Title: Trisomy of Human chromosome 21 enhances Aβ deposition independently of an extra copy of APP

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Abbreviations: amyloid-β (Aβ), full-length APP (FL-APP)
Abstract

Down syndrome, caused by trisomy of chromosome 21, is the single most common risk factor for early-onset Alzheimer’s disease. Worldwide approximately six million people have Down syndrome, and all these individuals will develop the hallmark amyloid plaques and neurofibrillary tangles of Alzheimer’s disease by the age of forty and the vast majority will go on to develop dementia. Triplication of \textit{APP}, a gene on chromosome 21, is sufficient to cause early-onset Alzheimer’s disease in the absence of Down syndrome. However, whether triplication of other chromosome 21 genes influences disease pathogenesis in the context of Down syndrome is unclear. Here we show, in a mouse model, that triplication of chromosome 21 genes other than \textit{APP} increases amyloid-β aggregation, deposition of amyloid-β plaques and worsens associated cognitive deficits. This indicates that triplication of chromosome 21 genes other than \textit{APP} is likely to have an important role to play in Alzheimer’s disease pathogenesis in people who have Down syndrome. We go on to show that the effect of trisomy of chromosome 21 on amyloid-β aggregation correlates with an unexpected shift in soluble amyloid-β 40/42 ratio. This alteration in amyloid-β isoform ratio occurs independently of a change in the carboxypeptidase activity of the γ-secretase complex, which cleaves the peptide from APP, or the rate of extracellular clearance of amyloid-β. These new mechanistic insights into the role of triplication of genes on chromosome 21 other than \textit{APP}, in the development of Alzheimer’s disease in people who have Down syndrome may have implications for the treatment of this common cause of neurodegeneration.
Introduction

Approximately 6 million individuals worldwide have Down syndrome. Changes in social attitudes and improvements in medical care have led to a significant rise in life-expectancy for people who have Down syndrome (Glasson et al., 2016). In particular, neonatal survival rates of individuals who have Down syndrome rose dramatically between 1950-1970 (Wu and Morris, 2013), such that today more people with the condition than ever before are approaching late middle age. Down syndrome is the biggest single genetic risk factor for Alzheimer’s disease (Wiseman et al., 2015). A proportion of people with Down syndrome start to accumulate amyloid-β (Aβ) within their brain in childhood (Lemere et al., 1996; Leverenz and Raskind, 1998), and the vast majority of individuals will have accumulated substantial amounts of Aβ by their mid-twenties (Mann, 1988). By the age of 40, people who have Down syndrome will also have universally developed neurofibrillary tangles in a pattern broadly similar to that of Alzheimer’s disease in the general population (Lemere et al., 1996; Leverenz and Raskind, 1998; Mann, 1988). The vast majority of people who have Down syndrome will develop early-onset Alzheimer’s disease dementia, for example, it is estimated that by their sixties, approximately two-thirds of individuals will have clinical dementia (McCarron et al., 2014). The first site of Aβ accumulation in people with Down syndrome is within the cell (Gouras et al., 2000; Gyure et al., 2001; Hirayama et al., 2003; Mori et al., 2002) in the endo-lysosomal system (Cataldo et al., 2004; Cataldo et al., 2000). It is clear that one of the earliest Alzheimer’s disease-associated pathological changes in people who have Down syndrome is the accumulation of Aβ. Understanding the genetic factors that influence Aβ accumulation in the context of trisomy of chromosome 21 may assist with the development of novel treatments for Alzheimer’s disease in this important population.
Duplication of APP alone, in the absence of chromosome 21 trisomy, is a cause of early onset Alzheimer’s disease (Rovelet-Lecrux et al., 2006; Sleegers et al., 2006) making it likely that three copies of APP are important in the development of Down syndrome - Alzheimer’s disease. However, the influence of three copies of the other >600 Hsa21 protein-coding and non-coding genetic elements (Ensembl GRCh38.p10) on Alzheimer’s disease in people who have Down syndrome is largely not understood.

Here, we report for the first time that triplication of chromosome 21 genes other than APP, increases Aβ aggregation, plaque formation, and cognitive deficits in a novel Down syndrome - Alzheimer’s disease (Aβ deposition) mouse model. In our unique model system, we show that trisomy of chromosome 21 lowers the ratio of soluble Aβ40 to Aβ42, a known pro-amyloidogenic change, and that this alteration in the peptide ratio correlates with Aβ aggregation in the brain. Importantly, we show that the effect of trisomy of chromosome 21 on soluble Aβ40/Aβ42 ratio occurs despite unaltered γ-secretase complex carboxypeptidase activity.
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Materials and Methods

Experimental Design

For animal studies end-points were determined by animals’ age, as defined below (cohort A-I). All experiments were undertaken blind to genotype. Genotype was decoded after experimental analysis and reconfirmed using an independent DNA sample isolated from post-mortem tail, apart from instances of sudden death when tissue could not be recovered. Group sizes were calculated using power calculations, based upon estimates of error in the literature. Animals in which a reduction of copy number of the APP transgene was observed at initial genotyping at 3-4-weeks of age were excluded from further analysis. No outliers were excluded from the study. Individual animals were treated as the experimental unit.

Experiments using human post-mortem tissues were undertaken blind to euploid/trisomy status. Samples sizes were determined by the availability of tissues and appropriate matched controls at the suppling brain banks. Individual patients were treated as the experimental unit.

Animal Cohorts

Cohort A (longitudinal behaviour and 16-month old for Aβ deposition studies)

   wildtype n = 29, Trisomic n = 30, tgAPP n = 19, Trisomic;tgAPP n = 17

Cohort B (2-3-month old behaviour and aged to 15-months)

   wildtype n = 17, Trisomic n = 13, tgAPP n = 17, Trisomic;tgAPP n = 9

Cohort C (aged to 15-months, mice used in another study)
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wildtype n = 14, Trisomic n = 11, tgAPP n = 20, Trisomic;tgAPP n = 6

Cohort D (6-month old for Aβ deposition and fractionation studies)

wildtype n = 12, Trisomic n = 12, tgAPP n = 12, Trisomic;tgAPP n = 12

Cohort E (2-month old for Aβ fractionation and multimeric Aβ)

wildtype n = 24, Trisomic n = 22, tgAPP n = 25, Trisomic;tgAPP n = 27

Cohort F (3-month old for RNA, proteins, and biochemical activity assays)

wildtype n = 55, Trisomic n = 43, tgAPP n = 53, Trisomic;tgAPP n = 43

Cohort G (3-month old females for in vivo microdialysis)

tgAPP n = 8, Trisomic;tgAPP n = 6

Cohort H (3-month old for γ-secretase enzymatic assays)

wildtype n = 10, Trisomic n = 10

Cohort I (4.5-6.5-month old for IHC APP and Aβ study)

wildtype n = 10, Trisomic n = 10, tgAPP n = 12, Trisomic;tgAPP n = 10

Statistical Analysis

Data were analysed as indicated in figure legend by either two-tailed students T-test (single variable study) or univariate ANOVA (to control for multiple variables). For ANOVA between-subject factors for mouse work were tgAPP, trisomy, and sex; for experiments using human post-mortem material, age at death, sex, post-mortem interval, and disease/syndrome status. Additional between-subjects factors were included in ANOVA as follows, for study of Aβ (82E1) and APP (22C11) staining on samples aged between 4.5-6.5 months, age in days was included and for study of tris soluble Aβ38, 40 and 42 preparation batch and age in days of mouse were included. Repeated measures ANOVA was used for technical replicates when every
sample had the same number of replicates run, including immunohistology studies where two sections were stained from each mouse, plaque counts made by the two independent scorers, and technical replicates in the γ-secretase carboxypeptidase activity study. For cases when the number of technical replicates varied between subjects, subject means were calculated and used in the ANOVA. For the longitudinal analysis of T-maze alternation (short-term memory) performance at different ages, a repeated measures ANOVA was used (between-subject factors tgAPP, trisomy, sex, batch of mice cohort A(i) or cohort A(ii)); within-subject factor age). A repeat-measures ANOVA was used for analysis of habituation to the open field (factors tgAPP, trisomy, sex, batch of mice cohort A(i) or cohort A(ii)). Univariate ANOVA was used for the other behavioural tasks (factors tgAPP, trisomy, sex, batch of mice (cohort A(i), cohort A(ii), and/or cohort B). The identity (i.e. physical location) of “other arm” used during the exposure phase was included as an additional between-subjects factor in the analysis of the spatial Y-maze data.

All analysis was performed in SPSS.

