Subtle behavioral changes and increased prefrontal-hippocampal network synchronicity in APP^{NL-G-F} mice before prominent plaque deposition

Amira Latif Hernandez[1*], Disha Shah[2*], Kathleen Craessaerts[3], Takaomi Saido[4], Takashi Saito[4], Bart De Strooper[3], Annemie Van der Linden[2] and Rudi D'Hooge[1]

[1] Laboratory of Biological Psychology, University of Leuven (KU Leuven), Belgium

[2] Bio-Imaging Lab, University of Antwerp, Belgium

[3] VIB center for the biology of disease, KU Leuven, Belgium.

[4] Laboratory for Proteolytic Neuroscience, Riken Brain Science Institute, Japan

* Equal contribution

Corresponding author: Rudi D'Hooge, PhD; Laboratory of Biological Psychology, KU Leuven, Tiensestraat 102, 3000 Leuven, Belgium; rudi.dhooge@kuleuven.be

Abstract

Amyloid-β (Aβ) peptides occur in the brains of patients with Alzheimer's disease (AD), but their role in functional impairment is still debated. High levels of APP and APP fragments in mice that overexpress APP might confound their use in preclinical research. We examined the occurrence of behavioral, cognitive and neuroimaging changes in APP^{NL-G-F} knock-in mice that display Aβ42 amyloidosis in the absence of APP overexpression. Female APP^{NL-G-F} mice (carrying Swedish, Iberian and Arctic APP mutations) were compared to APP^{NL} mice (APP Swedish) at 3, 7 and 10 months. Mice were subjected to a test battery that referred to clinical AD symptoms, comprising cage activity, open field, elevated plus maze, social preference and novelty test, and spatial learning, reversal learning and spatial reference memory performance. Our assessment confirmed that behavior at these early ages was largely unaffected in these mice in accordance with previous reports, with some subtle behavioral changes, mainly in social and anxiety-related test performance. Resting-state functional MRI (rsfMRI) assessed connectivity between hippocampal and prefrontal regions with an established role in flexibility, learning and memory. Increased prefrontal-hippocampal network synchronicity was found in 3-month-old APP^{NL-G-F} mice. These functional changes occurred before prominent amyloid plaque deposition.

Introduction

Alzheimer's disease (AD) is characterized by the progressive brain deposition of extracellular 40–42 residue amyloid-β peptide (Aβ) [1–3], and neurofibrillary tangles [4]. Transgenic mice overexpressing APP and Tau have been instrumental to recent AD research, but these mice may have artificial phenotypes because they overproduce APP fragments [5,6]. Models that endogenously overproduce $A\beta_{42}$ without overexpressing APP have been generated by knock-in (KI) of a humanized Aβ sequence [7]. Characterization of the functional consequences of the KI strategy on complex behavioral and cognitive abilities and brain circuitry is still limited, and previous reports showed only mild behavioral defects at the age examined in the present report [8].

Patients that are eventually diagnosed with clinical AD show problems in executive functioning and attention at early stages of the disease [9]. The present study evaluates the validity of APP knock-in (KI) mice as models of clinical AD. $APP^{NL\text{-}G\text{-}F}$ mice carrying Iberian and Arctic mutations in the Aβ sequence were compared to APP^{NL} mice carrying only the Swedish mutation to dissociate the effects of aggressive Aβ pathology. We investigated these mice using behavioral tasks that assess higher-order functions (such as cognitive flexibility), which relate to defects observed in AD patients [10–12]. Behavioral flexibility is required when faced with environmental changes, which starts declining in early phases of AD pathology. Behavioral assessment and reversal learning included in the present study models neuropsychological testing in patients [13–17]. In addition, resting-state functional MRI (rsfMRI) was used as a non-invasive imaging method, based on fluctuations in blood oxygen level-dependent (BOLD) signals [18], to assess connectivity between cortical regions and brain network integrity [19]. Measuring fMRI during the brain's resting state has been used to define early disease biomarkers, since changes in connectivity underlie different neuropsychiatric disorders [19,20], and rsfMRI is a clinically feasible tool for early diagnosis [21].

