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Sex-related dimorphism in dentate gyrus atrophy and behavioral phenotypes in an inducible tTa:APPsi transgenic model of Alzheimer's disease

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Abstract

Sex differences are a well-known phenomenon in Alzheimer's disease (AD), with women having a higher risk for AD than men. Many AD mouse models display a similar sex-dependent pattern, with females showing earlier cognitive deficits and more severe neuropathology than males. However, whether those differences are relevant to human disease is unclear. Here we show that in AD mouse models that overexpress amyloid precursor protein (APP) under control of the prion protein promoter (PrP), female transgenic mice have higher APP expression than males, complicating interpretations of the role of sex-related factors in such models. By contrast, in a tTa:APPsi model, in which APP expression is driven by the tetracycline transactivator (tTa) from the CaMKII α promoter, there are no sex-related differences in expression or processing of APP. In addition, the levels of A β dimers and tetramers, as well as A β peptide accumulation, are similar between sexes. Behavioral testing demonstrated that both male and female tTa:APPsi mice develop age-dependent deficits in spatial recognition memory and conditional freezing to context. These cognitive deficits were accompanied by habituation-associated hyperlocomotion and startle hyper-reactivity. Significant sex-related dimorphisms were observed, due to females showing earlier onsets of the deficits in conditioned freezing and hyperlocomotion. In addition, tTa:APPsi males but not females demonstrated a lack of novelty-induced activation. Both males and females showed atrophy of the dentate gyrus (DG) of the dorsal hippocampus, associated with widening of the pyramidal layer of the CA1 area in both sexes. Ventral DG was preserved. Sex-related differences were limited to the DG, with females showing more advanced degeneration than males. Collectively, our data show that the tTa:APPsi model is characterized by a lack of sex-

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Appendix A. Supplementary data

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related differences in APP expression, making this model useful in deciphering the mechanisms of sex differences in AD pathogenesis. Sex-related dimorphisms observed in this model under conditions of equal APP expression between sexes suggest a higher sensitivity of females to the effects of APP and/or A β production.

Keywords

gender differences; sex dimorphism; APP expression; A β oligomers; dorsal dentate gyrus; contextual fear; habituation-associated hyperactivity; novelty-induced activation; tTa effects

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory loss, cognitive deficits and behavioral changes (Sperling et al., 2011). β -amyloid (A β) plaques, neurofibrillary tangles, and neuronal degeneration are main neuropathological hallmarks of the disease (Hyman et al., 2012; Nelson et al., 2012). Although there is no consensus yet as to what causes the disease, one of the risk factors that is universally accepted is female gender with women making up over two thirds of AD patients (Hebert et al., 2013). Epidemiological studies indicate that women bear a greater burden of AD, due to increased prevalence and, possibly, incidence of the disease rev in (Lin and Doraiswamy, 2014). Women with mild cognitive impairment (MCI) or AD have greater longitudinal rates of cognitive and functional progression than men (Li and Singh, 2014; Lin et al., 2015; Mielke et al., 2014; Pusswald et al., 2015). Studies that utilize imaging techniques revealed significant sex-related dimorphism in the development of atrophy as assessed by MRI in various gray matter regions in patients with AD or MCI (Hua et al., 2010; Skup et al., 2011). Importantly, the imaging studies indicate that atrophy develops at an earlier stage of cognitive deterioration in women than in men. These findings are in agreement with earlier neuropathological studies showing a stronger relation between pathology and clinical AD in women than in men (Barnes et al., 2005; Kraszpulski et al., 2001). With each additional unit of global AD pathology (neuritic plaques) there is a >2-fold increase in the odds of clinical AD in men compared with a >11-fold increase in women (Barnes et al., 2005). In addition, the reduction in the global cognitive score is twice as large in women as in men (Barnes et al., 2005). Understanding why the association between AD pathology and dementia differs in men and women could yield important clues about the pathophysiology of AD and eventually lead to sex-specific preventive or therapeutic strategies.

Genetic and pathologic studies of AD have implicated changes in A β amyloid processing and deposition as early events in the disease (Golde et al., 2011; Hardy and Selkoe, 2002; Herrup, 2010; Hutton and McGowan, 2004; Sperling et al., 2011)}. A number of transgenic mouse models have been created that overexpress amyloid precursor protein (APP) and successfully reproduce A β deposition and age-associated cognitive deficits (Ashe and Zahs, 2010; Eriksen and Janus, 2007; Savonenko and Borchelt, 2008; Webster et al., 2014). Interestingly, in APP transgenic mice with different strain backgrounds, promoters, and AD-linked mutations, female mice tend to show more severe plaque pathology than males (Callahan et al., 2001; Hirata-Fukae et al., 2008; Hutter-Paier et al., 2004; Kulnane and

Lamb, 2001; LaClair et al., 2013; Lee et al., 2002; Perez et al., 2011; Wang et al., 2003). There have been relatively few studies addressing sex differences in cognitive deficits; these have pointed to the development of more severe or earlier declines in female APP transgenic mice than in males (Berger-Sweeney et al., 1995; Clinton et al., 2007; King and Arendash, 2002; King et al., 1999; Pistell et al., 2008). However, in most AD models, sex differences in cognitive deficits are not well documented, making it difficult to relate their cause to dimorphism in plaque load, oligomeric A β or levels of expression from the transgenes. The latter is particularly important since sex-related differences in transgene expression, if present, introduce an additional factor that might not be related to the sex differences observed in human disease.

In this study, we used a number of different lines and models that overexpress mutated APP to analyze sex-related differences in transgene expression. We demonstrated that models in which an APP transgene is driven by the prion protein (PrP) promoter show significant sex dimorphism, with females expressing higher levels of APP. A model that expresses APP under conditional control from the CaMKII α promoter lacked sex-related differences in APP expression and A β accumulation. This model, however, demonstrated sex differences in AD-related phenotype, with females showing greater extent of atrophy in the dentate gyrus of the hippocampus and an earlier onset of hyperactivity than males. These data indicated that, in a model providing equal expression of APP between sexes, females accumulated an equal amount of A β but had higher sensitivity to the toxic effects of APP/A β production than males.

2. Methods

2.1. Animals

All experimental procedures involving animals were conducted according to NIH guidelines for animal care and approved by the JHU Medical School Institutional Animal Care and Use Committee.

Two transgenic APP mouse models expressing transgenes under the control of mouse prion protein (PrP) promoter were generated as described (Borchelt et al., 1996a, 1996b; Jankowsky et al., 2004). Briefly, line C3–3 express single chimeric mouse/human mutant amyloid precursor protein transgene with the Swedish mutations (APP695swe) (JAX MMRRC: B6.Cg-Tg(APP695)3Dbo). Line 85 co-expresses the APP695swe transgene together with PS1 E9 mutant gene (independently regulated by PrP) to facilitate A β production (JAX MMRRC: B6.Cg_Tg (APPswe, Psen1dE9) 85Dbo).

Two inducible transgenic APP models that express transgenes under the control of tetracycline-responsive promoter were generated as described (Jankowsky et al., 2005b). Line 8–85 (JAX MMRRC: B6.Cg-Tg(tetO-APPSwInd)885Dbo) and line 107 (JAX MMRRC: B6.Cg-Tg(tetO-APPSwInd)107Dbo) express the same chimeric mouse/human APP695 transgene bearing the Swedish (KM570/571NL) and Indiana (V617F) mutations (APPsi) under the control of a tetracycline-responsive promoter. Both lines were crossed with mice expressing tetracycline-controlled transactivator (tTa) under regulation of the calcium calmodulin-dependent kinase II (CaMK2 α) promoter (JAX MMRRC: B6.Cg-

Tg(Camk2a-tTA)1Mmay/DboJ). Due to specificity of the CaMK2 α promoter, expression of APPsi in both 8–85 and 107 lines is limited to the forebrain neurons. APPsi expression in CaMKII-tTa:APPsi mice can be inhibited by administration doxycycline (DOX) in diet (200 mg of DOX/kg, Bio-Serve, Flemington, NJ). Mice from line107 that were used for behavioral testing were kept on DOX diet throughout development until 4 weeks of age. Preventing expression of the transgenes during development has been shown to ameliorate unwanted side effects of APP and/or tTa on behavioral phenotypes and hippocampus morphology (Han et al., 2012; Melnikova et al., 2013; Rodgers et al., 2012). All mice had free access to food and water and were housed in automatically controlled light conditions (light 7:00 A.M. to 9:00 P.M.).