**Human tissue ethics**

The procurement and use of human tissues in this study was in accordance with the UK Human Tissue Act 2004. The study was reviewed and approved by NHS Research Ethics committee, London-Queen Square. All samples were supplied, anonymized by UK Brain Banks, as indicated in Supplementary Table 1, and had full research consent.
Animal welfare and husbandry

Mice were housed in controlled conditions in accordance with guidance issued by the Medical Research Council in Responsibility in the Use of Animals for Medical Research (1993) and all experiments were carried out under License from the UK Home Office and with Local Ethical Review panel approval. Tc1 mice were taken from a colony maintained by mating Tc1 females (MGI: 3814712) to F1 (129S8 x C57BL/6) males. J20 (B6.Cg-Tg(PDGFB-APPSwInd)20Lms/2J (MGI: 3057148) animals were maintained by mating J20 APP transgenic mice to C57BL/6J. Tc1 females were mated to J20 males to generate cohorts A-J, all cohorts were mixed sex unless otherwise stated. All mice were co-housed throughout the study as lone-housing is known to modify APP-related phenotypes (Kang et al., 2007), mice were housed with littermates and/or animals of the same sex weaned at the same time, thus mice of differing genotypes were cohoused pseudo-randomly.

Mice had access to a mouse house with bedding material and wood chips. All animals had continual access to water and RM1 (Special Diet Services) (stock animals) or RM3 (Special Diet Services) (breeding animals) chow. Mice in cohorts (B-F, H-J) were housed in individually ventilated cages (IVCs) in a specific pathogen free (SPF) facility. Mice in cohorts A and G were bred in IVCs in an SPF facility prior to transportation to another facility. Mice from cohort A were then housed in open cages in a non-SPF facility. Mice in cohort A-F and H-J, were housed at 21 ± 2 °C and 55 % ± 10 humidity. Animals were euthanized by exposure to rising carbon dioxide, followed by confirmation of death by dislocation of the neck in accordance with the Animals (Scientific Procedures) Act 1986 (United Kingdom).
DNA extraction and genotyping

DNA was extracted from tail tip or ear biopsy by the HOTSHOT method (Truett et al., 2000). Mice were genotyped using polymerase chain reaction (PCR) for the presence of human chromosome 21 (Tc1 specific primers f: 5’-GGTTTGAGGGAACACAAAGCTTAACCTCCA-3’ r: 5’-ACAGAGCTACAGCCTCTGACACTATGAACT-3’, control primers f: 5’-TTACGTCCATCGTGACAGCAT-3’ r: 5’-TGGGCTGGGTGTTAGTCTTAT-3’) as described in reference (O’Doherty et al., 2005). Presence of the human APP transgene was tested by PCR using primers (APP f: 5’-GGTGAGTTTGTGAAGTGATGCC-3’ r: 5’-TCTTCTTCTCCACCTCAGC-3’, control primers f: 5’-CAAATGTTGCTTGTCTGGTG-3’ r: 5’-GTCAGTCGAGGTGCACAGTTT-3). Relative copy number of the human APP transgene was checked by quantitative PCR using a Taqman Fast (ABI) (human APP transgene primers f: 5’-TGGGTTCAAACAAAGGTGCAA-3’ r: 5’-GATGAAGATCAGTCGTGTTATGAC-3’ probe FAM-CATTGGACATCGTGGGCGGTG-3’ control primers f: 5’-CACGTGGGCTCCACAGATT-3’ r: 5’-TCACCAGTCATTCTGCGCTT-3’ control probe VIC-CCAATGGGTCGCGACTGCTCA-3’).

RNA extraction and quantitative RT-PCR

Total hippocampal RNA was extracted using miRNeasy mini kit (Qiagen). Tissue was disrupted using a Tissue Ruptor (Qiagen), and the protocol followed as per manufacturer’s instructions, samples were defrosted and homogenised on ice. Final extracted RNA was eluted in DNase- and RNase-free water. Amounts of RNA were equalised and cDNA was generated using the
QuantiTect Rev. Transcription Kit (Qiagen). Quantitative PCR was undertaken to determine expression of human mutant APP (primers f: 5’-CGACCGAGGACTGACCCTC-3’ r: 5’-TGTCGGAATTCTGCATCCAGA-3’ probe FAM-CCAGGTTCGTTGGTGAATAATCGAAGACG) and mouse App (primers f: 5’-CTCCAGCCGTGGCACC-3’ r: 5’-AGTCCTCGGTCAGCGCAGC-3’ probe FAM-ACTCTGTGACCCAGCAATACCGAAAATGA). Mouse β-actin (4352341E-Vic Life Technologies) and GAPDH (4342339E-Vic Life Technologies) were used as endogenous controls. Minus reverse-transcriptase controls were run for every sample for all reactions, no evidence of genomic amplification was detected.

Tissue preparation and western blotting

For analysis of protein abundance in hippocampus and cortex, tissue was dissected under ice cold PBS before snap freezing. Samples were then homogenised in RIPA Buffer (150 mM sodium chloride, 50 mM Tris, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate) plus complete protease inhibitors (Calbiochem) by mechanical disruption. Total protein content was determined by Bradford assay. Samples from individual animals were run separately and were not pooled.

Equal amounts of total brain proteins were then denatured in LDS denaturing buffer (Invitrogen) and β-mercaptoethanol, prior to separation by SDS-PAGE gel electrophoresis using precast 4-12 % Bis-Tris gels (Invitrogen). Proteins were transferred to nitrocellulose or PVDF membranes prior to blocking in 5 % milk/PBST (0.05 % Tween-20) or 5-10 % bovine serum albumin
(BSA)/PBST. Primary antibodies were diluted in 1 % BSA/PBST, HRP-conjugated secondary anti-rabbit, anti-mouse and anti-goat antibodies (Dako) were diluted 1:10,000 in 1% BSA/PBST. Linearity of antibody binding was confirmed by a 2-fold dilution series of cortical protein samples. Band density was analysed using Image J. Relative signal of the antibody of interest compared to the internal loading control was then calculated, and relative signal was then normalized to mean relative signal of control samples run on the same gel. Mean of technical replicates were calculated and used for ANOVA, such that biological replicates were used as the experimental unit.

Primary antibodies against C-terminal APP (Sigma A8717, 1:10,000), BACE1 (Abcam ab108394, 1:1000), IDE (Abcam ab32216, 1:1000), Neprilysin (R&D Systems AF1126, 1:1000), β-actin (Sigma A5441, 1:60,000), and GAPDH (Sigma G9545, 1:200,000), were used.

**BACE-1 β-secretase activity assay**

BACE-1 β-secretase activity was measured as described in reference (Ahmed et al., 2010). Briefly, whole cortex was lysed in extraction buffer (10 mM sodium acetate, 3 mM NaCl, 0.1 % Triton X-100, 0.32 M sucrose, pH 5.0) and an anti-BACE1 antibody (Abcam 108394) was used to capture endogenous BACE1, and cleavage of a β-secretase fluorogenic peptide substrate was measured over 2 hours at 37 °C.
Carboxypeptidase γ-secretase activity assay

CHAPSO detergent resistant membranes (DRMs) were prepared from brain cortices after careful removal of leptomeninges and blood vessels, as previously described (Szaruga et al., 2015). Briefly, tissue was homogenized in approximately 10 volumes of 10% sucrose in MBS buffer (25mM MES, pH 6.5, 150mM NaCl) containing 1% CHAPSO (Sigma); separated by a sucrose density gradient and the DRM fraction (interface of 5%/35% sucrose) was collected and rinsed twice in 20mM PIPES, pH 7, 250mM sucrose, 1M EGTA. The resultant pellet was resuspended with the above buffer and used as source of enzyme. Activity assays were carried out for 1 or 2 hours for mouse or human derived DRMs, respectively, as described before in (Szaruga et al., 2015).

Expression and purification of wild type C99-3xFLAG substrate

Human wild type APPC99-3xFLAG substrate was expressed in COS1 or HEK cells and purified as previously described (Chavez-Gutierrez et al., 2008). Purity was assessed by SDS-PAGE and Coomassie staining (GelCode reagent, Pierce).

Quantification of Aβ production by ELISA

Aβ38, Aβ40 and Aβ42 product levels were quantified on Multi-Spot 96 well plates pre-coated with anti-Aβ38, Aβ40, and Aβ42 antibodies obtained from Janssen Pharmaceutica using multiplex MSD technology, as described before in (Szaruga et al., 2015).
Tissue fractionation for Aβ or sAPP Assays and Aβ ELISA

Total cortical proteins were fractionated based on the method in (Shankar et al., 2009). Total cortex was homogenized in 5 volumes of ice-cold Tris-buffered saline (TBS) (50 mM Tris-HCL pH 8.0) plus complete protease and phosphatase inhibitors (Calbiochem). Homogenates were centrifuged at 175,000 g at 4 °C for 30 minutes, and the resultant supernatant (the soluble TBS fraction) was stored at – 80 °C. The resultant pellet was homogenized in 5 volumes of 1 % Triton X-100 in TBS plus complete protease inhibitors and centrifuged at 175,000 g at 4 °C for 30 minutes, and the resultant supernatant (the triton soluble fraction) was stored at − 80 °C. The resultant pellet was homogenized in 8 volumes of 50 mM Tris–HCl buffer, pH 8.0, containing 5 M guanidine-HCl plus complete protease inhibitors (Calbiochem). This re-suspension (the guanidine HCl soluble fraction) was incubated at 4 °C for a minimum of 14 hours with shaking and was stored at − 80 °C. Protein concentration was determined by Bradford assay (Biorad).