Methods

Animals

APP^{NL} and APP^{NL-G-F} mice were derived from the Riken Institute colony (Japan). APP^{NL-G-F} mice co-express Swedish (KM670/671NL), Beyreuther/Iberian (I716F) and Arctic (E693G) mutations, whereas APP^{NL} mice only express the Swedish mutation and were used as controls in all tests performed. The behavioral test battery was carried out in homozygous female mice aged of 3, 6 and 10 months old. There were 14 APP^{NL} and 14 APP^{NL-G-F} mice in the 3-month-old group, 8 APP^{NL} and 8 APP^{NL-G-F} mice in the 6-month-old group, and 12 APP^{NL} and 12 APP^{NL-G-F} mice in the 10-month-old group. Saito and colleagues observed age-dependent Aβ amyloidosis in homozygous APP^{NL-G-F} mice. Notably, cortical deposition began by 2 months and was saturated from around 7 months.

Immunostaining

The amyloid plaque load was measured in brain sagittal vibratome sections (60 µm) from mice transcardially perfused with PFA. The sections were stained for amyloid plaques using immunofluorescence with an Aβ primary antibody (6E10, against $Aβ_{1–17}$, Sigma) after antigen retrieval in sodium citrate buffer. Antibody-antigen complexes were revealed using a DyLight 650-conjugated goat anti mouse secondary antibody. DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen) was used as counterstain. Digital images were taken on a Nikon A1R Eclipse Ti microscope.

Cage and exploratory activity assessment

Mice were placed in small animal cages between 3 infrared beams to monitor 23 h spontaneous activity as previously described [22]. After 15 min habituation, registration of beam crossings started at 4pm with lights being switched off at 8pm (12 h on/off cycle). Open field (OF) locomotor behavior was monitored in observation areas with walls and floor consisting of transparent PVC (w × d × h: 50 × 50 × 30 cm), and placed on translucent shelves inside an isolation cabinet. Indirect lighting was applied from underneath the setups. Cameras mounted above the arenas transmitted images to computers equipped with ANYMAZE™ video tracking software (Stoelting Co., IL, USA). Animals were placed in the left corner of the OF arena proximal to the experimenter and allowed to explore the open arena freely for 1 h. The arena was cleaned between animals with a dry towel. The open field was virtually divided into three different zones: an outer periphery (0–5 cm from OF walls), inner periphery (5–10 cm from OF walls) and center square. Exploration parameters such as distance travelled, time spent and number of entries were analyzed for 10min. Anxiety-related exploration was evaluated in the elevated plus maze (EPM) as described before [22]. Briefly, the EPM comprised two arms (5 cm wide, 20 cm long, elevated 40 cm above table top) closed by side walls, and two arms without walls. Mice were placed at the center of the maze, and were allowed to explore freely for 11 min (1 min habituation and 10 min recording). Exploratory activity was recorded by 5 IR beams (4 for arm entries, and 1 for open arm dwell) connected to a computerized activity logger.

Sociability/preference for social novelty task

A social novelty and recognition task was adapted from Nadler and colleagues (2004) as described in detail elsewhere [23]. Setup consisted of a rectangular transparent Plexiglas box (w × d × h: 94 × 28 × 30 cm) divided into three chambers. Mice could circulate between left, right (29 × 28 × 30 cm) and central chamber (36 × 28 × 30 cm) via openings (w × h: 6 × 8 cm) in division walls between chambers. Openings could be manually closed to limit access to chambers. The

setup had an opaque floor and was illuminated indirectly from underneath the setup. It was placed inside an enclosure to limit environmental distractions. Two cameras were located 60 cm above the setup and ANY-maze^{TM} Video Tracking System software (Stoelting Co., IL, USA) was used to record and analyze movements of animals. Cylindrical wire cups (height × diameter:11 × 12 cm) that contained stranger mice were placed in the left and right chamber. The procedure consisted on three consecutive phases, between the phases the animal was maintained in the middle compartment. During the first phase (acclimation phase) mice were habituated to the apparatus and placed in the middle chamber with both divider doors closed and left to explore for 5 min. During this trial, empty wire cages were present in left and right chambers visible from the middle chamber. In the second phase (sociability phase) one stranger mouse (S1) was placed in wire cage in either left or right chamber, the other wire cage was left empty. Exploratory behavior (exploring and sniffing) towards S1 and the empty cage was recorded for 10 min. Finally during the third phase (social recognition phase) a second stranger mouse (S2) was placed in empty wire cage with S1 mouse remaining in its cage. Exploratory behavior towards S1 and S2 was again recorded for 10 min. We calculated preference ratio ($Ratio_{Pref}$) as $Time_{S1}/(Time_{S1} + Time_{empty})$, and recognition ratio ($Ratio_{Rec}$) as $Time_{S2}/(Time_{S1} + Time_{S2})$. The position of S1 and S2 was counterbalanced between animals. The setup was thoroughly cleaned with water and paper towel between animals. At the end of each testing day, test setup was cleaned with 30% ethanol. Stranger mice were 3-month old, group-housed (2 per cage) female C57BL/6J mice that had served as stranger mice in other SPSN experiments before. Distance travelled in each chamber was also calculated.

