After five minutes of incubation (figure 11) with ^{3}[H]-choline the choline uptake of non-transfected CTb cells was 41.5% of the level detected in the non-trisomic CNh cells. The choline uptake of AS-transfected CTb cells was 40.7%, 46.3% and 59.3% of the level detected in CNh cells at 24, 48 and 72 hr post-transfection, respectively. A one way ANOVA rejected the equality of the means of the distributions (F_4=21.05; p < 0.0001; figure 11) and Dunnett's test showed that the difference between CNh and CTb in all conditions remained significant (p < 0.01; figure 11).

A relevant negative result was obtained from the data, showing that the knockdown of SOD1 alone does not normalize the uptake of choline in the trisomic CTb cells.

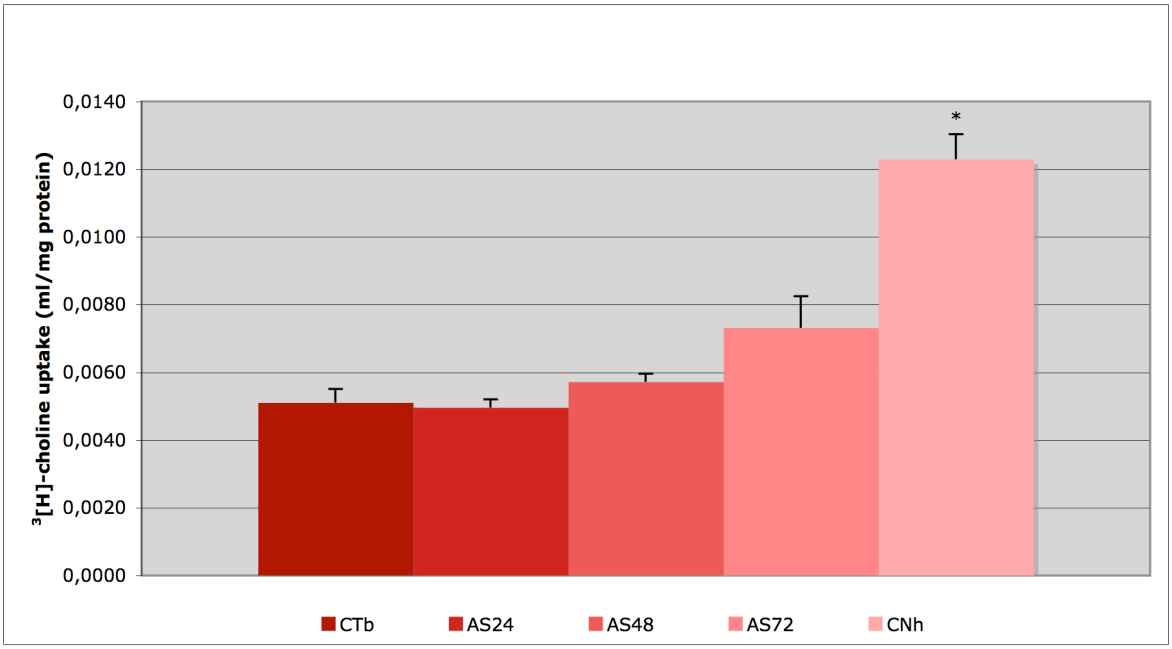

Figure 11.

Uptake of ^{3}[H]-choline in controls (CTb and CNh) and CTb cells transfected with AS after incubation with 1 µCi ^{3}[H]-choline for 5 min. Values are mean ± SEM (n=5 for AS24; n=6 for other samples). *Indicates a significant difference (Dunnett's test: $p < 0.01$) compared to non-transfected CTb cells.

The choline uptake experiment was also carried out with cells transfected with SCR. The data (not shown) obtained for 24 and 48 hr post-transfection showed no effect from transfection with SCR on ^{3}[H]-choline uptake (Dunnett's test: $p < 0.05$). No conclusive data was obtained for 72 hr post-transfection.

4.3 Fractional Release of Acetylcholine

The preliminary acetylcholine release experiments (data not shown) did not yield sufficient data to be statistically valid, but they indicate that the knockdown of SOD1 does not affect the fractional release of acetylcholine in trisomic CTb cells. Further repetitions of the experiment should be carried out to verify this.