All mice were maintained on C57BL/6 background. Animals of both sexes were included in the study; sex- and age-matched littermates were used as controls.

2.2. Brain extraction and tissue processing

Mice were intracardially perfused with cold PBS under deep anesthesia; the brains were removed and cut along the midline. The right half of the brain was taken for histology and fixed in 4% paraformaldehyde solution in PBS for 24 h at 4 °C. The left hemisphere was dissected to isolate the cortex and hippocampus for biochemical analyses. The brain samples were immediately frozen on dry ice, and stored at –80 °C until use. Frozen samples of the cortex were homogenized by sonication in 10 volume (*w/v*) of cold PBS containing protease inhibitors cocktail (Roshe, Indianapolis, Ind) and the homogenates were aliquoted for further biochemical analyses. Samples of the hippocampus were prepared in RIPA buffer (Sigma) as described (Melnikova et al., 2013).

2.3. Immunoblot analyses

For western blot analysis homogenates from the cortex and hippocampus were centrifuged at 13,000 rpm for 15 min at 4 °C and supernatant was used for immunoblotting and BCA protein assay. Proteins were separated on NuPAGE® Bis-Tris 4–12% gradient gels in MES-SDS running buffer (Life Technologies) and transferred to nitrocellulose membranes by using iBlot® Dry Transfer System (Life Technologies). Membranes were blocked with 3% non-fat milk and incubated with anti-human 6E10 antibody (Covance); anti-human sAPP β -Swe, 6 A1 antibody (IBL, Japan); anti-APP, C-term antibody (Millipore, Billerica, MA), or anti-human amyloid β (N-term) 82E1 (IBL, Japan) primary antibodies. For loading control anti-actin, anti-tubulin or anti-GAPDH antibodies (Cell Signaling, Danvers, MA) were used. Images were taken using Chemo-Doc imaging system (Bio-Rad) or film developer and signals quantified using an open-source image processing package Fiji ([Fiji Is Just] ImageJ) (Schindelin et al., 2012).

2.4. A β ELISA

For measuring total A β level, an aliquot of PBS homogenate was mixed with 8 M guanidine HCl, 50 mM Tris buffer to get final 5 M guanidine solution. The samples were incubated for 4 h at room temperature. Human A β 40 and A β 42 were measured by the β -Amyloid (1–40) or (1–42) ELISA Kit (Invitrogen) following the manufacture protocols.

2.5. Assessment of degeneration of the Dentate Gyrus of the hippocampus

For histological study, the right hemisphere was fixed in cold 4% paraformaldehyde for 24 h, washed in PBS and cryoprotected in 15% and 30% sucrose solutions and then frozen on a dry ice cooled isopentane, and stored at -80°C until use. Brain was serially cut on a cryotome. Frozen sagittal sections ($30\text{ }\mu\text{m}$) were mounted onto slides for cresyl violet staining and processed in the following steps: a lipid removal by xylene, rehydration in alcohols (100%, 95%, and 70%) and water, then 30 s staining in warm cresyl violet solution. Sections were differentiated in 70% alcohol, dehydrated and mounted for further analysis. For each animal 6–7 sections were analyzed using the Stereo Investigator Optical Dissector software from MBF Biosciences (Stereo Investigator System, Version 7, 2007 MicroBrightField, Inc., Williston, VT). Sections were sampled with a random start position (around 0.24–0.36 lateral coordinates, determined using a mouse brain atlas) (Franklin and Paxinos, 2007) and evenly distributed between 0.24 and 2.5 mm lateral coordinates (a mean of 11 sections per animal). Per section, the width of the granular layer of the dentate gyrus (DG) was measured at three different points for each the supra- and intra- blades and the average was calculated. In addition, the length of both blades of the DG was measured. The width of the pyramidal layer of the CA1 area (at three different points for each section) and the width of the cortex (above the middle point of DG) were also recorded. For each variable, raw measures were standardized across all genotypes and sexes.

2.6. Behavioral Testing

2.6.1. Experimental design—Mice were handled 5 min daily for three days each before start of the behavioral testing. Behavioral testing was conducted during the light phase of circadian cycle in cohorts of 10–15 mice balanced by genotype. Before each test, mice were moved to the testing room and allowed to habituate to the new location for at least 1 h before behavioral testing. Tests were conducted for mice of 5–6 or 11–12 months of age with a 2–3 days interval between different tasks. The tasks were run in the order described below. After the end of behavioral testing mice were sacrificed and brains harvested for quantification of A β peptide levels and hippocampal morphometry.

2.6.1.1. Novelty-induced and habituation-associated motor activity. Locomotor activity of mice was tested in a set of environments differing in size and familiarity. Home cage represented a familiar environment. 2 days after bedding change, cage mates were removed from the home cage into a waiting cage. 20 min later, one of the mice was returned to the cage and its activity was registered for 2 min. The mouse was then removed to a second waiting cage until all mice were tested. Two open fields with different levels of aversiveness were used to allow for a dissociation of changes in hyperactivity and emotionality as described in (Melnikova et al., 2006). A small open field consisted of squared arena (Lucite, $55 \times 55\text{ cm}$) surrounded by walls (30 cm high) and placed in a room with dimmed light. The second, big open field was a white highly illuminated circular arena (diameter 100 cm) with 60 cm high walls. Each animal performed one daily trial in each of the fields. The trial began with the mouse being released near the wall of the open field and lasted for 30 or 10 min for small and big open fields, respectively. Following each trial, the fields were cleaned with a dilute alcohol solution and dried with a paper towel. Performance was recorded by a computer-based video tracking system (HVS Image Analysis VP-200,

HVS Image, Hampton, England). Activity measures included distance travelled, percent time spent in active exploration (episodes of movement ≥ 5 cm/s), and speed of movement during active exploration. To analyze anxiety levels, the activity measures were broken down into two zones: central and periphery. The number of entries and time spent in the zones of the Open field was calculated. Percent of time spent in periphery (thigmotaxis) was used as a measure of anxiety.

2.6.2. Spatial Recognition Memory in a Two-Trial Y maze—The two-trial Y maze task was conducted as described (Melnikova et al., 2013, 2006). Y maze measured 48×7 cm with 2 cm high borders. During trial 1, one of the arms of the maze was blocked, allowing exploration of only two arms of the maze. After a 30-min delay, trial 2 was conducted in which all three arms were available for exploration. Trial 2 took advantage of the innate tendency of mice to explore novel areas, and mice with intact spatial recognition memory will prefer to explore a novel arm over familiar arms, whereas mice with impaired spatial memory will enter all arms equally. Thus, trial 2 represented a classic test for spatial recognition memory as described in (Dellu et al., 2000). Motor activity was recorded as number of arms visited for a whole duration of the trials (5 min each). Behavior was scored by trained observers blind to genotype using a computer-assisted data acquisition system (Stopwatch+; <http://www.cbn-atl.org/organization/stopwatch.html>).