Morris Water Maze Performance

Spatial memory was assessed in the Morris water maze (MWM) [24], using a training protocol adapted for mice [25]. The maze had a diameter of 150 cm and contained water (23°C) that was made opaque with non-toxic white paint. The pool was located in a brightly lit room with distal

visual cues, including computer, tables and posters with geometric figures attached to the walls. Images were recorded with a PC-interfaced camera located above the water maze and analysed with EthoVision software (Noldus, Wageningen, The Netherlands). During acquisition trials, a small platform (diameter 15 cm) was hidden beneath the surface at a fixed position. Mice were placed in the water at the border of the maze and had to reach the platform after which they were transported back to their home cage. Mice that did not reach the platform within 2 min were gently guided towards the platform and were left on it for 10 s before being placed back in their cages. Four of such daily training trials (inter trial interval: 15–30 min) were given on 5 subsequent days (Monday to Friday; acquisition days 1–5); the week after the same procedure was repeated (acquisition days 6–10). Data were averaged per trial day. Starting positions in the pool varied between four fixed positions (0◦, 90◦, 180◦ and 270◦) so that on every training day, each position was used. The 4 starting positions define 4 quadrants: (i) the target quadrant where the escape platform is placed, (ii) the opposite quadrant which is at the opposite side of the target quadrant, (iii) the first adjacent quadrant and (iv) the second adjacent quadrant. During intertrial intervals, mice were placed under IR lamps to dry. Two probe trials were interspersed with training trials: probe 1 before start of training trials on acquisition day 6; probe 2 was run on the third day after acquisition day 11. During probe trials, the platform was removed from the pool and mice were allowed 100 s to search for the platform. This way, it could be verified whether mice showed a preference for the area where the platform used to be hidden. After acquisition trials, 3 daily reversal trials were performed on 5 subsequent days. The reversal phase consisted on placing the platform to the opposite quadrant.

Resting state magnetic resonance imaging

MRI acquisition and imaging data analyses was done as previously described in [26]. Briefly, resting-state imaging (rsfMRI) was performed on a 9.4T Biospec MRI system (Bruker BioSpin,

Germany) with Paravision 5.1 software (www.bruker.com). Three orthogonal multi-slice Turbo RARE T2-weighted images were acquired to allow uniform slice positioning (repetition time 2000 ms, echo time 15 ms, 16 slices of 0.4 mm). Field maps were acquired for each animal to assess field homogeneity, followed by local shimming, which corrects for inhomogeneity in a rectangular brain VOI. Resting-state signals were measured during a T2*-weighted single shot EPI sequence (repetition time 2000 ms, echo time 15 ms, 16 slices of 0.4 mm, 150 repetitions). Analysis consisted of two major steps. First, seed-based analysis was performed using right prefrontal cortex as seed region. A statistical difference map was obtained showing all voxels that were significantly different between the two groups (i.e., voxels that show differential FC with the right prefrontal cortex between sham and lesioned animals). This difference map was shown as an overlay on the EPI template. Next, the REST toolbox was used to compute z-transformed FC matrices for each subject using cortical regions that had shown different FC between the groups during seed-based analysis (i.e., prefrontal cortex, motor cortex, cingulate and retrosplenial cortex, somatosensory cortex, hippocampal CA1 region and thalamus). The time course of BOLD signals were extracted for each of these regions, and z-transformed correlation coefficients between time traces of each region pair were calculated and represented in a correlation matrix. Additionally, these matrices were used to calculate FC strength for each cortical region (i.e., mean strength of the correlation between a specific region and all other regions in the matrix). In the present study, the size of each group was as follows: 3 months APP^{NL} (n= 10), and APP^{NL-G-F} (n=12); 6 months APP^{NL} (n= 10), and APP^{NL-G-F} (n=10); 11 months APP^{NL} (n= 11), and APP^{NL-G-F} (n=12).