Total hippocampal proteins were homogenized in 5 volumes of ice-cold TBS plus complete protease and phosphatase inhibitors (Calbiochem) based on the method in reference (Holtta et al., 2013). Homogenates were centrifuged at 16,000 g at 4 °C for 30 minutes. The resultant supernatant (the soluble TBS fraction) was stored at − 80 °C. Protein concentration was determined by Bradford assay (Biorad).

Samples were then analyzed by human Aβ40 and Aβ42 ELISA (Life Technologies), and/or Aβ 6E10 Triplex, sAPPβ or sAPPα Assay (Meso Scale Discovery) following the manufacturer’s protocols. Briefly the TBS, triton, and guanidine HCl soluble fractions were diluted into reaction buffer (Dulbecco’s phosphor-buffered saline 5 % BSA 0.0003 % Tween, complete protease
inhibitors) or Diluent 35 (Meso Scale Discovery) and added to a pre-coated plate prior to
addition of Aβ detection antibody and incubation overnight at 4 °C (Invitrogen) or 2 hours at
room temperature (Meso Scale Discovery). After washing, either a HRP-secondary antibody
(Life Technologies) was applied prior to application of a chromogenic reagent (Life
Technologies) and plates were read on a Sunrise plate-reader (450 nm), or Read Buffer (Meso
Scale Discovery) was applied immediately prior to plate reading on a Meso Scale Discovery
Sector Imager.

**Multimeric Aβ ELISA**

Multimeric Aβ was measured as described before in detail (Holtta et al., 2013). In brief, the Aβ
N-terminal-specific antibody 82E1 (IBL international, Hamburg, Germany) was used for both
capture and detection, which results in selective quantification of oligomerised Aβ (no signal
from monomers due to epitope-blocking). A synthetic dimer consisting of two Aβ1–11 peptides
with an added C-terminal cysteine through which the peptides were coupled via a disulfide
bridge (Caslo, Kongens Lyngby, Denmark) was used to create the standard curve. All samples
were measured on one occasion using one batch of reagents. The intra-assay coefficient of
variation was 7%.

**Mass-spectrometry analysis of Aβ in fractionated cortical proteins**

Aβ was immunoprecipitated using the KingFisher magnetic particle processor
(ThermoScientific, Waltman) and mass spectrometric analysis using MALDI-TOF MS were
performed as described in (Mustafiz et al., 2011). Briefly, anti-Aβ antibodies 6E10 and 4G8 (Signet Laboratories), were separately added to magnetic Dynabeads M-280 Sheep Anti-Mouse IgG (Invitrogen). These coated beads were mixed and added to fractionated brain homogenate diluted in 0.025% Tween 20 PBS (pH 7.4). After washing, using the KingFisher magnetic particle processor, bound Aβ was eluted using 0.5% formic acid. MALDI-TOF MS measurements were performed using an Autoflexinstrument (Bruker Daltonics). Each spectrum represents an average of 10,000. The MALDI samples were prepared with the seed layer method using cyano-4-hydroxycinnamic acid as the matrix. The area of each form of Aβ in the spectra was normalized to the sum of all areas of the Aβ peptides detected in the spectra, such that relative changes in the abundance of the different Aβ peptides can be calculated. It should be noted that the ratio between the different isoforms detected in the mass spectrum cannot be interpreted as a direct reflection of their absolute abundance in the brain since the ionization efficiency might be different for the different peptides and since different peptides are more hydrophobic and less soluble than others.

**In vivo microdialysis**

*In vivo* microdialysis was used to assess brain interstitial fluid (ISF) Aβ-40 half-life in awake mice as previously described (Castellano et al., 2011). Briefly, unilateral guide cannula were implanted into the hippocampus and used to insert a 2 mm microdialysis probe (38 kDa molecular weight cut-off, BR-2, Bioanalytical Systems). Artificial CSF containing 0.15 % bovine serum albumin was used as perfusion buffer. Hourly ISF samples were collected for 6 hours, using a 1 μl/min flow rate to determine basal level of ISF Aβ, prior to the administration
of compound E by intraperitoneal injection (20 mg/kg body weight). After compound E administration, which is a gamma-secretase inhibitor that prevents further Aβ production, samples were collected hourly for a further 6 hours. Levels of Aβ40 were determined for each time-point using a sandwich ELISA (anti-Aβ35-40 HJ2 capture antibody and anti-Aβ13-18 HJ5.1-biotin as detecting antibody). The elimination of Aβ from the ISF followed first-order kinetics; therefore, for each mouse, t\(1/2\) for Aβ was calculated with the slope, \(k'\), of the linear regression that included all fractions until the concentration of Aβ stopped decreasing (t\(1/2 = 0.693/k\), where \(k = 2.303k'\) (Castellano et al., 2011).

**Immunohistochemistry of mouse brain**

The brains were immersion fixed in 10 % buffered formal saline (Pioneer Research Chemicals) for a minimum of 48 hours prior to being processed to wax (Leica ASP300S tissue processor). The blocks were trimmed laterally from the midline by ~0.9-1.4 mm to give a sagittal section of the hippocampal formation. Two 4 μm sections 40 μm apart were analysed. The sections were pretreated with 98 % formic acid for 8 minutes, then antigen retrieval was undertaken by incubation for 30 minutes in Tris Boric acid EDTA buffer (pH 9.0). Slides were then blocked prior to the application of directly biotinylated mouse monoclonal IgG1 antibodies against either full length APP (22C11, MAB348B, Millipore, 1:3000), or Aβ (82E1, IBL, 0.2 μg/ml) for 8 hours. This was followed by treatment with the Ventana DabMap kit (iView DAB, Ventana Medical Systems) using a Ventana XT automated stainer (Ventana Medical Systems, Tuscon, AZ, USA). Alternatively, for staining of Beta-amyloid, were incubated with mouse monoclonal 6F/3D (Dako 1:50) followed by Iview Ig secondary antibody (Ventana Medical Systems). The
sections were counterstained with haematoxylin, scanned (Leica SCN400F scanner) and analyzed using Definiens software. 6F/3D stained slides were photographed (ImageView II 3.5 Mpix digital camera) and composed with Adobe Photoshop so that the entire cortex could be analysed. The same thresholds for staining intensity were then used in quantify the area covered by DAB stain using Volocity image analysis software (Perkin Elmer). Plaque numbers were counted by two independent scorers, using the counting objects feature of ImageJ.

**Behavioural study design**

Cohort A was longitudinally tested in the following order: test 1) spontaneous alternation in the T-maze at 2-3-months of age; test 2) spontaneous alternation in the T-maze at 6-7-months of age; test 3) spatial novelty preference in the Y-maze at 6-7-months of age; test 4) habituation to an open field at 6-7 months of age; test 5) spontaneous alternation in the T-maze at 15–16 months of age. Cohort A was batched into two groups to facilitate testing (batch i and ii). Cohort B was tested at 2-3-months of age in the spatial novelty preference Y-maze task only, before being aged to 15-months. All behavioural testing was undertaken between 8.30-16.30 hours (light 7.00-19.00 and dark 19.00-7.00). Cohort A and B were tested at independent facilities, such that site of test compounded analysis of effect of age in the Y-maze task.

**Spontaneous alternation in the T-maze**

A T-shaped maze made of wood painted dark grey with 30x10x29 cm arms, with a central partition extending 7 cm into the start arm from the back of the maze, was used to assess
spontaneous alternation as previously described in reference (Deacon and Rawlins, 2006). Mice were placed into the maze facing the wall of the start arm and allowed to make a free choice of either goal arm. The mouse was then restricted to that goal arm for 30 seconds by use of a guillotine door. The central partition was then removed and all doors reopened. The mouse was again placed at the end of the start arm facing the wall and allowed to make a further free choice of either goal arm. This was performed twice a day, once prior to 12.30 and once after 12.30 for 8 days, with an approximate interval of 4 hours between the two daily sessions. Whether or not the animal chose the novel arm on the second run was recorded and summed across 16 trials.

**Spatial novelty preference Y-maze task**

Spatial novelty preference was assessed in an enclosed Perspex Y-maze as described previously (Sanderson et al., 2007). Briefly, a Perspex Y-maze with arms of 30x8x20 cm was placed into a room containing a variety of extra-maze cues. Mice were assigned two arms (the “start” and the “other” arm) to which they were exposed during the first phase (the exposure phase), for 5 minutes. This selection of arms was counterbalanced with respect to genotype. Timing of the 5 minute period began only once the mouse had left the start arm. The mouse was then removed from the maze and returned to its home-cage for a 1 minute interval between the exposure and test phases. During the test phase, mice were allowed free access to all three arms. Mice were placed at the end of the start arm and allowed to explore all three arms for 2 minutes beginning once they had left the start arm. An entry into an arm was defined by a mouse placing all four paws inside the arm. Similarly, a mouse was considered to have left an arm if all four paws were
placed outside the arm. The times that mice spent in each arm were recorded manually and a novelty preference ratio was calculated for the time spent in arms (novel arm/(novel+other arm)).