2.6.3. Prepulse Inhibition of Acoustic Startle reaction—Prepulse Inhibition of acoustic startle reaction was conducted in a startle soundproof chamber (Model SR-LAB, San-Diego Instruments, CA) as described in (Savonenko et al., 2008). Briefly, after a 6-min acclimation period, the mouse was exposed to three 25-msec startle pulses of 120 dB white noise to determine the initial level of acoustic startle reaction (ASR). Mice then received 6 blocks of 8 trials each to measure the prepulse inhibition (PPI). Each block of trials consisted of 6 different types of trial presented pseudo randomly across blocks: two types of trial were with startle pulse only (120 dB or 110 dB conducted 2 times in each block of trials); and four different types of trials in which prepulses were followed by the startle stimuli (one trial for each of the pre-pulse and startle intensities). The pre-pulses were 25 ms weak stimuli of white noise with intensities of 4 or 8 dB above the background, a 63 dB white noise. The time interval between the pre-pulse and the startle pulse onsets was 100 ms. Trials were presented at a variable-interval schedule of 20–40 s. The maximal amplitude of the reaction and its latency was recorded for each pre-pulse (during the interval 0–100 ms) and pulse stimulus (during the interval 100–200 ms). The average value for every type of trial across 6 blocks was used for the statistical analysis. Startle reactivity was analyzed by using maximal amplitudes of reactions to pre-pulse (68 dB and 72 dB) and pulse stimuli (110 dB and 120 dB). PPI was measured as a percentage of ASR inhibition induced by each prepulse and was calculated as $[100 \times (\text{Startle amplitude in the startle alone trial} - \text{Startle amplitude in the prepulse trial}) / \text{Startle amplitude in the startle alone trial}]$.

2.6.4. Contextual fear conditioning—Contextual fear conditioning was conducted in a mouse training chamber (Stoelting Co., Wood Dale, IL) as described in (LaClair et al., 2013). Testing and data collection were automated by ANY-Maze 4.72 software (Stoelting Co., Wood Dale, IL) according to the following protocol parameters: Light in the chamber

1.5 visible +1.5 Infrared, background noise 65 dB, smell 30% Ethanol, Minimum Freezing Duration = 1000 msec. In the Training Session, mice were placed in the test chamber and allowed to explore for 30 s, during which the level of “pretraining” freezing was measured. Mice were then exposed to the unconditioned stimulus (US, 0.6 mA) for two seconds. Mice remained in the chamber for an additional 35 s to measure immediate freezing response. 24 (session 1) and 48 h (session2) later, mice were placed in the same test chamber for four minutes without US administration, and time spent freezing was measured. Levels of freezing were measured throughout all sessions and averaged over blocks of 20 s.

2.7. Statistical Analyses

The data were analyzed using the statistical package STATISTICA 12 (StatSoft, Inc., Tulsa, OK) and a minimal level of significance $p < 0.05$. The data from Western blot quantification were analyzed using a two-sided t -test to assess a significance of sex effect. Correlations between levels of different APP metabolites were initially assessed using Pearson correlations with p levels adjusted by a number of correlations tested (the classical Bonferroni correction, $p < 0.005$) (McDonald, 2014; Morikawa et al., 1996). Then, to control for the effects of multiple correlations (details of statistical results are presented in Supplementary Table 1), the semi-partial correlations were computed using General Regression (GR) models. The squared semi-partial correlation is the proportion of variance accounted for by the predictor, relative to the total variance. Adjusted p level for semi-partial correlations was 0.005. The data from ELISA measurements of A β 40 and A β 42 levels were analyzed using analyses of covariance (ANCOVA) with main effect of sex and age as a continuous covariate. The morphometric measures of the hippocampus and cortex were analyzed using two-way (main effect) ANCOVA with Sex and Genotype as independent factors and Age as a covariate. An HSD post-hoc test (Tukey HSD test for unequal N) was applied to significant main effect or their interaction to find differences between a particular set of means.

The data from behavioral tests in 5–6 or 11–12 month-old mice were analyzed using two or three-way repeated measures ANOVA with Sex and Genotype as independent factors and a repeated measure, RM (arms, trials or blocks, etc). Sex $\times$ Genotype, Genotype $\times$ RM, and Sex $\times$ RM interactions were set as orthogonal. LSD post-hoc test was applied to significant Sex $\times$ Genotype or Genotype $\times$ RM interactions to evaluate differences between a particular set of means. For testing of novelty-induced and habituation-associated motor activity we performed planned between-genotype comparisons for each sex separately using the first two and the last two time periods in the open fields (adjusted p level < 0.0125). The details of statistical results for behavioral data are presented in Supplementary Tables 1–2. Based on our previous experience, the behavioral measures may be highly correlated making direct application of Bonferroni correction potentially very conservative and resulting in a high false negative rate. At the present, early phase of discovery, we have opted to interpret findings relative to the number of spurious findings expected under a global null hypothesis (see legend to Supplementary Tables 2–3). Data in figures represent means $\pm$ standard error of means (SEM) unless otherwise noted.

3. Results

3.1. APP expression and processing in PrP-APP^{swe} and CaMKII α :APP^{si} models

Transgenic models of AD-related amyloidosis express amyloid precursor protein (APP) under control of different promoters, resulting in patterns of expression that are not necessarily fully congruent with expression of APP from its natural promoter. Here we used different APP models in which APP expression is driven by prion protein promoter (PrP) or calmodulin kinase II promoter (CaMKII α) and analyzed the effect of sex on APP expression from these promoters. PrP-APP models had significant sex-related differences, with females showing higher levels of full-length APP (FL-APP) than males (Fig. 1). These differences were observed consistently across different lines, as shown here for transgenic lines C3–3 and 85 (Fig. 1A–B,E), indicating that higher APP expression observed in females of the PrP-APP models is not likely an effect of random gene-insertion. APP can be sequentially processed by β - and γ -secretases, resulting in the generation of A β peptides (Vassar et al., 2014). Importantly, the first cleavage of APP by β -secretase, which produces N- and C-terminal fragments of APP (β CTF), is a rate-limiting step in A β generation (Vassar et al., 2014). Higher levels of FL-APP in females may thus result in increased levels of β CTF and, in turn, in increased levels of A β . Indeed, as demonstrated for line 85 (Fig. 1B,E), female PrP-APP transgenic mice have higher levels of β CTF and A β peptides.

These findings prompted us to evaluate the extent to which sex differences can be observed in mice that have APP driven by tTa transactivator expressed from CaMKII α promoter (Jankowsky et al., 2005b). We found that there were no sex-related differences in the levels of FL-APP as documented here in two different CaMKII-tTa:APP^{si} lines, 8–85 and 107 (Fig. 1C–E). Thus, in contrast to the PrP-APP model, CaMKII-tTa:APP^{si} mice can serve as a model in which sex-related differences in A β pathology can be studied without complicating effects of sex-dependent variability in expression of APP.

We next focused on using line 107 of CaMKII-tTa:APP^{si} mice and addressed possible sex effects on APP processing by β - and γ -secretases. Given the equal levels of FL-APP in males and females in this model (see Figs. 1D and 2A for cortical and hippocampal samples, respectively), the effect of sex on β -secretase activity was evaluated by measuring levels of soluble N-terminal fragments of APP (NTF), namely β APPs. To characterize β APPs derived from transgenic APP rather than native APP we used an antibody raised against NTFs and specific to the Swedish mutation present in transgenic APP (sAPP β -sw, 6 A1, IBL Co, Japan). As shown in Fig. 2, levels of β_{swe} APPs were similar between males and females. Another product of β -secretase activity, C-terminal fragment CTF- β , was detected by an antibody specific to a C-terminus of APP (A8717, Sigma, St. Louis, MO). This antibody also detects a product from α -secretase cleavage, CTF- α . Western analyses of CTFs with this antibody revealed no significant differences between males and females (Fig. 2A–B). These data suggest that, in the CaMKII-tTa:APP^{si} model, APP processing by β - and α -secretases is not significantly affected by sex-related factors.