Statistics

For behavioral tests, all data are shown as means ± SEM. Differences between mean values were determined using 1-way or 2-way analysis of variance (ANOVA), or 2-way repeated

measures (RM) ANOVA procedures with Tukey tests for post hoc comparison. ANOVA on the probe trial results used factors group and quadrant. In all statistical tests, differences of $p<0.05$ were considered significant.

Results

Aβ plaques in brains of APP^{NL-G-F} and APP^{NL} mice

Antibodies to the N and C termini appeared to bind to both Aβ species in a similar manner. Using a combination of antibodies, we observed Aβ amyloidosis in APP^{NL-G-F} mice in an age dependent manner. We also observed early accumulation of Aβ plaques starting at the age of 2-2.5 months with full-blown pathology by 6 months in the cortex and hippocampus of APP^{NL-G-F} mice. In brains of APP^{NL} mice, we did not observe any plaques at the time points tested (Figure 1).

Cage activity and exploration in APP^{NL-G-F} mice

APP^{NL} and APP^{NL-G-F} mice were tested in the cage activity device to investigate spontaneous activity of these mice. Over a 23-hour period, the spontaneous activity of 3-month-old APP^{NL-G-F} mice (Figure 2A, left panel) was significantly higher than the activity of APP^{NL} mice (RM-ANOVA: $F_{1, 1170} = 11.56$; $p=0.002$). However, this difference was not measured at 6 months (Figure 2A, middle panel), and 10-month-old APP^{NL-G-F} mice (Figure 2A, right panel) showed significantly increased overall activity across the 23h period (RM-ANOVA: $F_{1, 1034} = 6.406$; $p= 0.019$). Marked activity changes occurred between 8pm (after lights were switched off) and 9 am (RM-ANOVA: $F_{22, 506} = 9.682$; $p= 0.005$).

The open field task was used to investigate anxiety-related exploratory activity in APP^{NL} and APP^{NL-G-F} mice. In other AD mouse models, this test already highlighted anxiety and exploration disturbances [27]. In the open field test, the time spent in the arena center is a parameter that

reflects anxiety, whereas total distance moved represents exploratory activity. As depicted in figure 2B (right panel), 6-month-old APP^{NL-G-F} mice spent significantly more time in the arena center compared to APP^{NL} mice (t=2.818; p= 0.0258). This increase of time spent in arena center indicates decreased anxiety, which is consistent with anxiolytic behavior in other AD mouse models [27,28]. Moreover, no differences were found in APP^{NL-G-F} mice exploration compared to APP^{NL} mice in the other age groups (Figure 2B, right panel). In addition, we found that the total distance moved was consistently reduced in APP^{NL-G-F} mice (Figure 2B, left panel), but not significantly between groups. A study performed in wild-type C57BL/6 mice [29] has shown that performance in the open field task is affected by increasing age. For example, Shoji et al. showed that subjects in older age groups travelled shorter distances than those in younger age groups [29]. The difference in time spent in arena center and distance moved found between younger and older APP^{NL} and APP^{NL-G-F} mice seem, therefore, to be an effect of ageing, unrelated to their AD pathology.

The elevated plus maze test allows evaluation of anxiety-related behaviors, since increased or decreased exploration of the open arms can indicate anxiolytic or anxiogenic behavior, respectively [28]. At 3 months of age (Figure 3 left panel), the number of entries in the open arms (defined as number of beam crossings) was significantly increased in APP^{NL-G-F} mice (crossings: 30 ± 5, n=7) compared to APP^{NL} mice (crossings: 45 ± 4, n=8), whereas APP^{NL-G-F} entered the closed arm less frequently (81 ± 7) than APP^{NL} mice (99 ±11). Non parametric t-test with Welch's corrections indicated a significant difference in the number of beam breaks between the two genotypes (t= 2.53; p= 0.0297), which is consistent with the anxiolytic behavior in other AD mouse models, likely induced by disinhibition resulting from AD pathology [27,28]. This decreased anxiety was obvious during the open field test as well (see above). At a later time point (6 months; Figure 3, middle panel), APP^{NL-G-F} mice entered the open arms 46 ± 7 times, and the closed arm 76 ± 5 times, whereas APP^{NL} mice entered the closed arm 126 ± 8, and the open arm 27 ± 3 times. RM-ANOVA confirmed the different preference of APP^{NL-G-F} mice for the open versus closed arms:

a main effect of arm (open v. closed) on number of beam breaks was found ($F_{1, 13}$= 146, p< 0.0001), a main effect of genotype ($F_{1, 13}$ = 4.8, p= 0.0464) and a genotype by arm interaction effect ($F_{1, 13}$ = 42, p< 0.0001). Indeed, t-test with Welch's correction indicated a significant difference in the number of beam breaks in the open arm between the two genotypes (t= 2.456; p=0.0396). Surprisingly, APP^{NL-G-F} mice displayed a significant reduction in the number of entries in the closed arm compared to APP^{NL} (t= 5.114; p= 0.0003). At 10 months (Figure 3, right panel), both groups visit the open arm equally often, whereas the close arms are significantly less visited by the APP^{NL-G-F} mice (t= 2.593; p= 0.0223). It should be noted in this respect that old C57BL/6 mice have been shown to exhibit a significantly higher percentage of open arm entries compared to younger animals [29].

Sociability and social recognition behaviors

Social memory was assessed in APP^{NL} and APP^{NL-G-F} mice by means of the Social Preference Social Novelty (SPSN) test. Social recognition was found to be impaired in several AD mouse lines [30,31]. During social preference (Figure 4B) and recognition phases (Figure 4C), statistical comparison of the data sets with an unpaired t test (Welch's correction two-tailed) revealed no significant differences between the two groups at any of the ages tested (neither $Ratio_{Pref,}$ nor $Ratio_{Rec}$). However, during the social preference trial, 10 months-old APP^{NL-G-F} mice exhibited a reduced $Ratio_{Pref}$ compared to APP^{NL}. APP^{NL-G-F} mice showed a non-significant reduction in $Ratio_{Rec}$ during the recognition trial at 3, 6 and 10 months, which suggests that these mice display some mild social impairment. To investigate this further, time spent in the small periphery (closer to S1 or S2) was analyzed in both phases for every group at 3 (Figure 4A, left panel), 6 (Figure 4A, middle panel) and 10 months of age (Figure 4A, right panel). RM-ANOVA of social preference trial indicated a main effects of *arena side* at 3 months ($F_{(1, 15)}$ = 28.02; p< 0.0001). Figure 4A (left panel) shows that both groups prefer to approach mouse S1 to an empty cage, APP^{NL-G-F} to a

higher degree that APP^{NL} mice. At 6 months, we found a similar effect of *stranger side* ($F_{1,13}$ = 7.203; p= 0.0188), but the preference of APP^{NL-G-F} mice for S1 over the empty side is much smaller than at 3 months, possibly due to increased variability at this age.

Ten-month-old APP^{NL-G-F} mice display increased preference for the empty side over the S1, with a "*stranger side" x "genotype"* interaction effect ($F_{1, 16}$ = 5.044; p= 0.0392). In the second trial, during the recognition phase, main effect of *stranger side* was present at 3 months ($F_{(1, 15)}$ = 11.24; p= 0.0044) and at 6 months of age ($F_{(1, 13)}$ = 41.79; p < 0.0001), whereas no effect was found at 10 months. In fact, as displayed in Figure 4E, there is no preference in none of the groups towards S2 over S1. There is a tendency indicating that APP^{NL-G-F} mice explore the novel S2 mouse less than the known S1, although the difference is not significant. The fact that 10-month-old APP^{NL-G-F} displayed no interest in exploring S1 during the social preference trials might have influenced their performance in the social recognition trials.

