Brief Communication

App Gene Dosage Modulates Endosomal Abnormalities of Alzheimer’s Disease in a Segmental Trisomy 16 Mouse Model of Down Syndrome

Anne M. Cataldo,1,2,3 Suzana Petanceska,1 Corrinne M. Peterhoff,1 Nicole B. Terio,1 Charles J. Epstein,4 Angela Villar,4 Elaine J. Carlson,3 Matthias Staufenbiel,6 and Ralph A. Nixon1,5,7

1Mailman Research Center, McLean Hospital, Belmont, Massachusetts 02478, 2Department of Psychiatry, Harvard Medical School, Boston, Massachusetts 02115, 3Center for Dementia Research, Nathan Kline Institute, Orangeburg, New York 10962, 4Department of Pediatrics, University of California, San Francisco, San Francisco, California 94143, 5Genomics Core Facility, University of California, San Francisco, San Francisco, California 94143, 6Novartis Institute of Biomedical Research, Nervous System, CH-4002 Basel, Switzerland, and 7Departments of Psychiatry and Cell Biology, New York University School of Medicine, New York, New York 10016

Altered neuronal endocytosis is the earliest known pathology in sporadic Alzheimer’s disease (AD) and Down syndrome (DS) brain and has been linked to increased Aβ production. Here, we show that a genetic model of DS (trisomy 21), the segmental trisomy 16 mouse Ts65Dn, develops enlarged neuronal early endosomes, increased immunoreactivity for markers of endosome fusion (rab5, early endosomal antigen 1, and rabaptin5), and endosome recycling (rab4) similar to those in AD and DS individuals. These abnormalities are most prominent in neurons of the basal forebrain, which later develop aging-related atrophy and degenerative changes, as in AD and DS. We also show that App, one of the triplicated genes in Ts65Dn mice and human DS, is critical to the development of these endocytic abnormalities. Selectively deleting one copy of App or a small portion of the chromosome 16 segment containing App from Ts65Dn mice eliminated the endosomal phenotype. Overexpressing App at high levels in mice did not alter early endosomes, implying that one or more additional genes on the triplicated segment of chromosome 16 are also required for the Ts65Dn endosomal phenotype. These results identify an essential role for App gene triplication in causing AD-related endosomal abnormalities and further establish the pathogenic significance of endosomal dysfunction in AD.

Key words: endosomes; endocytic pathway; Ts65Dn; Ts1Cje; Down syndrome; Alzheimer’s disease; amyloid precursor protein; basal forebrain neurons

Introduction

Early endosomes of the endocytic pathway are the first intracellular, protease-rich sites involved in the internalization, recycling, and catabolic modulation of various macromolecules required for the normal maintenance of cells. The importance of the endocytic pathway to Alzheimer’s disease (AD) pathogenesis is amply evidenced by its critical role in the processing and function of various proteins relevant to AD, including amyloid precursor protein (APP), amyloid β (Aβ) peptide, apolipoprotein E (ApoE), low-density lipoprotein, and low-density lipoprotein receptor-related protein (LRP), and by the localization of APP secretases or their activities, at least in part, within endosomes (Vassar et al., 1999; Huse et al., 2000).

Early endosomal alterations are the earliest known pathology in sporadic AD (SAD) and Down syndrome (DS), appearing in DS before birth, and in SAD, developing before β-amyloid is deposited and as soluble Aβ peptide levels first rise. Endosomal abnormalities, which are influenced by ApoE genotype (Cataldo et al., 1997), do not develop in familial AD caused by presenilin mutations (Cataldo et al., 2001), underscoring that they are AD subtype-specific and are also not a response to amyloid deposition. Similar endosomal abnormalities, when modeled in cells, are associated with increased Aβ production (Grbovic et al., 2002). Endosomal alterations in AD and DS develop in otherwise normal-appearing neurons of regions that become the most severely affected in the disease (Cataldo et al., 1997, 2000a), including hippocampus, neocortex, and basal forebrain. In light of the suspected pathogenic importance of endosomal pathology in AD, an animal model that reproduces these cellular alterations would be valuable.