3.2. CaMKII α Ta:APPsi model displays no sex dimorphism in levels of A β monomers or oligomers

We then evaluated the effect of sex on levels of A β monomers using Western blotting with an end-specific anti-A β _{1–5} antibody (82E1, IBL Co, Japan). No differences related to sex were detected (Fig. 3). In addition to the band of monomeric A β , 82E1 antibody revealed a series of low molecular mass species of sizes consistent with β CTF (~14 kDa, marked with asterisks in Fig. 3A–C), A β dimers and tetramers (Fig. 3A–B). Detection of A β trimers was not possible due to an overlap with the β CTF band (Fig. 3A–B). Densitometry of bands with sizes consistent with A β dimers and tetramers revealed a consistent trend for males to have lower levels of these A β species; however, this change did not reach significance (Fig. 3D). Similar results were obtained with anti-A β _{1–6} antibody (6E10, Covance, Emeryville, CA) (Fig. S1 A–B).

Detection of A β monomers, dimers, tetramers and CTFs was done in the same ~20 brain samples that in addition to a relatively high within-group variability allowed for analyses of correlations between these species. Pearson correlation revealed that most of these species were significantly correlated with each other in both sexes (Supplementary Table 1). The strength of the correlations varied, with strongest correlation observed between A β dimers and tetramers (93% of variability explained, R^2) (Fig. 3E–F) and weakest correlation between β CTF and A β monomers (31% of variability explained, R^2) (Fig. S1).

3.3. Age-related accumulation of A β peptides and plaques in the CaMKII α Ta:APPsi model (Line 107)

An inspection of A β plaques in the cortex and hippocampus revealed comparable levels of plaques in males and females (Fig. 4A). Given a large set of brains used in this study ($n = 48$), statistical confirmation of this observation would require a quantitative stereological analysis and be very time-consuming. Instead we used ELISA assays (Biosource, US) that better fit for a high-throughput analysis (Fig. 4B–C). The ELISA analyses showed no sex-related differences in accumulation of A β peptides (ANCOVA with age as a covariate, effect of sex $p > 0.6$, see inserts Fig. 4B–C). A “sensitivity” analyses in which the three oldest subjects were removed (see Fig. 4B–C) yielded results similar to the original analysis. As aging progressed, significant increases were observed for both A β ₄₀ and A β ₄₂ peptides (ANCOVA with age as a covariate, effect of covariate $F(1,50) = 28.73$, $p < 0.0001$ and $F(1,56) = 28.65$, $p < 0.0001$ for A β ₄₀ and A β ₄₂, respectively) (Fig. 4B). As expected from the presence of the Indiana mutation in transgenic APP and in agreement with previous publications on CaMKII α Ta:APPsi models (Fowler et al., 2014; Jankowsky et al., 2005b), levels of A β ₄₂ peptides were significantly higher than those of A β ₄₀ peptides (one-way ANCOVA, $F(1,56) = 481$, $p < 0.00001$).

3.4. Atrophy of the Dentate Gyrus of the hippocampus and its relation to sex and A β accumulation

Previous studies in line 102 of the CaMKII α Ta:APPsi model demonstrated a dramatic degeneration of the dentate gyrus (DG) of the hippocampus in tTa:APP transgenic mice (Han et al., 2012). Therefore, we tested whether line 107 of the same model expresses a similar phenotype, and whether it shows any sex differences or correlations with

accumulation of A β peptides. Serial sagittal brain sections from non-transgenic controls (NTg), single tTa transgenic and tTa:APP transgenic mice were stained with cresyl violet. Single APP transgenic mice were not included in this study, since our pilot study showed similar morphology between NTG and APP mice (not shown).

An inspection of the dorsal hippocampus revealed an apparent atrophy of DG in tTa:APP mice as expected from the previous studies (Fig. 5). However, morphology of DG of the ventral hippocampus was preserved (Fig. S2), a finding indicating a subregion-specific selectivity in the DG atrophy. We focused our morphometric studies on the dorsal hippocampus. The width and length of the infra and supra blades of DG, width of the pyramidal layer of the CA1 area of the hippocampus, and width of the cortex above the hippocampus were analyzed in the range of lateral coordinates from 0.2 to 2.4 mm (Fig. 6A). Raw data (as shown for the DG width, Fig. 6A) were segregated into intervals on the lateral axes, transformed to z scores within each interval and then an average of Z scores was calculated for each mouse across all lateral intervals (Fig. 6B). This approach allowed for pulling out the variability due to different lateral levels while preserving the variabilities due to genotype, sex and/or age. ANCOVA with age as a covariance and genotype as a main factor revealed significant effect of genotype for measures of DG and CA1 area ($F(2,68) = 26.58 \div 110.8$, $p < 0.0000$). The tTa:APPsi transgenic mice demonstrated narrower and shorter DG blades (a granular cell layer) than NTg and tTa mice (HSD post-hoc test, $p < 0.00001$) (Fig. 6C). The atrophy observed in DG of the tTa:APP mice coincided with the increased width of the pyramidal cell layer of the CA1 area as compared to non-transgenic controls (HSD post-hoc test, $p < 0.0002$) (Fig. 6C). Importantly, the effect of covariate (age) was not significant for any variable ($F(1,68) < 2.40$, $p > 0.13$). These results indicate that the genotype-related differences in morphological measures reach their statistically maximal expression already at the youngest age investigated (4–5 months of age) (Fig. 6B). In agreement with these findings, analyses of correlations between measures of DG in the tTa:APPsi mice and levels of A β 40 and A β 42 peptides in guanidine fraction of the hippocampus (ELISA) yielded no significant correlations ($p > 0.13$). An inspection of the hippocampus in mice shortly after transgene activation revealed normal hippocampal morphology in all genotypes (Fig. S3) indicating that DG atrophy developed already after the initiation of APP expression.

To examine contribution of sex-related factors to the morphological changes of the hippocampus, two-way genotype $\times$ sex ANOVAs were utilized. More pronounced DG atrophy was revealed in female tTa:APPsi mice, as assessed by width and length of the DG granular cell layer [Fig. 6C; significant effects of genotype ($F(2,66) = 88.81 \div 108.43$, $p < 0.00000$) and genotype $\times$ sex interactions ($F(2,66) = 6.49 \div 6.63$, $p < 0.003$)].

In agreement with previous findings (Han et al., 2012), single tTa transgenic mice on a C57BL6/J background were not different from non-transgenic controls (NTg) in width of the DG granular layer (Fig. 6C). However, length of the DG granular cell layer was shorter in tTa mice as compared to NTg mice, reaching significance in male mice (Fig. 6C). In addition, male tTa mice were different from male NTg in the width of the pyramidal cell layer of the CA1 area (Fig. 6C). These findings indicate that if tTa mice are used as controls, male tTa:APPsi mice would demonstrate significant morphological changes only in one

measure, width of the DG. In contrast to males, female tTa:APPsi mice show significant differences from tTa controls in both measures of the DG and in the width of the CA1 area.

3.5. Behavioral phenotypes as related to sex and A β load

Cohorts of male and female mice were tested in a set of cognitive and behavioral tasks at 5–6 or 11–12 months of age. tTa:APPsi mice were compared to control groups of sex-matched littermates that included non-transgenic, tTa and APP single transgenic mice. Behavioral characteristics of the three control genotypes are presented in Supplementary Fig. 4.