Recent results (unpublished), from experiments carried out in the laboratory of Dr. Pablo Caviedes by Ignacio Diaz Franulič in the University of Chile after the work for this

thesis was done, have verified that the knockdown of SOD1 indeed had no significant effect on the fractional release of ACh in CTb cells (Mikkola et al., 2008).

4.4 Summary

The analysis, via Western Blot, of the expression of Cu/Zn superoxide dismutase SOD1 in the trisomic CTb cells shows a clear overexpression, approximately 88% higher, compared to the normal CNh cells.

The results show that the knockdown of SOD1, using phosphorothioate mRNA oligonucleotides with a specific antisense sequence, was successful. The levels of SOD1 expression were reduced to normal levels (such as expressed in CNh cells) at approximately 72 hr post-transfection with the AS oligonucleotides.

The results indicate that both the trisomic CTb and normal CNh cells take up choline and metabolize it into acetylcholine, which is released from the cells constantly at a basal level and in larger amounts in response to depolarization with K^+ or stimulation with glutamate or nicotine. The uptake of ^{3}[H]-choline used in the experiments was significantly lower in CTb cells, only 41.5% of the normal uptake seen in CNh cells.

The knockdown of SOD1 did not cause significant changes in the uptake of ^{3}[H]-choline in CTb cells at 24, 48 or 72 hr post-transfection, as shown in figures 9-11. The difference remains significant between CNh and CTb in all conditions (Dunnett's test: $p < 0.01$; figures 9-11), indicating that the overexpression of SOD1 is not by itself responsible for the cholinergic dysfunction seen in Ts16 and DS.

5 Discussion

Although the results from the cholinergic experiments were negative, they remain relevant, and the results of the study as a whole lead to several conclusions. Some propositions can also be made as to how the results and the reasons potentially underlying them could be further explored in the future.

5.1 Knockdown of SOD1

The relatively slow rate of decrease in the amount of SOD1 present in the transfected cells is readily explained by the stability of the protein (discussed in chapter 1.4). This is also the apparent reason why the difference between SOD1 expression in non-transfected CTb cells and transfected CTb cells 24 hr post-transfection appears to be very small (figure 6). In comparison, Opazo et al. (2006) have reported that it took approximately 48 hr for APP expression to reach normal levels in CTb cells after transfection with antisense oligonucleotides. The expression of APP was also significantly reduced already at 24 hr post-transfection.

The successful knockdown also verifies that the use of cationic liposomes (Lipofectamine reagent, Invitrogen, USA) is an effective method in inducing endocytosis of oligonucleotide sequences in CTb cells, as has been previously described by Opazo et al. (2006).

5.2 Cholinergic experiments

The results from CTb and CNh cell lines are in agreement with the initial studies using these cell lines by Allen et al. (2000), and also indicate that the cell lines continue to retain cholinergic function. Likewise, the results support the findings of Fiedler et al. (1994), who demonstrated a 65% reduction in choline uptake in primary cultures of brain tissue from Ts16 foetuses compared to similar primary culturues from normal mouse foetuses.

Since the knockdown of SOD1 had no significant effect on the uptake of ^{3}[H]-choline in CTb cells, it appears that either lipid peroxidation in the cell membrane or direct damage resulting from oxidative stress are not sufficient to have an adverse effect on the function of the high affinity ChT, or the damage caused cannot be immediately repaired in the cells after normalization of SOD1 expression. In either case, SOD1 alone is not a suitable target for the normalization of choline uptake in Ts16.

The results obtained from this study agree with the findings from electrophysiological studies carried out by Ault et al. (1989), using murine neuronal cells transgenic for human SOD1, which showed no significant changes in electrical properties between controls and the transgenic cells containing several extra copies of the SOD1 gene.

It remains possible, however, that SOD1 overexpression has a combinatory effect with other genes that are triplicated in Ts16 and Ts21. APP, in particular, contributes to the oxidative stress, resulting from elevated levels of hydrogen peroxide and hydroxyl radicals caused by SOD1 overexpression, by reducing antioxidant formation (Takahashi et al., 2000), and may thus contribute to a slow rate of recovery from oxidative stress even after normalization of SOD1 expression.

The findings of Opazo et al. (2006), which show that the normalization of APP expression, using a knockdown protocol, had a significant normalizing effect on the uptake of choline and the release of acetylcholine in CTb cells, support the results from both of the cholinergic experiments. It would have seemed strange if SOD1 and APP, so profoundly different in form and function, had a very similar effect on these cholinergic functions. The possibility of a cooperative effect by the two still remains, however, and should be investigated further.