**Habituation to an open field**

Mice were habituated to a grey arena (40x40x40 cm) under low light levels (20 lux), for 10 minutes/day on three consecutive days (at a similar time each day (± 30 minutes)). Distance travelled was measured using Ethovision. The "outer" zone was defined as 10 cm from the edge of the box and the inner zone as the 20 cm² area in the centre.
Results

Trisomy of chromosome 21 genes other than APP promotes the deposition of Aβ

Having three copies of chromosome 21 genes other than APP may influence the development of Alzheimer’s disease in people who have Down syndrome. This is partly based on mouse studies using single gene over-expression models, rather than animal models that are aneuploid (Wiseman et al., 2015). To take an unbiased approach and investigate if triplication of genes other than APP can modulate the development of Down syndrome - Alzheimer’s disease in vivo, we crossed a model of Down syndrome that is aneuploid for chromosome 21 with a mouse that deposits Aβ in the brain. Thus, we have generated a new model system to understand the early stages of Down syndrome - Alzheimer’s disease, when Aβ starts to accumulate.

The Down syndrome mouse was the Tc(Hsa21)1TybEmcf (Tc1) model (O’Doherty et al., 2005), which carries a freely segregating chromosome 21 and is trisomic for 75 % of the genes on this chromosome but, importantly, is not functionally triplicated for APP (Gribble et al., 2013; Sheppard et al., 2012). As with a small percentage of Down syndrome individuals, the mouse is mosaic; on average approximately 66% of brain nuclei (neurons and glia) retain chromosome 21 (O’Doherty et al 2005). This model has well-defined Down syndrome-associated deficits, including defects in nervous system function such as in long term potentiation, short-term memory, dendritic spine morphology and connectivity in the hippocampus (Hall et al., 2016; Morice et al., 2008; O'Doherty et al., 2005; Witton et al., 2015).
To determine if trisomy of chromosome 21 sequences other than APP is sufficient to modify Alzheimer’s disease-related phenotypes we crossed Tc1 mice with the APP transgenic mouse strain Tg(PDGFB-APPSwInd)20Lms (J20-tgAPP). J20-tgAPP mice over-express a human APP transgene with Alzheimer’s disease mutations and accumulates Aβ within the brain from around 4-5-months of age (Mucke et al., 2000). This model has been widely used to study amyloid deposition. Our ‘Trisomic x tgAPP’ (Tc1 x J20-tgAPP) cross is an in vivo model system that allows us to investigate if amyloid deposition (from the tgAPP transgene) is modified in the presence of an additional copy of human chromosome 21, by producing four types of progeny: (i) wildtypes (WT), (ii) those inheriting the APP transgene only (‘tgAPP’), (iii) those inheriting human chromosome 21 only (‘Trisomic’), and (iv) those that inherit both the APP transgene and human chromosome 21 (‘Trisomic;tgAPP’).

We observed that early intracellular Aβ deposition within the CA3 pyramidal cells of the hippocampus was significantly enhanced by trisomy of chromosome 21 at 4.5-6.5-months of age in Trisomic;tgAPP mice compared to tgAPP littermates (Fig. 1A,B). This is consistent with the site of earliest deposition of Aβ occurring intracellularly in the brains of people who have Down syndrome (Cataldo et al., 2004; Lemere et al., 1996; Mori et al., 2002). A similar but non-significant increase in Aβ accumulation within Trisomic;tgAPP granular cells of the dentate gyrus and in the cortex was also observed, but no change in full-length APP could be detected using similar methods (Supplementary Fig. 1). Consistent with this we saw a trend for an increase in the abundance of soluble aggregated Aβ, as measured by a multimeric Aβ ELISA, at 2-months of age in Trisomic;tgAPP hippocampus as compared with tgAPP littermates (Fig. 1C).
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We quantified plaque number at 6- and 16-months of age in the hippocampus in the Trisomic x tgAPP model system. Trisomic;tgAPP progeny had significantly more extra-cellular plaques at both ages than tgAPP littermates (Fig. 1D,E,F,G,H). Similarly, the area of Aβ deposition was significantly greater in Trisomic;tgAPP hippocampus and cortex compared with tgAPP controls at 16 months of age (Fig. 1I, and Supplementary Fig. 2).

**Trisomy of chromosome 21 genes other than APP does not alter the ratio of Aβ isoforms that aggregate**

To determine which forms of Aβ peptide have increased deposition in the Trisomic;tgAPP model, cortical proteins from 6- and 16-month old animals were biochemically fractionated by homogenization in progressively more chemically disruptive solutions followed by ultracentrifugation (tris buffer; then 1 % triton X-100; then 5 M guanidine hydrochloride) (Shankar et al., 2009). The abundance of Aβ42, Aβ40 and Aβ38 in each fraction was then determined (Fig. 2A-C and Supplementary Fig. 3). At 16-months of age more Aβ42 was observed in the least soluble fraction (5 M guanidine hydrochloride) in Trisomic;tgAPP compared with tgAPP controls, as measured using two independent assays (Fig. 2C and Supplementary Fig. 3F). No changes in the Aβ40/42 or Aβ38/42 ratios were observed in the aggregated (5 M guanidine hydrochloride soluble) fraction at either 16-months or 6-months of age (Fig. 2D,E and Supplementary Fig. 3). Consistent with this observation, we found no difference in the relative abundance of Aβ species, as determined by mass-spectrometry, between
Trisomic;tgAPP and tgAPP in the 5 M guanidine hydrochloride fraction isolated from 16-month cortex (Fig. 2F). Thus, trisomy of chromosome 21 sequences other than APP, are sufficient to greatly increase intracellular and extracellular Aβ deposition independently of changing the ratio of Aβ that aggregates.

**Trisomy of chromosome 21 genes other than APP exacerbates APP/Aβ associated cognitive deficits**

To determine if the chromosome 21 trisomy-associated increase in Aβ aggregation contributes to changes in APP/Aβ-associated cognitive deficits, a series of behavioural tests were undertaken on cohorts of wildtype (WT), Trisomic, tgAPP and Trisomic;tgAPP littermates. These experiments were designed to avoid potential floor-effects in test performance; so any interaction between the influence of trisomy and tgAPP status could be identified. Therefore tasks in which the Trisomic mice had near-wildtype performance were employed. This approach is also being developed to improve the assessment of cognitive decline in adults who have Down syndrome (Startin et al., 2016). Trisomy of chromosome 21 significantly exacerbated APP/Aβ-associated hyperactivity at both 2-3 and 6-7-months of age, and Trisomic;tgAPP animals specifically failed to habituate to an open field at 6-7-months of age (Fig. 3A-C, Supplementary Fig 4A,B). Typical habituation, as measured by a decline in activity caused by increased familiarity with the new environment, was observed in wildtype, Trisomic and tgAPP littermates (Fig. 3B). In this task we also observed that both Trisomic and Trisomic;tgAPP mice spent significantly more time
in the centre of the open field than wildtype or tgAPP animals (Supplementary Fig. 4C). However, Trisomic;tgAPP mice did not spend any additional time in the centre of the field compared with Trisomic controls. Both groups of animals spent ~20% of the time available in the centre of the field thus a ceiling effect is not biasing this task. These data suggest the failure to habituate in the Trisomic;tgAPP is not driven by a modification of anxiety. We note that this task was not designed to measure anxiety; the open field and lighting-conditions were designed to be non-anxious. However, these data indicate the Tc1-trisomic mice may have lower anxiety than wildtype controls, and further tests are warranted to explore this.

To further understand the observed failure to habituate in the Trisomic;tgAPP mice we undertook two tests of immediate memory. For the Y-maze task, two independent cohorts of mice were tested, one at 2-3-months of age and one at 6-7-months of age. The effect of genotype was similar in both cohorts so they were combined for analysis. For the T-maze task, one cohort of mice was tested longitudinally, at 2-3-months, 6-7-months and 15-16-months of age. We note that the performance of wildtype mice declined with age in this task as has been previously reported (Lalonde, 2002). No deficit as measured by spatial novelty preference in the Y-maze (1-minute trial interval) was observed in tgAPP or Trisomic mice (Fig. 3D). However, Trisomic;tgAPP mice had a poorer test performance than wildtype and Trisomic animals in this task, such that a main effect of trisomy of chromosome 21 and of tgAPP was observed, and a significant interaction of these genetic factors was seen (Fig. 3D). Although some residual memory was retained, as Trisomic;tgAPP had above chance performance in the task at the three ages studied. The poorer performance of Trisomic;tgAPP was apparent despite these animals having a greater opportunity to experience extra-maze cues during the training phase than
Trisomic controls because of a greater time spent in the training (other) arm during the initial exposure to the maze (Supplementary Fig. 4B). An independent immediate memory task, a discrete-trial spontaneous alternation in the T-maze, was used to validate these observations. In this task, a main effect of chromosome 21 trisomy and an interaction of trisomy with tgAPP was observed by ANOVA, such that Trisomic;tgAPP mice performed the task significantly worse than wildtype controls (Fig. 3E). Consistent with our observations in the Y-maze task, some residual memory was retained, as Trisomic;tgAPP did have above chance performance at the three ages studied.