3.5.1. Contextual fear memory—We used a one-trial contextual fear paradigm as a task that is particularly sensitive to disruptions in the trisynaptic pathway (DG-CA3-CA1) of the hippocampus (McHugh and Tonegawa, 2009; Nakashiba et al., 2008). Mice were placed in a novel chamber and after one minute of exploration a single mild footshock was delivered through a grid floor. Freezing as a measure of fear was increased at the end of the training session; however, no age-, sex- or genotype-related differences were observed (data not shown). 24 and 48 h later mice were tested for long-term fear memory of context by placing them in the same apparatus (Fig. 7A–F). At 5–6 months of age, there were no deficits in the levels of freezing in tTa:APPsi male or female mice as compared to their sex-matched control littermates (Fig. 6A–B). However, levels of freezing in tTa:APPsi females were significantly lower than in tTa:APPsi males ($p < 0.006$, Fig. 7B) (significant effect of sex and sex-genotype interaction in ANOVA, see Table S2 for statistics). Similar differences were observed in the average duration of freezing reaction, with tTa:APPsi females showing shorter freezing reactions than tTa:APPsi males ($p < 0.007$; Fig. 7C). Thus, at 5–6 mo of age, a stage with rare A β plaques (Fig. 4A) and practically undetectable levels of SDS-soluble A β oligomers (not shown), tTa:APPsi females demonstrated weaker long-term contextual fear memory as compared to tTa:APPsi males. At an older age (11–12 months), when high levels of A β plaques and oligomers had accumulated in the cortex and hippocampus (Fig. 3,4), tTa:APPsi mice of both sexes demonstrated significant deficits in total levels of freezing (Fig. 7D) and average duration of freezing reaction (Fig. 7F). These impairments were not significantly modulated by the effect of sex (Fig. 7E–F; Table S2).

3.5.2. Spatial Recognition Memory—A two-trial Y maze task was used to assess spatial recognition memory after a short-term delay. Performance in this task can be affected by lesions in a number of brain structures, including cortex and hippocampus, and requires the CA3 area of the hippocampus in particular (Shipton et al., 2014). At 5–6 months of age, tTa:APPsi mice differed from control mice in their arm preferences (Fig. 7G). The control mice, as expected, showed a clear preference for the new arm of the maze. tTa:APPsi mice, however, investigated equally the new and start arms, so that they spent a lower percentage of time in the new arm compared to controls (Fig. 7H). This deficit was not modulated by the effect of sex (Fig. 7H, Table S2).

Preference for the start arm observed in tTa:APPsi mice was reminiscent of that in tTa single transgenic females (Fig. S4B). tTa females also showed deficits in preference for the new arm as compared to non-transgenic or APP single transgenic females (Fig. S4C). However, tTa:APPsi females with low A β levels were not deficient in this measure, implying that tTa

expression in tTa:APPsi mice might not result in the same phenotype as in tTa single transgenic mice. In addition, since male tTa mice did not show a preference for the start arm, tTa expression is an unlikely reason for such a preference in tTa:APPsi males. We incorporated testing for anxiety (see below) to analyze whether preference for the start arm observed in tTa:APPsi mice coincided with any alterations in anxiety levels.

Testing of tTa:APPsi mice at 11–12 months of age revealed significant deficits in spatial recognition memory, documented by their lack of preferences in the maze (Fig. 7K–L). Some of these mice were sacrificed shortly after the end of behavioral testing. Analysis of performances in mice with high and low A β load detected lack of any spatial preferences in both groups (Fig. S4C–D).

3.5.3. Novelty-induced and habituation-associated locomotion—Locomotor activity of mice was tested in a set of environments differing in size and familiarity. Big open field (OF), small OF and home cage represented progressively less anxiogenic environments. Home cage, in contrast to open fields, represented a familiar environment. In both age groups, tTa:APPsi and control mice demonstrated different dynamics of motor activation in the open fields, with only controls showing within-session changes in locomotion consistent with habituation (Fig. 8A,C,E–F, Table S3). Between-genotype differences were consistently observed in the first 1–2 min of exploration of open fields due to a lack of novelty-induced activation in tTa:APPsi mice. This deficit was statistically confirmed only in male mice (Fig. 8A,C,E–F). Initial motor activity of male tTa:APPsi mice was not decreased when tested in a familiar home cage (Fig. 8G), supporting the role of novelty in expression of the phenotype. A similar phenotype was observed in tTa single transgenic males (Fig. S4D–E), implying that the lack of novelty-induced activation in tTa:APPsi males is a result of tTa expression.

Between-genotype differences in motor activity were also revealed at the end of the testing period in the small open field. In this small environment, the period of novelty exploration was shorter than in the big OF (as judged by the performance of control mice), allowing for observation of motor activity after habituation to the environment (Fig. 8A–F). tTa:APPsi mice demonstrated age-related hyperactivity, with statistical significance confirmed in 11–12 months-old mice (Fig. 8F). Hyperactivity was more pronounced in tTa:APPsi females, starting earlier during the testing period. Testing in a familiar home cage also revealed hyperactivity in tTa:APPsi mice, with females showing an earlier onset of hyperactivity (at 5–6 months of age) than males (Fig. 8G).

3.5.4. Anxiety levels—Testing of anxiety in the big open field revealed increased anxiety levels in tTa:APPsi mice in both ages and sexes (Fig. 7B,D). To test whether increased anxiety in tTa:APPsi mice might result in a lack of novelty-induced activation, we analyzed correlations between activity levels in 1st minute of exploration and thigmotaxis in this task. No significant correlations were found in either age or genotype ($R = -0.19 \div 0.34$, $p > 0.15$). In contrast to novelty-induced activation, anxiety levels were not affected by expression of tTa in tTa single transgenic mice. These findings suggest that the increased anxiety observed in tTa:APPsi mice is a phenotype independent from the deficit in novelty-induced activation.

In addition, a possible contribution of increased anxiety in tTa:APPsi mice to their preference for the start arm of the Y maze (Fig. 6G,K) was assessed by correlations between thigmotaxis and time spent in the start arm. No significant correlations were found in either age or genotype ($R = -0.28 \div -0.10$, $p > 0.20$).

3.5.5. Startle reactivity and prepulse inhibition—Mice were tested for startle reactivity and plasticity to analyze possible changes in regulation of sensory input and its inhibition known to be affected in Alzheimer's disease (Thomas et al., 2010). Prepulse inhibition (PPI) represents the effects of a weak sound (prepulse) preceding a strong acoustic stimulus (pulse) on startle response and is a classical measure of sensorimotor gating in preclinical models (Cromwell and Atchley, 2015; Cromwell et al., 2008; Powell et al., 2009). tTa:APPsi mice of both sexes showed an age-related deficit in prepulse inhibition that was significant at 11–12 months of age (Fig. 8H, Table S3). This deficit was limited to trials that used a weaker prepulse (4 dB) and was characterized by inability to increase inhibition when intensity of the startle stimulus was decreased. This finding implies that tTa:APPsi mice were hyper-reactive, since the arousing effect of a weaker startle stimulus in PPI circuitry was similar to that of a stronger startle stimulus. In addition, the startle response in tTa:APPsi mice was characterized by increased amplitude (see Pulse 120 dB, Fig. 7H) and shorter latency than in control mice (Fig. 7H; effects of sex and sex-genotype interaction are not significant, see Supplementary Table 3). These data indicate that tTa:APPsi mice showed age-related hyper-reactivity in parameters of startle response and prepulse inhibition.

4. Discussion

Detailed analyses of sex-related differences in a conditional tTa:APPsi model revealed exaggerated atrophy in the dentate gyrus of the dorsal hippocampus and earlier-onset hyperactivity in females compared to males. These differences were observed on the background of equal expression of *APP* transgene, APP processing, and levels of A β peptides. In contrast, in some other models, levels of accumulated A β peptides and/or plaques are consistently reported to be higher in female APP transgenic mice (Callahan et al., 2001; Hirata-Fukae et al., 2008; Hutter-Paier et al., 2004; Kulnane and Lamb, 2001; LaClair et al., 2013; Lee et al., 2002; Perez et al., 2011; Wang et al., 2003). One of these models that we and others have used extensively is the APPs:PS1 E9 model, which is characterized by earlier-onset and more dramatic cognitive deficits in females compared to males (Jankowsky et al., 2005a; LaClair et al., 2013; Savonenko et al., 2005). These between-sex differences in A β accumulation and cognitive deficits in the APPs:PS1 E9 model cannot necessarily be attributed to sex-related factors due to unequal expression of the APP transgene. High APP levels in females coexisted with increased levels of its proteolytic fragments, β CTF and A β . Both of these fragments have been implicated in cognitive toxicities (Ashe and Zahs, 2010; Herrup, 2010; Mitani et al., 2012; Tamayev and D'Adamio, 2012; Tamayev et al., 2012) affecting a multitude of A β -dependent and -independent mechanisms including endocytosis, axonal transport, and APP processing (Kim et al., 2015; Pimplikar et al., 2010; Xu et al., 2015). The sex-associated differences in APP expression observed in APPs:PS1 E9 mice might be associated with the Prion promoter, since other transgenic models sharing the same promoter have demonstrated similar alterations in a

transgene expression (Lewis et al., 2000; Sahara et al., 2014). In contrast to the APPs:PS1 E9 model, APP expression in the tTa:APPsi model is driven by tTa from the CaMKIIa promoter, resulting in equal expression of the transgene in males and females. These data indicate that tTa:APPsi model has an important benefit, allowing utilization of this model for studies of sex-related differences in sensitivities to A β .