Two segmental trisomy mouse models of DS, Ts65Dn (Davisson et al., 1990), and Ts1Cje (Sago et al., 1998) mice, survive to adulthood and exhibit a number of the morphological, biochemical, and transcriptional changes seen in the human disease (Davisson et al., 1990; Reeves et al., 1995; Holtzman et al., 1996). These animals possess three copies of the segment of mouse chromosome 16 (MMU16) orthologous to the critical region of human chromosome 21 (HSA21) thought to be responsible for the
phenotype of DS. Ts65Dn mice with a segmental trisomy extending from App to Mx1 exhibit behavioral and cognitive abnormalities not unlike some of the abnormalities seen in individuals with DS (Reeves et al., 1995). Ts65Dn mice also exhibit age-related atrophy and neurodegeneration of basal forebrain cholinergic neurons (BFCNs) and extensive astrocytic hypertrophy resembling these aspects of the neuropathology in AD and DS (Holtzman et al., 1996; Cooper et al., 2001). Unlike what occurs in human disorders, however, Ts65Dn mice do not deposit β-amyloid. Ts1Cje mice are genetically similar to Ts65Dn, except that the region from App to Sod1 is not triplicated. These mice do not develop BFCN atrophy and degeneration, and they have less severe learning and behavioral deficits than Ts65Dn mice (Sago et al., 1998).

In the present study, we established that Ts65Dn mice develop aging-related endosomal enlargement and altered expression and distribution of early endosome markers, which strongly resemble the neuronal endosomal pathology in SAD and DS. In addition, we investigated the role of App in the development of endosomal pathology using two additional types of trisomic mice, Ts1Cje and Ts65Dn–App+/−/−, both of which possess only two copies of the App gene. These mice were also compared with transgenic mice expressing high levels of human mutated APP670/671 (Swedish mutation) or APP670/671 plus APP717 (London mutation). We demonstrate here that endosomal pathology in Ts65Dn mice is dependent on triplication of the App gene and that the ability of App to alter endosome function requires the participation of one or more genes within a small trisomic region of MMU16. The influence on neuronal endocytic function of App and ApoE, two genes that modify risk for AD, provides strong support for the view that the very early-appearing abnormalities of endosomes in AD and DS have pathogenic significance.

Materials and Methods

Mice. Three segmental trisomy mice were used in this study. All mice were age-matched and housed with 2N (diploid) littermates of the same sex. Ts65Dn mice (n = 6), which carry an extra copy of the distal end of MMU16 proximal to the App gene to Mx1 (Davidson et al., 1990), were maintained on a B6C3H background and identified by karyotyping. Ts1Cje mice (n = 4), which are trisomic for the segment of MMU16 spanning from Sod1 to Mx1, were identified by neo–PCR and fluorescence in situ hybridization (Sago et al., 1998). App–mutant mice (App−/−) were generated and maintained on a C57BL/6J background (Zheng et al., 1995) and mated with Ts65Dn mice to generate Ts65Dn–App−/+ and Ts65Dn–App−/− mice expressing two copies of the murine App gene (n = 6). Transgenic mouse lines that express the Swedish double mutation of App (K670M/N671L) (n = 6) or the Swedish mutation plus the London mutation of human APP751 (V717I) were generated using transgenic constructs that contain human or murine Thy-1 expression cassettes and human APP751 cDNAs, and mutant APP expression was confirmed as described previously (Sturchler-Pierrat et al., 1997). The presence of extracellular β-amyloid deposition was identified using thioflavin S. Transgenic mice carrying both human mutant APP and presenilin 1 (n = 6) transgenes were generated as described previously (Holcomb et al., 1998), and animals of both sexes ranging in age from 6 to 12 months were used in this study. The presence of transgene mRNAs was confirmed by PCR, and the presence of AD-like neuropathology was verified using thioflavin S histofluorescence.

Tissue. Trisomic, transgenic, and normal 2N controls were fixed by transcardiac perfusion with aldehydes. Vibratome sections 30–40 μm thick that included regions of the medial septal nucleus (MSN), nucleus basalis magnocellularis (NBMB), hippocampus, neocortex, basal ganglia, and cerebellum were collected from each animal.

Antibodies and immunocytochemistry. Immunocytochemical studies were performed as described previously (Cataldo et al., 1997, 2000a) using commercial antibodies to human rab5, rab4, and rabaptin5 and a purified polyclonal antibody raised against anti-early endosomal antigen 1 (EEA1) (Dr. S. Corvera, University of Massachusetts Medical School, Worcester, MA).

Western blot analysis. Brain hemispheres were homogenized in diethylamine (DEA)/50 mM NaCl at 1:10 w/v ratio and neutralized to pH 8.0. The pellets of the DEA extracts were solubilized in a 2% SDS–PAGE mixture of protease inhibitors, sonicated, and boiled. Equal amounts of protein were sized by SDS–PAGE, and membranes were immunoblotted using anti-human rab4 (Cataldo et al., 2000a).