These data show that trisomy Hsa21 exacerbates APP/Aβ associated cognitive changes, and that Trisomic;tgAPP mice exhibit specific immediate memory deficits in two tasks compared to the matched controls. Additionally, Trisomic;tgAPP mice were less likely to survive to 15 months of age than matched tgAPP control animals (Supplementary Fig. 4D). Thus, the chromosome 21 trisomy-associated increase in Aβ accumulation described here correlates with changes in multiple tests of cognition and is associated with an increased risk of mortality.

**Trisomy of chromosome 21 genes other than APP does not alter APP or β-CTF/α-CTF ratio in the brain**

To determine if a change in the abundance of APP in the brain of trisomic mice contributes to the increase in Aβ aggregation, we compared levels of full-length APP (FL-APP) in cortex and hippocampus from progeny of the Trisomic x tgAPP cross (3-months of age) (Fig. 4A,B). As
expected, animals with tgAPP had higher levels of FL-APP protein, compared with the lower levels observed in both wildtype and trisomic controls. However, no significant increase in the abundance of FL-APP in the Trisomic;tgAPP progeny compared with tgAPP controls was observed. Additionally, Trisomic;tgAPP mice have comparable levels of human APP transcript compared with tgAPP in both the cortex and hippocampus (Supplementary Fig. 5). Mouse App mRNA levels in the Trisomic, tgAPP and Trisomic;tgAPP hippocampus were similar to wildtype mice (Supplementary Fig. 5), consistent with previous reports (Gribble et al., 2013; Sheppard et al., 2012). Thus, trisomy does not cause exacerbated Aβ deposition, in our model system by increasing the abundance of FL-APP protein in vivo.

Alterations in the processing of APP by α- then γ-secretase or β- then γ-secretase, can modulate Aβ deposition (Haass et al., 2012). Cleavage of FL-APP by β-secretase generates soluble β-APP and a membrane bound β-C-terminal-fragment (β-CTF), which can then be cleaved by γ-secretases, forming Aβ. Whereas cleavage of APP by an α-secretase, produces soluble α-APP and α-C-terminal-fragment (α-CTF), in a process that prevents β-cleavage and hence the formation of Aβ. We found that chromosome 21 trisomy causes a significant increase in both α- and β-CTFs relative to FL-APP, in both the hippocampus and cortex at 3-months of age in male mice (Fig. 4B,C). However, no significant change in α- or β-CTF/FL-APP ratios occurs in female mice (Supplementary Fig. 6). Moreover, trisomy of chromosome 21 did not alter the β-CTF/α-CTF ratio in either sex in the cortex or hippocampus (Fig. 4D). This indicates that although trisomy of chromosome 21 increases APP-CTF abundance in males, it does not alter the relative balance of the amylogenic versus non-amylogenic APP processing pathway in the brain. Additionally, trisomy-associated increases in Aβ accumulation are observed in both males...
and females, suggesting that the specific effect of trisomy in male mice on APP-CTF abundance is unlikely to be the cause of enhanced Aβ deposition observed in our mouse model system.

**Trisomy of chromosome 21 genes other than APP does not elevate APP-CTF production**

To determine if the elevation in APP-CTFs in males is caused by an increased rate of CTF-production; we analysed the abundance of soluble β-APP, the other APP fragment produced by β-secretase activity. Trisomy of chromosome 21 did not alter the abundance of soluble β-APP in the cortex of the Tc1 mouse (Fig. 5A), indicating that the production of β-CTF is not upregulated in vivo by trisomy of chromosome 21. Consistent with this, BACE1 level and BACE1-β-secretase activity (as measured by a BACE1 capture enzymatic assay) were not altered by trisomy at 3-months of age, in the cortex (Fig. 5B-D). Similarly, trisomy of Hsa21 did not affect levels of soluble α-APP in the cortex at 3 months of age (Fig. 5E). Therefore, the elevated β-CTF and α-CTF/FL-APP ratios observed in male Trisomic;tgAPP compared with tgAPP male controls are likely the result of impaired turn-over of these fragments.

**Trisomy of chromosome 21 does not alter the extracellular clearance of Aβ**
The increase in abundance of β-CTF and α-CTF in trisomic male mice indicates that trisomy of chromosome 21 may alter the clearance of APP derivatives in some circumstances. To determine if trisomy causes increased Aβ aggregation by impairing clearance of the peptide in vivo, we measured the half-life of extracellular Aβ40 in the hippocampus at 3-months of age by microdialysis of interstitial fluid (ISF). In order to follow Aβ clearance kinetics in vivo, a potent inhibitor of γ-secretase (compound E) was injected to halt Aβ generation. Chromosome 21 trisomy did not significantly alter Aβ40 half-life (Fig. 6A,B), nor did it alter the expression of the key Aβ extracellular clearance enzymes, insulin degrading enzyme and neprilysin in the brain (Supplementary Fig. 7). Thus changes in the extracellular clearance of Aβ do not contribute to trisomy-associated increases in deposition.

Trisomy of chromosome 21 genes other than APP causes a shift in the soluble Aβ40/42 ratio

The aggregation rate of Aβ in vitro is influenced by the relative abundance of more aggregate prone Aβ species, such as Aβ42, compared with forms of the peptide which are less able to seed aggregation such as Aβ40 (Vandersteen et al., 2012). To determine if chromosome 21 trisomy associated increases in Aβ deposition are the result of changes in the ratio of Aβ species in vivo, we investigated the relative abundance of soluble Aβ prior to accumulation of detectable Aβ deposits, in the hippocampus of 2-3-month old progeny from the Trisomic x tgAPP cross. Levels of tris-soluble total hippocampal Aβ42 were not affected by trisomy, but a significant decrease in
both tris-soluble Aβ38 and Aβ40 was observed, leading to a significant decrease in the soluble Aβ38/42 and Aβ40/42 ratios (Fig. 7A-C). Moreover the soluble Aβ40/42 ratio significantly correlates with Aβ aggregation in young hippocampus, as measured using a multimeric Aβ ELISA (Fig. 7D), indicating that the effect of trisomy on soluble Aβ ratios may underlie the increased accumulation of the peptide observed within the brain.

**Trisomy of chromosome 21 genes other than APP does not alter intrinsic γ-secretase carboxypeptidase activity**

Impairment of γ-secretase carboxypeptidase activity, such as is caused by some familial Alzheimer’s disease mutations in APP or presenilin 1 or 2, can result in similar changes to the Aβ isoform ratios (Chavez-Gutierrez et al., 2012). We isolated a membrane fraction containing γ-secretase from the cortex of our mouse model and used this for an *in vitro* enzymatic activity assay using a recombinant APP-CTF substrate. No change in γ-secretase carboxypeptidase activity was detected in the trisomic mouse model (Fig. 7E-F). Similarly using the same method no difference in enzymatic activity was observed in people who have Down syndrome - Alzheimer’s disease compared with aged-matched healthy controls (Fig. 7G-H, Supplementary table 1). Trisomy of chromosome 21 therefore alters the Aβ ratio *in vivo* independently of a direct effect on γ-secretase activity.
Discussion

The extra copy of APP, encoded on chromosome 21, has a central role in Down syndrome - Alzheimer’s disease pathogenesis. This includes increasing the level of APP protein and its derivatives, such as Aβ, and causing specific alteration to endolysosomal biology (Cheon et al., 2008; Jiang et al., 2010; Lemere et al., 1996; Salehi et al., 2006). Here we show that in addition, trisomy of other chromosome 21 sequences is sufficient to promote the aggregation and deposition of Aβ within the brain and worsen associated-cognitive deficits. Our data suggest that the increase in Aβ aggregation caused by trisomy of chromosome 21 may be mediated by an alteration in the ratio of soluble Aβ isoforms that occurs independently of alterations in the activity of α-, β- or γ-secretases, or a change in the rate of extracellular clearance of Aβ.

Our research uses animal models created to study Down syndrome and Aβ accumulation, to investigate the effect of triplication of chromosome 21 genes on Aβ generation and deposition within the brain. As with all work using animal models it is essential to recognize that each model gives us a limited view of a complex human disease. Down syndrome mouse models in which App is triplicated (e.g. Ts65Dn or Dp(10)1Yey;Dp(16)1Yey;Dp(17)1Yey triple trisomic mice) do not form Aβ plaques even in very old mice (26 months) (Reeves et al., 1995; Yu et al., 2010). Thus, in order to understand the development of amyloid pathology in the context of chromosome 21 trisomy we work with a widely-used transgenic APP mouse model. This model over-expresses human APP with Alzheimer’s disease associated point mutations, which promote the formation of aggregation prone Aβ42, and develops robust amyloid pathology. Therefore in
this model system, the relative abundance of Aβ42 is higher than would be typically observed in individuals who have Down syndrome.