In a setting of equal APP expression, tTa:APPsi mice demonstrated no sex-related differences in β -secretase processing of APP as judged by levels of C- and N-terminal fragments of APP. However, interpretation of these findings as a lack of sex-related effects on β -secretase should be exercised with caution because of mutations present in APP. The Swedish double mutation inserted in the APP construct of this model results in ~100 fold more efficient processing of APP by β -secretase (Vassar et al., 2014). This mutation-induced increase in β -secretase activity might obscure possible effects of sex-related factors if they have a smaller size effect. Similar considerations must be taken into account while interpreting γ -secretase activity in this model, due to the presence of an Indiana APP mutation. Utilization of transgenic mice expressing wild type APP would represent a model more closely related to sporadic AD and help to delineate in what extent sex-related factors can affect secretase activities if no mutations are present in APP. The tTa:APPsi model affords an experimental condition in which effects of sex factors on APP expression, processing and levels of monomeric A β are negligible. In these conditions, levels of A β dimers and tetramers as well as total levels of A β peptides at different ages were similar between males and females, suggesting that sex-related factors do not significantly modulate A β accumulation.

Contrary to our expectations based on studies in APP transgenic mice with the prion promoter, analyses of sex-related differences in the tTa:APPsi model did not yield numerous findings. One of the main outcomes was increased degeneration of the dentate gyrus (DG) in females compared to males. Dramatic loss of neurons in the granular layer of DG was in line with what has previously been reported in mixed groups of males and females of the tTa:APPsi model (Han et al., 2012). DG atrophy has been detected as early as 4.5 months of age and does not change across the range of ages covering the development of A β pathology from its onset to full blown amyloidosis (from ~4.5 to 12–13 months of age). Such early appearance of DG atrophy raises the possibility that it is attributable to neurodevelopmental changes. However, at 2 weeks of age, the age of activation of the tTa promoter (Kojima et al., 1997), tTa and tTa:APPsi mice have been shown to have no detectable changes in DG (Han et al., 2012). In this study, tTa:APPsi mice were on a doxycycline diet until 1 month of age, and inspection of the hippocampus shortly after transgene activation (Fig. S3) did not reveal any obvious changes in its morphology. In addition, analyses of adult neurogenesis in this model revealed no effects of APP expression on its markers (Yetman and Jankowsky, 2013) that in contrast to other APP transgenic models (Verret et al., 2007) might be related to transgene activation only in mature neurons (Yetman and Jankowsky, 2013). Altogether these data implicate neurodegeneration as a primary cause of the DG atrophy described in this model. The window of this degeneration might take <3 months and corresponds to a period with low levels of accumulated A β . Whether APP expression or A β are driving the DG degeneration is unclear and requires further studies. Our observation that the DG atrophy is limited to the dorsal hippocampus is a new finding for this model. Distinct

structural (Vuksic et al., 2008) and functional characteristics (Fanselow and Dong, 2010; Sahay and Hen, 2007) of dorsal and ventral hippocampus might mediate the differential sensitivity of granule cells to APP and/or A β . However, other factors such as differences in sub-regional promoter efficiency or APP proteolysis may account for selective expression of DG atrophy in the dorsal hippocampus.

One of the factors that have been shown to modify morphology of DG in transgenic models is overexpression of tTa (Han et al., 2012). The effects of tTa depend on strain background, with a C57BL/6 background being protective against tTa-induced degeneration of DG (Han et al., 2012). We had expected that single transgenic tTa mice with a C57BL/6 strain background would demonstrate no changes in DG measures. Indeed, width of DG was not affected by tTa expression in either sex, in line with previously reported findings in groups of mice of mixed sexes (Han et al., 2012). However, the length of supra- and infra-blades of DG, a measure that was not previously analyzed, was significantly shorter in single tTa transgenic mice than in non-transgenic controls. When tested in each sex separately, these changes reached significance only in male tTa mice. Importantly, tTa-induced shortening of DG was comparable between single tTa and double tTa:APPsi transgenic males. In contrast, the length of DG in tTa:APPsi females was significantly shorter than in single tTa transgenic females. These data suggest that DG generation in tTa:APPsi females can mainly be attributed to the effects of APP/A β production rather than tTa expression.

An unexpected finding in this study was an increase in the width of the pyramidal layer of the CA1 field of the hippocampus. The directions of changes in the CA1 width were opposite to those observed in the length of DG. Namely, tTa:APPsi mice of both sexes had shorter blades of DG and increased width of CA1 as compared to nontransgenic controls. As seen in the DG, the effects of tTa expression in CA1 were sex-dependent, with only males being affected. Taking into account observations in both the DG and the CA1 fields of the hippocampus, one can conclude that the changes in hippocampal morphology can be mainly attributed to tTa expression in males and to APP/A β production in females. The opposite character of morphological changes in DG and CA1 calls for a circuit adaptation as a likely explanation. The CA1 field receives inputs from both trisynaptic and monosynaptic pathways from the entorhinal cortex (Amaral et al., 2007). Neurons of the DG form the first synapse in the trisynaptic loop, and in case of their degeneration the monosynaptic pathway might become dominant in sustaining CA1 neurons and overall output from the hippocampus. An alternative but not exclusive explanation might involve rearrangement of CA neurons as a result of shortening of the hippocampus proper along the transverse axis. We noticed that in most cases tTa:APP mice had a consistently more circular shape of the hippocampus as a result of its short transverse dimensions. Although the mechanisms of shape formation in the hippocampus are not fully understood, it is intriguing to hypothesize that mossy fibers, a main output of the DG, might serve a structural function by virtue of their transverse orientation (Amaral et al., 2007; Amaral and Witter, 1989). In the case of DG degeneration, a loss of mossy fibers might then result in collapse of the hippocampus proper along the transverse axis.

Degeneration of the DG, as noted above, primarily affects the trisynaptic pathway (DG-CA3-CA1) in the dorsal hippocampus, leaving the monosynaptic pathway from the

entorhinal cortex as the main, relatively intact, circuit. Studies testing functional significance of these parallel pathways by acute inhibition of synaptic transmission indicated that the monosynaptic pathway sufficiently supports multi-trial incremental learning and memory, such as in the Morris water maze (Kesner, 2007; Nakashiba et al., 2008). The trisynaptic pathway, on the other hand, is required for one-trial learning, as in the contextual fear paradigm (Nakashiba et al., 2008). We thought that cognitive tasks based on such quick learning paradigms would be the most sensitive to functional consequences of the DG degeneration seen in our model. Fear conditioning, in which a new context was paired with a mild foot-shock, demonstrated age-dependent deficits in fear memory, with 12-month-old tTa:APPsi mice of both sexes showing low levels of freezing to context. At 5–6 months, an age by which the DG degeneration had already developed, contextual fear memory was mostly preserved. No changes were observed in the fear memory of single tTa transgenic mice. Although it is possible that the remaining functionality of the DG could be sufficient to support fear conditioning, the extent of degeneration in tTa:APPsi mice makes it unlikely. The observation of preserved fear memory despite DG degeneration might rather indicate that in a situation of chronic failure of the DG, the structural basis for this behavior could be different and at least partially served by alternative circuitry (Nakashiba et al., 2008; Poulos et al., 2010).