To further investigate exploration patterns at 10 months, exploration time was analyzed in subsequent time bins of 2 minutes each per genotype condition and SPSN trial (Figure 4D-G). During the social preference trial, APP^{NL} mice showed preference for S1 over the empty side only during the first two time bins: RM-ANOVA indicated no effect of stranger side or time bin (Figure 4D). Once they have explored S1, from time bin 3 they spend equal time in the empty side and S1 side. However, APP^{NL-G-F} mice (Figure 4F) do not show any preference at all for the S1 during the time bin 1. On the contrary, from time bin two, they spent almost significantly more time in the empty side than with S1 (t= 2.023; p= 0.0641). This decreased interest for S1 persisted through the end of the trial (bins 3, 4 and 5), with a clear overall preference for the empty side (Figure 4F). During the recognition trial, the control animals show a preference for S2 over S1 only during the first time bins (Figure 4E), spending more time with the familiar mouse from time bin 3: RM-ANOVA indicated a main effect of *time bin* and *stranger side* interaction ($F_{4, 56}$ = 3.585; p= 0.0113). Interestingly, APP^{NL-G-F} mice showed slightly increased preference for S2 over S1 during the first time bin (Figure 4G), with a strong preference for the familiar mouse (S1) over the novel one (S2)

through the next 4 time bins (RM-ANOVA did not indicate significant effects). In summary, APP^{NL} mice showed pronounced sociability and preference for social novelty, especially during the first time bins, whereas such behavior was less pronounced or absent in APP^{NL-G-F} mice.

Spatial learning and memory

APP^{NL} and APP^{NL-G-F} mice were trained for 10 days to find the hidden platform in a large circular pool filled with opaque water in order to investigate spatial learning and memory as well as reversal learning. Probe trials were interspersed on day 6 and 11 after acquisition learning, and on day 6 after reversal learning to evaluate reference memory. The latter is used as a paradigm to study cognitive flexibility, commonly known as the ability to change behavior in response to changes in the environment [32]. Other AD mouse models have shown impairments in spatial and reversal learning [33]. A learning curve was obtained by plotting the path length to find the platform on each training day. During the acquisition phase, 3-4 month-old APP^{NL-G-F} and APP^{NL} mice learned the platform position at a different rate ($F_{1,207}$ = 4.798; p=0.04), but there was no main effect of *day* and *group* interaction ($F_{9,207}$ = 0.7290; p= 0.7; Figure 5A). Thus, APP^{NL} mice were slower than APP^{NL-G-F} during the first days of training. However, post-hoc comparisons using the Tukey HSD test during the second probe indicated that APP^{NL} mice showed more pronounced target quadrant preference (p= 0.0182) than their APP^{NL-G-F} littermates (p=0.2036). As depicted in Figure 5B, during probe 1, none of the groups displayed any preference for the target quadrant yet. Interestingly, at 6-7 months of age (Figure 5B), APP^{NL-G-F} and APP^{NL} mice performed equally well during 10 days acquisition learning in the MWM. Repeated measures (RM) ANOVA of the acquisition phase for factor day indicated that all animals learned to locate the hidden platform ($F_{9,117}$ =123.77, p<0.001). Reference memory performance was tested in probe trials 1 and 2, which indicated that both groups developed a preference for the target quadrant. Particularly,

Tukey post-hoc comparisons during probe 2 showed that APP^{NL} as well as APP^{NL-G-F} mice spent significantly more time searching the target quadrant than the other 3 quadrants (p=0.007, p=0.002). At 10-11 months (Figure 5C), we found very similar patterns of spatial learning and memory performance compared to 6-7 months. Two-way RM-ANOVA showed significant effects of *day* ($F_{9,181}$ = 31.34, p<0.001), but no effect of *group* ($F_{1,189}$ = 0.5625, p= 0.4616) or *group* by *day* ($F_{(9,181)}$ = 1.461, p= 0.1653) on time spent in the target quadrant. During the second probe, significant preference for the target quadrant was found in both APP^{NL}(p= 0.0012) and APP^{NL-G-F} mice (p= 0.005). Swimming velocity was not different between groups (data not shown).