Results

AD-like early endosomal abnormalities in neurons of Ts65Dn mice

To investigate the presence of endosome alterations in the Ts65Dn mouse, we examined brain tissue from trisomic and 2N control mice by immunocytochemistry with rab5, a specific marker for early endosomes. Neurons from Ts65Dn mice displayed rab5-positive early endosomes of abnormally large sizes (Fig. 1), which were similar morphologically to those seen in human DS brain as early as 2 months of age. In young Ts65Dn mice, these enlarged endosomes were prominent in a majority of the neurons in the MSN of the basal forebrain. Early endosomes in the same neuronal populations appeared as the typical, small, and uniform vesicular compartments in 2N littermate control mice (Fig. 1).

To assess the functional significance of the endosomal enlargement in Ts65Dn mice, we examined the cellular expression of two known markers of endocytic function: rabaptin5 and EEA1. These proteins are recruited to early endosomal membranes through interactions with the GTP-active form of rab5 and modulate early endosomal docking and fusion (Stenmark et al., 1995; Gournier et al., 1998). Using antibodies to rabaptin5 and EEA1, we found that the pattern of rabaptin5 and EEA1 in neurons from Ts65Dn mice resembled that of rab5, confirming the identity of the enlarged neuronal vacuolar profiles as early endosomes (Fig. 2) and implying that functionally, endosomal uptake and fusion were increased. As expected, the levels of rabaptin5 and EEA1 immunoreactivities were higher in Ts65Dn mice than in age-matched littermate controls (Fig. 2).

Because the GTPase rab4 plays a functional role complementary to rab5 in directing the recycling of internalized membrane back to the cell surface (van der Sluijs et al., 1992), we also determined the levels and distribution of rab4 immunoreactivity in the MSN, NBM, neocortex and hippocampus from Ts65Dn and control mice. Rab4 immunoreactivity was principally associated with small vesicular profiles consistent with the size and location of recycling vesicles. We found a qualitative increase of rab4 immunolabeling in Ts65Dn mice, which was most prominent in neurons of the septohippocampal system and neocortex of Ts65Dn mice compared with age-matched 2N controls (Fig. 2).
Gene dosage modulates early endosomal morphology in Ts65Dn mice. Neurons of the cingulate cortex of a 6-month-old Ts65Dn mouse labeled with rab5 (B) and rabaptin5 (D) show increased immunoreactivity (arrows) and enlarged endosomes (D, inset, arrow) compared with an age-matched 2N control mouse (A, C, C, inset, arrows). Serial adjacent sections immunolabeled with rab4 show increased immunoreactivity (E, arrow) in neurons of trisomic mice compared with controls (E, arrow), which is consistent with increased endosomal reflux to the plasma membrane. Western blot analysis (G) of whole-brain homogenates (50 μg per lane) prepared from 2N control mice (n = 4; lanes 1–4) and Ts65Dn mice (n = 4; lanes 5–8) confirm the immunocytochemical findings and revealed an increase in rab4-immunoreactive protein (M, ~25–28) in the Ts65Dn mouse brains.

Because of the importance of aging as a risk factor for AD, we evaluated the effects of aging on the neuronal endocytic pathway by examining Ts65Dn mice and 2N littermate controls at 6, 12, and 18 months of age. Immunocytochemistry showed that by 6 months of age, swollen, rab5-positive endosomes were present in most basal forebrain neurons of the MSN as well as the NBM. Endosomal enlargement was detected in fewer neurons within the amygdala, cingulate cortex, and hippocampus from Ts65Dn mice. The numbers of these neurons in these regions containing abnormally large endosomes increased in Ts65Dn mice 12 months of age and older, although the magnitude of enlargement did not appear to differ from that seen in the young, 2-month-old mice. Not all neuronal populations in the Ts65Dn mice exhibited enlarged endosomes; neurons in the caudate nucleus and putamen (Fig. 3) or in cerebellar Purkinje cells displayed normal-sized early endosomes.

**App gene dosage modulates early endosomal morphology in Ts65Dn mice**

Because of the importance of the **App** gene in AD pathogenesis, we investigated whether changes of the endocytic pathway detected in Ts65Dn were dependent on **App** gene dosage. We compared brain tissue from Ts65Dn mice with that from two different trisomic mouse strains. Rab5-positive endosomes (Fig. 4) and patterns of immunolabeling for the other endosomal markers EEA1, rab4, and rabaptin5, similar to those in 2N controls. Rab5 immunolabeling in neurons is associated with the typical small early endosomal profiles located in close proximity to the cell surface (left inset, arrows). IC, Internal capsule. A, Age-related endosomal alterations represented by enlarged rab5-positive endosomes and elevated levels of rab5 immunolabeling are apparent in neurons of the MSN as well as other regions of the septohippocampal system, including related populations in the neocortex and hippocampus and the NBM.