Spatial recognition memory in the Y maze is another task based on a single trial that requires the rapid formation of spatial representations. As with fear memory, spatial recognition memory was impaired in 12-month-old tTa:APPsi mice, who demonstrated a lack of any spatial preferences. Deficits observed in younger tTa:APPsi mice were difficult to interpret as pure memory deficits, since the mice were able to show clear spatial preferences to new and start arms of the maze. The inclination of tTa:APPsi mice toward the start arm is not likely to be explained by changes in anxiety levels, since there was a lack of correlation between anxiety measures and time spent in the start arm. Preferences to both new and start arms of the maze have also been observed in single tTa transgenic females. This implies that the behavioral phenotypes of tTa:APPsi females might be at least partially explained by the effects of tTa expression. Of note, young tTa:APPsi females with low levels of A β performed better in the Y maze than their sex-matched tTa controls. This observation indicates that effects of tTa expression in tTa:APPsi mice might not result in the same phenotypes as in tTa single transgenic mice.

To test whether levels of accumulated A β play a role in cognitive performance, we separated ~12-month-old tTa:APPsi mice into subgroups with high or low A β . These mice were behaviorally tested 6 months or 1 month before sacrifice, allowing us to estimate the effects of A β at different ages. At a younger age (5–6 months), the cognitive performances of subgroups that were bound to accumulate low or high levels of A β were dramatically different. The “low-to-be” subgroup showed a preference for the new arm similar to controls, whereas the “high-to-be” subgroup demonstrated a lack of spatial preferences. At an older age (11–12 months), both groups were significantly impaired. These data indicate that A β accumulation might be a better indicator of earlier cognitive impairments than those seen immediately preceding A β measurements.

The largest sex-related differences in the behavior of tTa:APPsi mice were seen in locomotor activity in environments with varying degrees of novelty. tTa:APPsi males demonstrated a lack of novelty-induced activation. This phenotype could not be explained by heightened anxiety in tTa:APPsi mice, since no significant correlations were detected between anxiety measures and activity in the open field. tTa:APPsi females also showed decreased initial activity in the open field; however, their deficits did not reach significance. The lack of novelty-induced activation was also observed in tTa single transgenic males, implying that tTa expression may mediate this phenotype. Considering that the hippocampus, and DG in particular, has been identified as a detector of novelty (Lever et al., 2010; Hunsaker et al., 2008) it is attractive to suggest that the structural basis of this phenotype is related to the DG degeneration observed in both tTa and tTa:APPsi males. Of note, the normal performance of tTa males in the spatial recognition task suggests that their novelty recognition was preserved enough to show preference to the new arm indistinguishable from non-transgenic controls. The lack of novelty-induced activation, then, might be a result of insufficient behavioral response to novelty (motor activation), rather than deficits in novelty perception itself.

Another sex-related difference in behavioral phenotypes was the exaggerated hyperlocomotion observed in tTa:APPsi females. In a familiar environment, this phenotype was expressed immediately at the start of testing, whereas in a novel environment hyperactivity was consistently detected at later stages of habituation to the environment. These data indicated that the hyperactivity of tTa:APPsi mice represents a steady-state increase in levels of locomotion rather than novelty-induced activation. Importantly, single tTa transgenic mice demonstrated normal motor activity in familiar environments, indicating that habituation-associated hyperlocomotion in tTa:APPsi mice was not related to tTa expression. Persistent hyperactivity has also been shown in a different line of the tTa:APPsi model in groups of mixed sexes (Rodgers et al., 2012). In our study, male tTa:APPsi mice were also hyperactive; however, tTa:APPsi females showed more inflated locomotion, with the onset at an earlier age than in males. The concurrent presence of hyperactivity and deficit in freezing behavior as we saw it in our tTa:APPsi model raises the question of their possible interference. Recent study utilizing optogenetic control of DG demonstrated that inhibition of dorsal DG disrupted encoding of contextual fear memory while increasing exploratory activity in the conditioning chamber (Kheirbek et al., 2013). Although the effects were observed after acute manipulations, these findings indicate that disruption in DG pathways can be responsible for both contextual fear deficits and increased exploration. The early-onset of hyperactivity in tTa:APPsi females that coincided with early mild deficits in freezing could be an example of such effects. It is tempting to speculate that the hyperactivity observed in the tTa:APPsi mice could be relevant to neuropsychiatric symptoms observed in AD such as agitation and wandering (Lyketsos et al., 2011; Dekker et al., 2015). However, such connections have to be made with caution, as phenotypic similarity of a behavioral response is not sufficient for interpretation of phenotypes in different species.

5. Conclusion

Sex differences are a well-known phenomenon in AD research, with women having a higher risk for developing AD than men. Many AD mouse models display a similar sex-dependent pattern in cognitive deficits and neuropathology. Although these experimental phenomena might look analogous to the sex differences seen in the human disease, the question that must be answered for each model is whether they are relevant. Here we show that the choice of animal models to address mechanisms of sex differences in AD can be critical. Given the relatively large magnitude of sex-related differences in APP expression observed in APP PrP models, increased A β peptide accumulation, plaque load, and cognitive/behavioral deficits associated with female sex in these models might not reflect mechanisms of sex-specific vulnerability to AD. Analyses of conditional TetOff:APPsi mice, in which the tetracycline activator is driven by the CaMKII α promoter, indicated that this model might be more suitable for studying sex-related mechanisms in the pathophysiology of AD. This model affords experimental conditions in which APP expression, A β accumulation and levels of SDS-extracted A β oligomers are equal between males and females. In this setting, females showed enhanced atrophy of the dentate gyrus of the hippocampus, earlier onset of hyperactivity, and earlier onset of a deficit in the conditioned freezing reaction than males. These sex-related differences might reflect a higher sensitivity of females to the effects of APP and/or A β production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APP	amyloid precursor protein
APPsi	APP with Swedish and Indiana mutations
sAPP	soluble N-terminal fragment of APP
CaMKIIα	calcium-calmodulin-dependent kinase II α
CTF	C-terminal fragment of APP
DG	dental gyrus of the hippocampus
FL-APP	full length APP
PPI	prepulse inhibition of acoustic startle reflex

PrP	Prion protein; SDS, sodium dodecyl sulfate
tTa	tetracycline transactivator

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Fig. 1. Levels of APP protein in the cortex of mice from PrP-APP and CaMKII-tTa:APP lines. Immunoblot analyses of crude PBS extracts from cortical samples of mice from PrP-APPswe line C3-3, 16 mo-old (A), PrP-APPswePS1 ΔE9 line 85, 3.5 mo-old (B), CaMKII-tTa:APPsi line 8-85, 5 mo-old (C), and CaMKII-tTa:APPsi line 107, 9 and 12 mo-old (D). Full-length APP (FLAPP; 98 kDa) was detected with C-terminal APP antibody (#A8717; Sigma). In PrP-APP lines, 6E10 antibody was also used, which, in addition to FL-APP, detects soluble APPα (86 kDa), βCTFs (14 kDa) and Aβ peptides (4 kDa) (A-B). Western blotting for tubulin (A), GAPDH (A), actin (C-D) or a non-specific band detected by C-terminal APP antibody (an asterisks in B) was used to check for equal protein loading. E. FL-APP, βCTFs (line 85) and Aβ (line 85) were quantified by densitometry, normalized to protein loading and presented as z scores. Box plots represent mean ± SEM and whiskers show confidence intervals with probability 0.95. Numbers under the boxes indicate number of cases per group. Asterisks indicate significant effect of sex in PrP-APP lines (** $p < 0.0001$; * $p < 0.002$, t-tests). No significant sex differences were detected in CaMKII-tTa:APP lines.