We crossed the tgAPP mouse with the Tc1 mouse model of Down syndrome that contains a freely-segregating copy of human chromosome 21. This model system allows us to address whether the additional copy of the chromosome 21 genes carried in the Tc1 mouse are sufficient to alter Aβ generation, deposition and associated cognitive changes. However, we note that as with all models of Down syndrome (including the triple trisomics (Yu et al., 2010)), the Tc1 mouse does not have the full complement of chromosome 21 sequences (Gribble et al., 2013). Indeed, here we have taken advantage of the absence of an additional copy of APP in this model to investigate APP triplication-independent effects. Within the Tc1 mouse model the transchromosome is lost during development from some cells; we previously determined that approximately 66% of brain nuclei retain the chromosome (O’Doherty et al., 2005).

Chromosome 21 mosaicism, in individuals who have Down syndrome, is associated with higher IQ, earlier acquisition of developmental milestones, less severe cardiac defects and reduced mortality compared to matched cases of non-mosaic chromosome 21 trisomy (Papavassiliou et al., 2009; Zhu et al., 2013). Similarly, in the Tc1 mouse, mosaicism may result in a reduction in phenotypic severity compared with that in non-mosaic Down syndrome model systems. Further studies in alternative mouse models of Down syndrome which are not mosaic, in particular mice with segmental duplications of the mouse genome orthologous to regions of human chromosome 21 may help investigate this (Brault et al., 2006; Lana-Elola et al., 2016; Lana-Elola et al., 2011; Yu et al., 2010).
Our results demonstrate that a number of cognitive changes are observed in Trisomic;tgAPP mice compared to wildtype controls. In particular, these mice display a profound hyperactivity, a failure to habituate to a novel open-field, and poorer immediate memory than wildtype controls. These changes may relate to the increase in Aβ aggregation observed in these mice or how trisomic neurons response to aggregating Aβ. Further studies, in which a trisomic model and wildtype control are exposed to equal quantity of Aβ are required to understand the mechanisms that cause the changes in cognition observed in our study.

Our findings suggest a number of new avenues of inquiry that warrant further investigation. Our work indicates that people who have Down syndrome may have exacerbated amyloid accumulation compared with individuals who have early onset Alzheimer’s disease caused by duplication of APP. Comparative pathological studies of the two causes of early onset disease are required to investigate this hypothesis. Notably, comparative studies of Alzheimer’s disease in people who have Down syndrome and sporadic Alzheimer's disease have suggested that higher levels of aggregated Aβ accumulate in people who have Down syndrome (Egensperger et al., 1999; Hyman et al., 1995; Wilcock et al., 2015), consistent with the finding in our mouse model system presented here. If amyloid deposition is found to be higher in people who have Down syndrome, understanding which gene on chromosome 21 other than APP contributes to this will provide novel insights into disease development and may provide a new target for drug therapy for individuals who have Down syndrome, who are at extraordinarily high risk of developing dementia.
Alzheimer’s disease in people who have Down syndrome first presents with memory impairment, behavioural changes, myoclonus and seizures; with a low incidence of cerebral haemorrhage and stroke. A recent review has noted that this pattern of clinical presentation is more similar to that seen in familial cases of Alzheimer’s disease caused by mutations in APP that decrease Aβ 40/42 ratio, than to cases of diseases caused by duplication of APP (Zis and Strydom, 2018). The authors hypothesised that a shift to a lower Aβ40/42 ratio in people who have Down syndrome might explain this apparent clinical disparity. Our animal work supports this hypothesis and suggests further studies of the ratio of Aβ40/42 in people who have Down syndrome, particularly at the earliest stages of disease is warranted.

We first observe a significant increase in Aβ accumulation within CA3 pyramidal hippocampal neurons of our mouse model (Figure 1B). A previous study using the Tc1 mouse showed that the CA3 has a particular vulnerability to trisomy of chromosome 21, which includes a specific reduction in synapse number, alterations to synapse architecture and related electrophysiological and behavioral deficits (Witton et al., 2015). The sensitivity of the CA3 to trisomy of chromosome 21 is also observed in an alternative Down syndrome mouse model (Popov et al., 2011). The specific effect of trisomy of chromosome 21 on the synapses of CA3 cells may contribute to their vulnerability to intracellular Aβ accumulation, as the synapse is proposed to be key site of Aβ formation (Das et al., 2013). Further work is required to determine if CA3 pyramidal cells in people who have Down syndrome also have a similar tendency to develop intracellular Aβ accumulation and the molecular changes that are responsible for this.
Additionally, this work also demonstrates that factors other than the intrinsic carboxypeptidase activity of the γ-secretase complex or the APP protein sequence can substantially alter the ratio of Aβ isoforms generated in vivo. Moreover, we show that an extra dose of a gene or genes encoded on chromosome 21 is sufficient to modulate this process. Identification of this gene/genes will provide novel insights into the pathways that can alter Aβ generation and how these processes could be modulated to prevent disease.

A number of proteins encoded on chromosome 21 have been suggested to influence Aβ biology and are trisomic in the Tc1 mouse model. These include SUMO3, which is conjugated to proteins to regulate their function and may influence APP processing (Dorval et al., 2007; Li et al., 2003). The kinase DYRK1A, that can phosphorylates APP and alter the protein’s stability and the formation of Aβ (Garcia-Cerro et al., 2017; Ryoo et al., 2008). Recently, a novel inhibitor of DYRK1A has been shown to reduce Aβ plaque load in an Alzheimer’s disease mouse model (Branca et al., 2017). BACE2, a homologue of BACE1 located on chromosome 21 may impair the formation of Aβ by cleaving APP within the Aβ region (Mok et al., 2014; Sun et al., 2006). An endogenous inhibitor of lysosomal cathepsins, CSTB, which reduces Aβ accumulation when knocked-out, is also found on chromosome 21 (Yang et al., 2011). Reduced activity of the enzymes inhibited by CSTB have been linked to altered clearance of Aβ and processing of APP; including a shift in the Aβ40/42 ratio (Hook et al., 2009; Mueller-Steiner et al., 2006; Wang et al., 2015).

Moreover, recent studies have highlighted that alterations in γ-secretase trafficking or modulation of the endo-lysosome system can profoundly affect Aβ generation (Sannerud et al.,
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2016; Xiao et al., 2015); other as yet unidentified gene/genes on chromosome 21 may affect these processes and mediate the effect of trisomy on Aβ ratios, aggregation and deposition observed here. Identification of the causal gene(s) on chromosome 21 will provide further novel insights into the new Down syndrome - Alzheimer’s disease mechanism described in this report.
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**Contributions:**

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Competing interests:


References


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Figures

Fig. 1: Trisomy of chromosome 21 promotes the intracellular and extracellular deposition of Aβ
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(A, B) Intracellular Aβ deposition (82E1) was increased by trisomy in hippocampal CA3 pyramidal neurons in 4.5-6.5 month-old mice (ANOVA trisomy-tgAPP interaction F(1,31) = 5.125, p = 0.031; Bonferroni pairwise comparisons Trisomic;tgAPP with tgAPP p = 0.012; wildtype (WT) (black circles) n = 10, trisomic (blue squares) n = 10, tgAPP (red triangles) n = 10, trisomic;tgAPP (purple inverted triangles) n = 10). (C) A trend for increased hippocampal tris soluble multimeric Aβ (82E1-82E1 ELISA) normalised to the sum of Aβ38, Aβ40 and Aβ42 in Trisomic;tgAPP was observed (trisomy F(1,24) = 3.928, p = 0.059; tgAPP n = 8, trisomic;tgAPP n = 11). (D-I) Aβ deposition (6F/3D) in the hippocampus was quantified at (D-F) 6- and (G-I) 16-months of age. Trisomy increases (E,H) the number of plaques (tgAPP-trisomy interaction F(1,77) = 6.744, p = 0.011, Bonferroni pairwise comparisons Trisomic;tgAPP with tgAPP 6-months p = 0.008, 16-months p = 0.003) and (F,I) the area covered by Aβ (tgAPP-trisomy interaction F(1,85) = 4.005, p = 0.049, Bonferroni pairwise comparisons Trisomic;tgAPP with tgAPP 6-months p = 0.097, 16-months p = 0.037) (6-months WT n = 8, Trisomic = 8, tgAPP n = 15, Trisomic;tgAPP n = 13; 16-months WT n = 16, Trisomic = 9, tgAPP n = 13, Trisomic;tgAPP n = 11). Data are represented as mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001. Both male and female mice were studied and sex was included as a factor in the ANOVA. (A) Scale bar = 50 µm (D,G) scale bar = 500 µm.
Fig. 2: Trisomy of chromosome 21 promotes the aggregation of Aβ