Figure 2. Endocytic uptake and recycling are increased in neurons of Ts65Dn mice. Neurons of the cingulate cortex of a 6-month-old Ts65Dn mouse labeled with rab5 (B) and rabaptin5 (D) show increased immunoreactivity (arrows) and enlarged endosomes (D, inset, arrow) compared with an age-matched 2N control mouse (A, C, C, inset, arrows). Serial adjacent sections immunolabeled with rab4 show increased immunoreactivity (E, arrow) in neurons of trisomic mice compared with controls (E, arrow), which is consistent with increased endosomal reflux to the plasma membrane. Western blot analysis (G) of whole-brain homogenates (50 μg per lane) prepared from 2N control mice (n = 4; lanes 1–4) and Ts65Dn mice (n = 4; lanes 5–8) confirm the immunocytochemical findings and revealed an increase in rab4-immunoreactive protein (M, ~25–28) in the Ts65Dn mouse brains.

Figure 3. Endocytic abnormalities target neurons of the septohippocampal system. A. In tissue sections from 2-month-old Ts65Dn mice, endocytic abnormalities are most prominent in neurons of the medial septal nucleus (MSN). Increased levels of rab5 immunoreactivity and atypically large rab5-positive endosomes (right inset, arrows) are detected in most neurons in this region, which is composed primarily of cholinergic neurons that undergo age-related neurodegeneration in Ts65Dn mice. In neurons of the basal ganglia (BG), in contrast, rab5 immunolabeling in neurons is associated with the typical small early endosomal profiles located in close proximity to the cell surface (left inset, arrows). IC, Internal capsule. B, Age-related endosomal alterations represented by enlarged rab5-positive endosomes and elevated levels of rab5 immunolabeling are apparent in neurons of the MSN as well as other regions of the septohippocampal system, including related populations in the neocortex and hippocampus and the NBM.

App gene dosage modulates early endosomal morphology in Ts65Dn mice

Because of the importance of the **App** gene in AD pathogenesis, we investigated whether changes of the endocytic pathway detected in Ts65Dn were dependent on **App** gene dosage. We compared brain tissue from Ts65Dn mice with that from two different trisomic mouse strains that carry the normal two copies of the **App** gene. The first, the Ts1Cje mouse, lacks the trisomic segment and expression of the **App** gene. The second, a sevenfold overexpression of the Swedish mutation of **App** in transgenic mice had normal-appearing vesicular rab5-positive early endosomes (Fig. 4) and patterns of immunolabeling for the other endosomal markers EEA1, rab4, and rabaptin5, similar to those in 2N controls.

**Discussion**

We identified previously morphological abnormalities in AD and DS brain consistent with increased endocytic pathway activation,
including increased volumes of early endosomes, a well established morphological event associated with increased endocytosis (Bucci et al., 1992), and elevated expression of proteins that regulate endocytosis (rab5, rabaptin5, EEA1) and recycling (rab4) (Cataldo et al., 1997, 2000a). We also showed that endosomal pathology in DS occurs as early as 28 weeks gestation, decades before β-amyloid is deposited in significant amounts (Cataldo et al., 2000a). In AD, the appearance of abnormal endosomes coincides with rises in soluble Aβ levels and the detection of Aβ immunoreactivity intraneuronally (Cataldo et al., 2000b). In this study, we observed that Ts65Dn mice also develop aging-related enlargement of rab5-positive endosomes, which immunolabel more strongly with antibodies to two other effectors of endocytosis, rabaptin5 and EEA1, consistent with the conclusion that endocytic uptake and subsequent endosomal docking and fusion are increased in these neurons. A compensatory rise in levels of the GTPase rab4, which stimulates endosome recycling to the plasmalemma, was also detected in the same neuronal groups. These similarities to DS and AD establish Ts65Dn mice as a useful model to study the nature and pathophysiological consequences of endosome dysfunction, the earliest known cellular pathology in AD (Cataldo et al., 1997, 2000a, 2001).