Fig. 2. Levels of full-length APP (FL-APP) and its proteolytic fragments generated by β - and α -secretases in the hippocampus of mice from line 107 of the CaMKII-tTa:APPsi model.

A. Immunoblot analysis of total protein extracts with RIPA buffer from hippocampal samples of CaMKII-tTa:APPsi mice (Line 107). Representative images of immunoblotting with C-terminal APP antibody (#A8717; Sigma), recognizing full-length APP (top panel) and the α -CTF (C83) and β -CTF (C99) fragments (bottom panel). Middle panel shows a representative image of immunoblotting with the 6 A1 antibody (IBL) that specifically recognizes soluble APP fragments with Swedish mutation cleaved by BACE1 (β sweAPPs).

Note that the levels of CTFs and β sweAPPs dramatically declined after doxycycline treatment (DOX, 3 months). Asterisks indicate non-specific bands. **B.** Quantification of signals for FL-APP, β sweAPPs, and CTFs in groups of mice of different sexes (top panel) or ages (bottom panel). The densitometry data for the proteins of interest were normalized to the actin levels, standardized within each blot and presented as z scores. Numbers under the boxes indicate number of cases per group. No significant differences related to sex or age were detected.

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Fig. 3. Levels of mono- and oligomeric Aβ species in CaMKII tTa:APPsi mice (Line 107). Immunoblot analyses were conducted using total protein extracts with RIPA buffer from hippocampal samples. **A.** Representative western blot with an end-specific anti-Aβ₁₋₅ antibody, 82E1. Spikes with synthetic Aβ₁₋₄₂ were used to facilitate the identification of bands corresponding to mono- and oligomers of Aβ. Three samples (9-mo old NTg control, lane 1; 9-mo old tTa:APPsi, lane 2; 12-mo old tTa:APPsi after 3 mo on DOX diet, lane 3) were run with addition of 0 (lanes 1–3), 2 (lanes 4–6) or 3 μg (lanes 7–9) of Aβ₁₋₄₂. **B.** Plot profiles of lanes 2, 5, and 11 shown in A. Molecular weights corresponding to monomeric, trimeric and tetrameric Aβ₁₋₄₂ are shown on X axes based on picks of densities in Lane 11. ×1, ×2, and ×4 Aβ denote bands with molecular weights corresponding to monomeric, dimeric, and tetrameric Aβ species, respectively. Asterisks in A–C indicate a band with molecular weights close to CTFβ and ×3 Aβ. **C.** Representative western blot of samples from male and female mice (82E1 antibody, Line 107). **D.** Quantification of signals in C (relative to actin) expressed as Z scores. No significant sex-related differences were detected. **E.** Scatterplot of Aβ dimers against Aβ tetramers. Solid and dotted lines represent

a linear regression line and its confidence limits, respectively. Squares and triangles show raw data for males and females, respectively, in Z score coordinates. r – Pearson correlation and corresponding p level; r^2 – proportion of variance explained. **F.** Graphic summary of semi-partial correlations for A β monomers, dimers, and tetramers ($n = 18$). Only significant correlations are included ($p < 0.005$). The width of the arrows is approximately proportional to the proportion of variance uniquely explained by the predictor. Exact values of semi-partial correlations and predictors included in multiple regressions are shown in Fig. S1 E–H. An empty arrow indicates that there was a notable portion of the variance of A β monomers, which was not explained by the variables included in the MR analysis.

Fig. 4. Age-related increase in Aβ accumulation and plaque load.

A. Representative sagittal brain sections through dorsal hippocampus and cortex stained with Thioflavin-S. Sections from male (left) and female (right) mice at 5.5 and 11.5 months of age. Scale bar 500 μm. **B-C.** ELISA analyses of Aβ40 (B) and Aβ42 (C) in a guanidine fraction of the prefrontal cortex in male (squares) and female (triangles) tTa:APPsi mice (Line 107). Lines represent data fitted by Distance Weighted Least Squares. Inserts show group averages for males and females as a result of ANCOVA, with age as a covariate.

Fig. 5. Atrophy of the Dentate Gyrus of the Hippocampus.

A. Representative examples of cresyl violet staining of sagittal section of the hippocampus at the lateral coordinate ~1.32 mm for 11–12 mo-old non-transgenic (NTg), single tTa and double transgenic tTa:APPsi female mice. Scale bars are 200, 100 and 50 μ m in A, B and C, respectively. See also Supplementary Fig. 2 for sections at different lateral levels.

Fig. 6. Morphometry of the Dentate Gyrus of the Hippocampus.

A. Scatterplot of lateral coordinate (X) against width of dentate gyrus supra-blade (in μm) (Y). Data for male and female mice are included. Similar sets of data were collected for all measures presented in C. **B.** Scatterplot of age of mice (X) against width of dentate gyrus supra-blade (in Z score) (Y). The range of lateral coordinates (shown in A) was divided into 11 intervals and for each interval raw measures were standardized across all genotypes and sexes. Average Z score was then calculated for each mouse. **C.** Measures of the dentate gyrus, CA1 area of the hippocampus and cortex expressed as Z scores and averaged for groups of mice of different genotypes and sexes. Asterisks and triangles indicate significant differences ($p < 0.01$) from NTg and tTa mice, respectively, as results of HSD for unequal N post-hoc tests applied to significant genotype $\times$ sex interactions (ANCOVAs with age as a covariate). Pound signs indicate significant differences ($p < 0.05$) between females (F) and males (M) of the same genotype.

Fig. 7. Contextual fear (A-F) and spatial recognition memory (G-J).

A, D. Dynamics of freezing to context in 5–6 (A) and 11–12 mo-old mice (D). Total duration of freezing (in sec) accumulated during blocks of 20 s is shown. Sessions 1 and 2 were conducted 24 and 48 h after fear conditioning, respectively. **B, E.** Dynamics of freezing to context are shown as in A, D but for male and female groups separately. **C, F.** Average duration of a freezing reaction in 5–6 (C) and 11–12 mo-old mice (F). Number of cases per group is shown at the bottoms of columns. **G, I.** Preference to different arms of the Y maze (sec/min) during Trial 2 of the test. Scheme of the test: in trial 1, a mouse is placed in the Y

maze with only 2 arms open for investigation. After a 30-min delay, the mouse is returned to the maze with all arms open, and its ability to recognize new and old arms of the maze is analyzed. Color-coded circles indicate differences in preference between arms ($p < 0.01$) as a result of LSD post-hoc test applied to significant genotype $\times$ arm interaction (ANOVA). **H,J.** Preference for a new arm (%) for groups of male and female mice. Solid lines indicate a chance level of performance (33%). * and ** indicate significant differences between genotypes, $p < 0.05$ and 0.01 , respectively (LSD post-hoc test). Results of ANOVAs are presented in Supplementary Table 2.

Fig. 8. Motor activity, anxiety and sensorimotor gaiting.

A,C. Dynamics of motor activity in a big (100 cm in diameter) open field assessed in 5–6 (A) or 11–12 (C) month-old mice naïve to the apparatus. **B,D.** Thigmotaxis (Time %) as a measure of anxiety levels in mice during testing in the open field (A,C). **E,F.** Dynamics of motor activity in a small (40 × 40 cm) open field assessed in 5–6 (E) or 11–12 (F) month-old mice naïve to the apparatus. **G.** Dynamics of motor activity after placement into home cage. **H.** Sensorimotor gaiting assessed by prepulse inhibition of acoustic startle reaction. Levels of pulses and pre-pulses are shown on X axis. Prepulse inhibition (%) and latency (msec) of

startle reaction are shown for males and females together. Asterisks (B,D, G-H) indicate significant between-genotypes differences, $p < 0.05$ as a result of significant Genotype effect or Genotype $\times$ Sex interaction in ANOVA. Pound signs (A, C, E, F) indicate significant differences between genotypes, $p < 0.01$ as a result of planned comparisons. Numbers at the bottoms of columns or next to the labels indicate number of cases per genotype.