(A-F) Cortical proteins from 16-month old mice were fractionated and 5 M guanidine hydrochloride soluble Aβ quantified by (A-E) Meso Scale Discovery Assay or (F) mass-spectrometry. (A,B) No effect of trisomy on Aβ38 (tgAPP-trisomy interaction F(1,28) = 0.385, p = 0.540) or Aβ40 (tgAPP-trisomy interaction F(1,28) = 0.962, p = 0.355) was observed, (C) but significantly more Aβ42 was detected in Trisomic;tgAPP mice (tgAPP-trisomy interaction F(1,28) = 5.573, p = 0.025) (Bonferroni pairwise comparisons Trisomic;tgAPP with tgAPP p = 0.005, WT n = 6, Trisomic = 6, tgAPP n = 13, Trisomic;tgAPP n = 11). (D,E) No change in the Aβ38/42 ratio (trisomy F(1,19) = 0.072, p = 0.792) or Aβ40/42 ratio (trisomy F(1,19) = 0.047, p = 0.831) was observed. (F) The relative abundance of different forms of Aβ peptides was not altered by trisomy of chromosome 21 (tgAPP n = 12, Trisomic;tgAPP n = 10). Data are represented as mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001. Both male and female mice were used and sex was included as a factor in the ANOVA.
Fig. 3: Trisomy of chromosome 21 exacerbates APP/Aβ-associated cognitive deficits

(A,B) Exposure to a novel open field was used as a test of activity and habituation. (6-months, WT n = 28, Trisomic n = 28, TgAPP n = 20, Trisomic;tgAPP n = 18) (A) Overall activity: ANOVA of distance travelled revealed a main effect of tgAPP (F(1,78) = 26.250, p < 0.001), trisomy (F(1,78) = 9.246, p = 0.003), and a tgAPP-trisomy interaction (F(1,78) = 7.818, p = 0.007) (Bonferroni pairwise comparisons Trisomic;tgAPP with WT p < 0.001, Trisomic p < 0.001, and TgAPP p = 0.008). (B) The total distance moved declined with exposure time (1-minute time-bins) (ANOVA: main effect of time-bin (F(29,2262) = 12.399 p < 0.001); an interaction of time-bin by trisomy (ANOVA F(29,2262) = 1.789 p = 0.006), time-bin by TgAPP (F(29,2262) = 1.560 p = 0.029) and time-bin by trisomy-tgAPP (F(29,2262) = 1.983, p < 0.001) was observed by ANOVA. (C,D) A Y-maze spatial novelty preference task (1-minute delay) was used as a test of activity and memory. (Cohort B 2-3-months and Cohort A 6-7-months, the effect of genotype was similar in both cohorts so data was combined for analysis, WT n = 45, Trisomic n = 43, TgAPP n = 36, Trisomic;tgAPP n = 26). (C) ANOVA of the number of arm entries (test phase), revealed a main effect of trisomy (F(1,89) = 50.360, p < 0.001), TgAPP (F(1,89) = 47.001, p < 0.001), and a TgAPP-trisomy interaction (F(1,89) = 31.720, p < 0.001) (Bonferroni pairwise comparisons Trisomic;tgAPP with WT p < 0.001, Trisomic p <
0.001, and tgAPP p < 0.001). (D) A preference ratio of 0.5 indicates chance performance (black dotted line). ANOVA of novelty preference revealed a main effect of trisomy (F(1,89) = 10.144 p = 0.002), tgAPP (F(1,89) = 9.312 p = 0.003) and a tgAPP-trisomy interaction (F(1,89) = 5.736, p = 0.019) (Bonferroni pairwise comparisons Trisomic;tgAPP with WT p < 0.001 and Trisomic p = 0.010). Performance of tgAPP-trisomic mice was above chance (one-sample t-test t = 3.287 p <0.001). (E) A discrete-trial, longitudinal spontaneous alternation task in a T-maze was used as a test of memory, 50% alternation represents chance performance (black dotted line) (2-3-months WT n = 29, Trisomic n = 30, TgAPP n = 21, Trisomic;tgAPP n = 17, 6-7 months WT n = 28, Trisomic n = 29, TgAPP n = 20, Trisomic;tgAPP n = 17, 15-16-months WT n = 27, Trisomic n = 26, TgAPP n = 27, Trisomic;tgAPP n = 11). ANOVA of alternation showed a main effect of trisomy (F(1,67) = 7.084 p = 0.010), and an interaction of tgAPP-trisomy (F(1,67) = 4.706, p =0.034) (Bonferroni pairwise comparison Trisomic;tgAPP with WT p = 0.032). Performance of tgAPP-trisomic mice was above chance (one-sample t-test, 2-3-months t = 5.884 p <0.001, 6-7-months t = 5.378 p <0.001, 15-16-months t = 6.495 p <0.001). Data are represented as mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001. Both male and female mice were used and sex was included as a variable in the ANOVA.
Fig. 4: Trisomy of chromosome 21 genes other than APP does not increase APP abundance nor alter β-CTF/α-CTF ratio

(A,B,D) Full-length APP (FL-APP), APP β-C-terminal fragment (β-CTF) and APP α-C-terminal fragment (α-CTF) was measured in cortex (WT n = 17, Trisomic n = 16, tgAPP n = 24, Trisomic;tgAPP n = 19) and hippocampus (WT n = 11, Trisomic n = 12, tgAPP n = 24, Trisomic;tgAPP n = 17) at 3-months of age. (A) FL-APP was higher in tgAPP and Trisomic;tgAPP compared with WT or Trisomic mice (cortex F(1,68) = 87.667, p < 0.001, hippocampus F(1,56) = 94.301, p < 0.001). Trisomy did not alter FL-APP (trisomy-tgAPP interaction cortex F(1,68) = 0.483, p = 0.489, hippocampus F(1,56) = 2.457, p = 0.123,). (B,C) In male mice, APP C-terminal fragment protein (APP-CTF)/FL-APP ratio was altered (cortex tgAPP n = 17, Trisomic;tgAPP n = 11, hippocampus tgAPP n = 14, Trisomic;tgAPP n = 8) β-CTF/FL-APP (T-test cortex p = 0.005, hippocampus p = 0.0217) and α-CTF/FL-APP (T-test cortex p = 0.005 hippocampus p < 0.001). (D) Trisomy did not alter the β-CTF/α-CTF ratio in the cortex (trisomy F(1,37) = 0.65, p = 0.799) or hippocampus (trisomy F(1,37) = 1.082, p = 0.305). (B) Cropped western blot, 4 lanes of an 8 lane gel. Data are represented as mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001.
Trisomy did not alter (A) soluble β-APP abundance (trisomy F(1,24) = 0.790, p = 0.383, trisomy-tgAPP interaction F(1,24) = 0.773, p = 0.388, WT n = 4, Trisomic n = 4, TgAPP n = 13, Trisomic;tgAPP n = 14), (B,C) cortical BACE-1 abundance (trisomy F(1,24) = 0.002, p = 0.963; trisomy-tgAPP interaction F(1,24) = 0.071, p = 0.792, WT n = 6, Trisomic n = 6, TgAPP n = 9, Trisomic;tgAPP n = 7), (D) BACE1 β-secretase activity (trisomy F(1,13) = 0.006, p = 0.941; trisomy-tgAPP interaction F(1,13) = 0.001 p = 0.971, WT n = 6, Trisomic n = 6, TgAPP n = 4, Trisomic;tgAPP n = 5), or (E) soluble α-APP abundance (trisomy F(1,27) = 0.041, p = 0.841; trisomy-tgAPP interaction F(1,27) = 0.002, p = 0.969, WT n = 4, Trisomic n = 4, TgAPP n = 13, Trisomic;tgAPP n = 14). (C) Cropped western blot, 4 lanes of an 8 lane gel. Data are represented as mean ± SEM. Both sexes were analysed and sex was included as a factor in the ANOVA.
Wiseman et al Trisomy of chromosome 21 enhances Aβ deposition

Fig. 6: Trisomy of chromosome 21 does not alter the half-life of extracellular Aβ

(A,B) The *in vivo* half-life of Aβ40, measured by microdialysis of hippocampal interstitial fluid (ISF), was not altered by trisomy of chromosome 21. Compound E injected at 6 hours to halt further Aβ generation (T-test p = 0.258, tgAPP n = 8, Trisomic;tgAPP n = 6 females only). Data are represented as mean ± SEM.
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Fig. 7: Trisomy of chromosome 21 modulates Aβ ratios in vivo independently of modulation of γ-secretase activity