We have shown that the development of early endosomal abnormalities in Ts65Dn mice is dependent on App gene triplication. Ts1Cje and Ts1Dn65—App<sup>+/−</sup> mice, which have two copies of App, showed normal endosomal morphology in the basal forebrain in contrast to Ts65Dn mice. These same populations of basal forebrain neurons typically develop less severe atrophy in the Ts1Cje (Sago et al., 1998) and Ts65Dn—App<sup>++</sup> mice (our unpublished observation) or older ages compared with that occurring in age-matched Ts65Dn mice. These findings are consistent with observations that endosomal abnormalities in DS develop in some neurons before birth, which links this phenomenon with dosage of particular genes on the triplicated segment of chromosome 21. It is relevant, in this regard, that another major genetic risk factor for AD, the ApoE4 allele, also accelerates the onset of endosome pathology in AD (Corder et al., 1993). ApoE, its receptor on neurons (LRP), and one of its ligands, cholesterol, which are trafficked through endosomes at increased rates during neuronal injury, have each been implicated in AD pathogenesis. Given the importance of the App gene to AD pathogenesis and the high disease specificity of neuronal endocytic pathology, our observation that App triplication is required for the endosomal phenotype in the mouse strongly supports the view that endocytic pathway dysfunction is a key pathogenic event in AD.

Although increased App gene dosage is necessary for endosome pathology to develop in Ts65Dn mice, it is not sufficient, because overexpressing APP alone did not cause endosome pathology or atrophy of basal forebrain neurons. These findings imply that one or more genes in the trisomic portion of MMU16 are also necessary. Of the ~150 genes on MMU16 that are syntenic with HSA21, 60% are expressed in brain, and most of these are present in postmitotic cells. Among these genes are several with potential relevance to AD. Oxidative stress is a possible contributory factor in DS and AD pathogenesis, and decreased viability of fetal DS neurons in culture has been linked to increased reactive oxygen species (Busciglio and Yankner, 1995). In this regard, Sod1 triplication may be contributory on the basis of evidence that increased Sod1 expression in DS occurs in the absence of a compensatory increase in catalase or glutathione peroxidase leading to an accumulation of hydrogen peroxide or free hydroxyl radical, both of which are cytotoxic. β-site APP cleaving enzyme (BACE)-2 is a transmembrane aspartic protease that is related to the major β-secretase BACE-1, an AD-relevant protein located in endosomes. Immunocytochemical studies of brain tissue from DS subjects have shown a direct correlation between the appearance of elevated levels of BACE-2 in brain and the presence of AD-like neuropathology (Motonaga et al., 2002). S100B is another triplicated gene on HSA21 that encodes a small acidic calcium-binding protein synthesized and released by astrocytes (Allore et al., 1988). S100B expression is increased as early as 17 weeks gestation in DS brain (Griffin et al., 1989, 1998), and it is believed to contribute to APP overexpression (Griffin et al., 1989, 1998), dendritic abnormalities, dystrophic neurites, and apoptosis (Sheng et al., 1997). Cholinergic neurons have been shown to be particularly vulnerable to the cytotoxic effects of inflammatory cytokines, including interleukins and S100B by astrocytes (Wenk and Willard, 1998). Finally, the ApoE gene, which is associated with increased risk of AD (Corder et al., 1993), is not located on the triplicated segment of MMU16, but it is overexpressed to higher levels in Ts65Dn mice than is predicted on the basis of gene dosage (Holtzman et al., 1996). It is possible that genes not on the trisomic segment could interact with App to promote endocytic dysfunction in Ts65Dn mouse, as ApoE4 does in AD.

Our findings show that Ts65Dn mice develop neuronal endosome abnormalities strongly resembling the highly AD-specific endocytic alterations that develop at the earliest stages of AD. Moreover, similar endosome alterations modeled in cells by overexpressing rab5 increase Aβ peptide generation (Grbovic et
al., 2002), which may account for the disproportionate elevation of Ab levels compared with APP expression in Ts65Dn mice. As seen in neurons from infants and fetuses with trisomy 21 (Cataldo et al., 2000b), we also found intraneuronal Ab within rab5-positive vesicular compartments of neurons from Ts65Dn mice. Although Ts65Dn mice (even at 24 months of age) do not exhibit extracellular Ab-amyloid-containing plaques (Holtzman et al., 1996) or neurofibrillary tangles, this late-stage AD pathology develops in mice only when human APP or tau in mutant form is expressed at high levels. This suggests differential effects of factors such as aging or diet among species on disease phenotype in the advanced stages. Despite the lack of development of late-stage pathology in Ts65Dn mice, the association between endosomal disturbances and basal forebrain neuronal atrophy and its close relationship to App gene dosage support the use of Ts65Dn mice as a valuable model in which to investigate the key early events of AD pathogenesis.

References