Spatial reversal learning defect in APP^{NL-G-F} *mice*

Reversal learning was investigated also in MWM by changing the platform position to the opposite quadrant. Studying reversal learning in mice allows the study of cognitive flexibility, which was altered in some other AD models [27]. During the reversal phase of learning at 3-4 months of age (Figure 6A), APP^{NL} and APP^{NL-G-F} mice perform equally well. RM-ANOVA revealed a main effect of the factor *day* ($F_{(4, 96)}$ = 51.49; p< 0.0001), and no effect of genotype. The probe trial showed that both APP^{NL} (p= 0.02) and APP^{NL-G-F} mice (p= 0.02) had a preference for the target quadrant. At 6-7 months of age (Figure 6B), reversal learning curves show that APP^{NL-G-F} and APP^{NL} learned the new platform location at a similar rate. RM-ANOVA indicates only main effect of *day* ($F_{4, 52}$ = 5.514; p= 0.0009). During the reversal probe trial, APP^{NL} mice spent more time in the target quadrant than in the other quadrants (p= 0.007), whereas APP^{NL-G-F} mice failed to show such a preference (p= 0.2). This marginal effect during the reversal retention test could be due to somewhat more variable performance in the APP^{NL-G-F} group, and not necessary to a robust cognitive defect as such. In effect, a previous report failed to show early cognitive defects in these mice [8], and in our report, at 10-11 months of age (Figure 6C), no differences were observed,

neither in reversal learning, nor in probe trial performance. We cannot exclude that more challenging testing might still reveal the robust occurrence of early cognitive changes in these mice.

Increased prefrontal network synchrony in APP^{NL-G-F} mice

We used rsfMRI to compare functional connectivity between APP^{NL-G-F} and APP^{NL} mice in telencephalic regions with an established role in spatial learning and reversal learning. We analyzed rsfMRI data with a seed-based strategy to investigate the synchrony of BOLD signals between specified brain regions. Synchrony of activity between regions connected to PFC was stronger in the APP^{NL-G-F} group than the APP^{NL} group. We analyzed regions with correlated patterns of neuronal activity at 3, 7 and 11 months of age. Seed-based analysis showed increased synchrony at 3 months in the PFC network in APP^{NL-G-F} compared to APP^{NL} mice ($p= 0.007$; figure 7B, right panel). This network comprised motor cortex, cingulate/retrosplenial cortex, somatosensory cortex and CA1 region of hippocampus (uncorrected, $p<0.001$; figure 7). However, we found no differences in PFC network synchrony at 7 and 11 months of age ($p= 0.99$ and $p= 0.85$, respectively; Sidak's multiple comparisons test, 2-way ANOVA; figure 7B, right panel).

Discussion

Mouse models of AD have been instrumental to investigate pathological mechanisms and pharmacological interventions [27]. In the presently studied APP^{NL-G-F} mouse model, plaque deposition starts early and saturates around 7 months of age. Neuro-inflammation and synaptic alterations, which constitute two other hallmarks of AD pathology, are observed in APP^{NL-G-F} mice as well [7]. APP^{NL-G-F} mice were constructed to control for some of the confounds of other AD mouse models, because the knock-in strategy used to generate this model induces less unwanted artifacts, and the phenotype of APP^{NL-G-F} mice would be more specifically related to AD pathology. At least part of the phenotypes reported in APP transgenic mouse model could be caused by APP overexpression. For example, APP overexpression perturbs axonal transport because APP interacts with kinesin via JIP-1 [7]. Therefore, early behavioral impairments observed in such transgenic mice might be induced by the interaction of overexpressed APP with a variety of molecular substrates, and not by AD pathology proper. However as it turned-out, APP^{NL-G-F} mice appeared to display a relatively mild behavioral phenotype, in accordance with previous reports, which becomes more manifest at a relatively advanced age [7,8].

Using a somewhat more detailed approach, we presently report that APP^{NL-G-F} mice already display some behavioral changes at an early age. Behavioral testing in APP^{NL-G-F} mice was carried out at three different time points to investigate the precise onset of cognitive or behavioral changes, using tests with reported sensitivity to age-related changes in wild-type C57BL/6 mice [29]. We observed increases in nocturnal cage activity in APP^{NL-G-F} mice already at 3 months of age. Increased locomotor activity and disturbances of circadian rhythm and activity have been observed in other AD mouse models [34,35], but Masuda et al. [36] observed impulsivity and enhanced compulsivity only from 6-7 months in APP^{NL-G-F} mice. It is important to note that their measures were not directly linked to spontaneous locomotor activity as they included cognitive

components that are not investigated in our cage activity test. In our study, mildly increased cage activity was specific to this task and not observed in other tasks.