(A,B,C) Tris soluble Aβ38, Aβ40 and Aβ42 were measured by Meso Scale Discovery 6E10 Aβ Triplex assay in 2-month old hippocampus (wildtype (WT) n = 24, Trisomic n = 22, TgAPP n = 25 and Trisomic;tgAPP n = 27). (A) Trisomy decreased Aβ38 (trisomy F(1,4) = 15.403, p = 0.017) and Aβ40 (trisomy F(1,11) = 6.359, p = 0.028), but Aβ42 was not changed (trisomy F(1,11) = 2.978, p = 0.112), (B) resulting in an alteration in the Aβ38/42 ratio (trisomy F(1,4) = 14.553, p = 0.019) and (C) the Aβ40/42 ratio (trisomy F(1,10) = 95.694, p < 0.001). (D) Hippocampal Aβ 40/42 ratio negatively correlates with the relative abundance of aggregated Aβ (multimeric 82E1-82E1 Aβ ELISA) (linear correlation, R^2 = 0.5485, p = 0.0003, tgAPP n = 8, trisomic;tgAPP n = 11). Trisomy did not alter the carboxypeptidase activity of the γ-secretase complex, as measured by (E,G) Aβ38/42 and (F,H) Aβ40/42 ratios produced in vitro by the complex isolated from cortex from (E,F) the Tc1 trisomic mouse (trisomy Aβ38/42 F(1,11) = 4.88, p = 0.499; Aβ42/40 F(1,11) = 0.799, p = 0.395, WT = 9, Trisomic = 9) and (G,H) people with Down syndrome and Alzheimer’s disease (AD-DS) (n = 6) compared with age- and sex-matched individuals who did not have Down syndrome or dementia (control n = 6) (trisomy Aβ38/42 F(1,5) = 0.102, p = 0.763; Aβ42/40 F(1,5) = 0.187, p = 0.684). (A-F) Data are represented as mean ± SEM, (G,H) individual cases plotted, horizontal line indicates mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001. Both male and females were studied and sex was included as a variable in the ANOVA.
Supplementary Fig. 1: Aβ and FL-APP staining in the hippocampus and cortex

(A-E) Aβ and APP staining in the brain of wildtype (WT), Trisomic, tgAPP and Trisomic;tgAPP mice was quantified by immunohistochemistry, using (A,B,C) anti-Aβ antibody (82E1) and (D,E) anti-APP antibody (22C11). Mice were between 4.5-6.5-months of age (mean age for groups: WT = 146 days, Trisomic = 162 days, tgAPP = 159 days, Trisomic;tgAPP = 144 days). Trisomy of chromosome 21 did not significantly alter the area covered by Aβ in (A) cortex (trisomy-tgAPP interaction F(1,31) = 1.267, p = 0.269), (B) the granule cells of the dentate gyrus (trisomy-tgAPP interaction F(1,31) = 2.781, p = 0.105), (C) or in CA1/CA2 pyramidal cells (trisomy-tgAPP interaction F(1,31) = 0.730, p = 0.399). (D,E) Trisomy of chromosome 21 did not significantly alter the area covered by APP staining in cortex (trisomy-tgAPP interaction F(1,31) = 0.053, p = 0.819), the granule cells of the dentate gyrus (trisomy-tgAPP interaction F(1,31) = 0.096, p = 0.756), CA1/CA2 pyramidal cells (trisomy-tgAPP interaction F(1,31) = 0.475, p = 0.496) or CA3 pyramidal cells (trisomy-tgAPP interaction F(1,31) = 2.931, p = 0.097). Data show mean ± SEM. Both sexes were studied and sex was included as a variable in the ANOVA.
Supplementary Figure 2

Supplementary Fig. 2: Aβ deposition in the cortex
Aβ deposition in the cortex of wildtype (WT), Trisomic, tgAPP and Trisomic;tgAPP mice was quantified using anti-β-amyloid antibody (6F/3D) at 16-months of age. Trisomy of chromosome 21 increases the area covered (ANOVA tgAPP-trisomy interaction F(1,37) = 5.803, p = 0.021). Data show mean ± SEM, p < 0.05 = *. Both sexes were studied and sex was included as a variable in the ANOVA.
Supplementary Figure 3
Supplementary Fig. 3: Human Aβ40 and Aβ42 in fractionated cortex

Total cortical proteins, from (A-F) 16-months or (G-L) 6-months of age wildtype (WT), Trisomic, tgAPP and Trisomic;tgAPP mice, were fractionated into (A,D,G,J) tris soluble, (B,E,H,K) 1 % triton soluble, and (C,F,I,L) 5 M guanidine hydrochloride (Gnd) soluble fractions. The amount of human (A,B,C,G,H,I) Aβ40 and (D,E,F,J,K,L) Aβ42 was quantified by ELISA. (F) Trisomy of chromosome 21 significantly increased the amount of 5 M guanidine hydrochloride soluble Aβ42 (ANOVA interaction of tgAPP-trisomy-age F(1,40) = 5.037 p= 0.030) at 16-months of age. No other statistically significant effects of genotype were observed. Data show mean ± SEM, p < 0.05 = *. Both sexes were studied and sex was included as a variable in the ANOVA.
Supplementary Figure 4

(A) The number of arm entries made during this first exposure to the Y-maze was increased in the Trisomic-tgAPP mice, ANOVA revealed a main effect of trisomy (F(1,89) = 17.604, p<0.001), of tgAPP (F(1,89) = 28.519, p < 0.001) and also an interaction of trisomy-tgAPP (F(1,89) = 4.705, p = 0.033) (Bonferroni pairwise comparisons; Trisomic;tgAPP with WT p < 0.001, Trisomic p < 0.001 and tgAPP p < 0.001, and tgAPP with WT p = 0.011). Cohort B 2-3-months of age and Cohort A 6-7-months of age, the effect of genotype was similar in both cohorts so data was combined for analysis, WT n = 45, Trisomic n = 43, TgAPP n = 36, Trisomic;tgAPP n = 26. (B) The total time spent in the other arm (non-start arm) during the Y-maze exposure phase showed a main effect of tgAPP (F(1,89) = 9.570, p = 0.003) and an interaction of tgAPP-trisomy (F(1,89) = 6.972, p = 0.010). (Bonferroni pairwise comparisons Trisomic with tgAPP p = 0.008 and Trisomic;tgAPP p = 0.002). (C) Time spent in the centre of the open field was affected by Trisomy at 6-months of age (ANOVA main effect F(1,78) = 39.044, p < 0.001, Bonferroni pairwise comparisons Trisomic with WT p < 0.001 and tgAPP p < 0.001, Trisomic;tgAPP with WT p < 0.001 and tgAPP p < 0.001). No interaction of Trisomy and tgAPP was found (F(1,78) = 0.074, p = 0.786). Day of exposure to the open field did not significantly alter time in the centre (F(1,78) = 3.106, p = 0.082) (D) Survival to 15 months of age was reduced in Trisomic;tgAPP compared with tgAPP mice (Chi-squared = 3.88, p = 0.048, Cohort A and C WT n = 60, Trisomic n = 53, TgAPP n = 58, Trisomic;tgAPP n = 34). Data are represented as mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001. Both sexes were studied and sex was included as a variable in the ANOVA.
Supplementary Figure 5

Supplementary Fig. 5: Human and mouse APP mRNA levels in cortex and hippocampus

(A, B, C) The amount of human APP (carrying the Swedish mutation) and mouse App transcript was quantified in total RNA isolated from (A) whole cortex or (B, C) hippocampus by quantitative reverse-transcription PCR. The geometric mean of the amount of APP/App transcript detected relative to ACTB and GAPDH transcript level was calculated. (A, B) Trisomy of chromosome 21 did not significantly alter the amount of human mutant APP transcript (cortex T-test p = 0.503, n = 6; hippocampus T-test p = 0.360, n = 6); human mutant APP transcript was not detected in wildtype or Tc1 littermates that did not carry tgAPP. (C) Trisomy of chromosome 21 (ANOVA F(1,26) = 0.243, p = 0.626) or presence of tgAPP (ANOVA F(1,26) = 0.727, p = 0.402, trisomy-tgAPP interaction F(1,26) = 0.781, p = 0.676) did not significantly alter the amount of mouse App transcript detected. Data show geometric mean ± SEM. Both sexes were studied.
Supplementary Figure 6

Supplementary Fig. 6: β-CTF/FL-APP and α-CTF/FL-APP in female cortex and hippocampus (A-D) The ratio of β-CTF/FL-APP and α-CTF/FL-APP in (A,B) cortex and (C,D) hippocampus from female mice was measured by western blot, using anti-APP-C-terminal antibody A8717. (A,C) No difference in the β-CTF/FL-APP ratio (T-test, cortex p = 0.903, tgAPP n = 7, Trisomic:tgAPP n = 9; hippocampus p = 0.796, tgAPP n = 10, Trisomic:tgAPP n = 9) or the (B,D) α-CTF/FL-APP ratio (T-test, cortex p = 0.396, tgAPP n = 7, Trisomic:tgAPP n = 9, hippocampus p = 0.312, tgAPP n = 10, Trisomic:tgAPP n = 7) was observed between tgAPP and Trisomic:tgAPP females. Data show mean ± SEM.
Supplementary Fig. 7: The amount of insulin degrading enzyme and neprilysin in cortex
(A, B) The amount of (A) insulin degrading enzyme (IDE) and (B) neprilysin in total cortical proteins was measured by western blot, an anti-β-actin antibody was used as a control for protein loading. Trisomy did not significantly alter the amount of the Aβ clearance enzymes (ANOVA IDE trisomy F(1,14) = 0.108, p = 0.748, trisomy-tgAPP interaction F(1,14) = 0.110, p = 0.745, neprilysin trisomy F(1,23) = 0.041, p = 0.841, trisomy-tgAPP interaction F(1,23) = 0.076, p = 0.786). Cropped western blots 4 lanes of a 8 lane gel. Data show mean ± SEM. Both sexes were analysed.
Supplementary table 1
Cases of human frontal cortex used for the study of γ-secretase activity

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