5.3 Errors

To obtain more reliable and accurate statistical data further repetitions of each phase (knockdown and its analysis through Western blot, ^{3}[H]-choline uptake, fractional acetylcholine release) of this study should be carried out.

As mentioned in chapter 4.2, no conclusive data was obtained from the ^{3}[H]-choline uptake experiment for 72h post-transfection with SCR. This was because the aliquotes taken from some samples for measuring protein content were contaminated and yielded protein counts far off the scale of the other samples, resulting in a number of processable samples too low to be statistically acceptable. For the acetylcholine release experiment too few repetitions were originally carried out to yield a statistically valid amount of data.

The main reason why these shortcomings could not be overcome in the scope of this thesis was the limited time to carry out the experiments at the University of Chile, of which a great part went into the optimization of the various protocols, particularly the Western blot protocol, that were used.

5.4 Conclusions

The results of this study show that the expression of SOD1 in trisomic cells can be returned to normal levels using a knockdown protocol. However, the normalization of SOD1 expression alone appears to have no significant effect on the cholinergic function, neither the uptake of choline nor the release of acetylcholine, of the neuronal trisomic CTb cells.

Although the overexpression of SOD1 has been shown to have several adverse effects, in light of this study it alone seems an unattractive target for the development of therapeutic agents against the cholinergic symptoms of DS. The role of SOD1 overexpression in the manifestation of the DS phenotype may be cooperative with some of the other genes that are triplicated in the aneuploidic condition, or it may have other effects by itself, such as the structural abnormalities demonstrated in mouse neuromuscular junctions by Avraham et al. (1988).

The effects of the combined overexpression of SOD1 and APP, both genes being overexpressed in DS as well as in Ts16, have been researched to a degree, with results indicating that more severe symptoms develop when they are overexpressed together than if only one of them is overexpressed (Harris-Cerruti et al., 2004). It would seem a logical continuation to this study to induce a simultaneous knockdown of these two genes in CTb

cells (as the knockdown effects of both have now been studied individually) to see what kind of results, in regard to cholinergic function, the combined normalization of their expression levels would produce.

Another possible way to further investigate the results of the present study would involve monitoring the level of oxidative stress during and after the knockdown of SOD1, using, for example, malondialdehyde as a marker of lipid peroxidation (Casado et al., 2007). The findings from such a study might reveal whether the cholinergic dysfunctions monitored in Ts16 and Ts21 can be attributed to oxidative stress.

The successful knockdown of SOD1 and the results obtained from this study show that gene knockdown remains a valuable tool in the study of DS-related dysfunction using cellular models such as CTb, and may contribute to the discovery of potential targets for therapeutic agents.

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7 Appendices

7.1 Solution formulas

PBS pH 7.4	**mM**
NaCl	130
KCl	5.4
KH_2PO_4	0.22
Na_2HPO_4	0.14

Solution D pH 7.2	**mM**
NaCl	130
Glucose	6
Sucrose	6
KCl	5.4
KH_2PO_4	0.22
Na_2HPO_4	0.14

RIPA lysis buffer	**mM**
EDTA	250
NaCl	150
Tris-base	100
Na-deoxycholate	24
Nonidet P40	1% (v/v)
Sodium dodecyl sulfate (SDS)	0.1% (w/v)

SDS-PAGE running buffer	
Glycine	14.4 g/l
Tris-HCl	3 g/l
10% (w/v) Sodium dodecyl sulfate (SDS)	8 ml/l

SDS-PAGE loading buffer	
Glycerol	20% (v/v)
β-mercaptoethanol	10% (v/v)
Sodium dodecyl sulfate (SDS)	6% (w/v)
Tris-HCl	250 mM
Bromophenol blue	1.5 mg/ml

Transference buffer	
SDS-PAGE running buffer	80% (v/v)
Methanol	20% (v/v)

Blocking solution (in PBS)	
Bovine serum albumin (BSA)	5% (w/v)
Tween-20	0.5% (v/v)

Krebs solution pH 7.4	**mM**
NaCl	120
HEPES	20
Glucose	10
KCl	5
$CaCl_2$	2.7
$MgSO_4$	1.2
Na_2HPO_4	1