APP^{NL-G-F} mice displayed reduced anxiety-related behavior in the open field as well as in the elevated plus maze from 3 months of age. The mice also displayed variable changes in social behaviors and memory. The open field test results were also somewhat more variable as 6 months-old APP^{NL-G-F} mice spent more time in the center of the open field, whereas 3- and 10-month-old APP^{NL-G-F} mice spent equal time exploring the center and the periphery. However, it should be noted that open field exploration is indeed reportedly variable, and might be less reliable to measure anxiety [37,38], compared to other anxiety-related tasks [39]. APP^{NL-G-F} mice showed anxiolytic-like behavior in the elevated plus maze, comparable to that of other AD mouse models, which could be attributed to disinhibition resulting from AD pathology [40].

Several genetic mouse models of AD that display amyloid pathology, for example APP/PS1 mice [41], display impairments in spatial-cognitive tasks such as radial-arm water maze or MWM [42]. These tasks are well-established to be hippocampus as well as mPFC dependent [43]. APP^{NL-G-F} and APP^{NL} mice performed very similarly in our MWM acquisition experiments, showing only marginal impairments in the reversal reference memory task at 6 months of age. This subtle defect could be due to somewhat more variable performance, and may not be a cognitive defect as such, which more challenging cognitive testing might reveal. Moreover this change in performance was not observed at later age, possibly overshadowed by the age-related decline in wild-type C57BL/6 mice [44]. Studies in other mouse models of Aβ accumulation have found more robustly impaired reversal learning [33,45–47], but these studies differ from ours in several ways. The more severe phenotypes mostly occurred in older animals (e.g., 12 months of age), when the pathology is more advanced compared to the early plaque stage in our mice. Also, they used mouse models that overexpress APP, whereas our model exhibits Aβ amyloidosis without APP overexpression (lacking its potential artifacts). Our mouse model exhibits relatively slow onset of pathology compared to other transgenic models of AD [7], and testing these animals at more advanced ages

might reveal more severe behavioral changes (however, testing at such senescent ages could be confounded as well).

Imaging techniques might actually be more sensitive to detect changes in brain function. Indeed, rsfMRI revealed hypersynchronized activity between memory-related areas in our mice, already at 3 months of age. The regions showing increased correlated patterns of neuronal activity were mainly those included in the prefrontal network. It still remains somewhat obscure what this hypersynchronized activity signifies or to which aspect of the pathology it could be related, but present findings are consistent with our previous observation of hypersynchronized activity in another amyloidosis model [48]. It remains difficult to relate hypersynchronous brain activity to behavioral performance, but we have previously shown that increased cortical connectivity coincides with impaired reversal learning in PFC-lesioned mice [26].

The observed changes that occur before prominent plaque deposition could be attributed to the neurotoxic effects of soluble Aβ, rather than actual Aβ plaques that mostly occur later [48]. The present report makes this even more likely as the knock-in model does not display any artifacts of APP overexpression. A previous study showed a reduction of mushroom spines at relatively early age in these mice [49], but they do not display any tau pathology or cell death, suggesting that the observed functional changes are entirely due to Aβ-induced effects. Thus, the observed rsfMRI changes could be an early sign of pathology, but we cannot exclude that the hypersynchronous frontal network could also be a neurobehavioral response to compensate for Aβ-induced dysfunction.

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Figure legends

Figure 1 - **Aβ deposition in APP^{NL} and APP^{NL-G-F} brains**. A) Brain sections from 1.5, 2, 3.5 and 6-month-old mice were immunostained using an $A\beta_{42}$ antibody. Cortical and hippocampal immunoreactive amyloid plaque load were measured using confocal microscopy revealing amyloid plaques already at the age of 3.5 months, although very minor compared to 6-month-old APP^{NL-G-F} mice (n = 7, 10, 5 and 6 mice per indicated time point, respectively). (B) absence of amyloid plaques in neocortex and hippocampus of APP^{NL} mice (left) in contrast to APP^{NL-G-F} mice, at 3.5 and 12 months of age (APP^{NL}: n= 14, APP^{NL-G-F}: n=16 at 12 months).

Figure 2 - **Locomotor activity at 3, 6 and 10 months.** (A) 23h activity patterns in APP^{NL} (black circles) and APP^{NL-G-F} mice (grey squares), 3-month-old APP^{NL-G-F} mice (left panel, n= 14) display increased locomotor activity compared to APP^{NL} mice (n=14); APP^{NL-G-F} (middle panel, n= 14) and APP^{NL} mice (n=14) at 6 months; APP^{NL-G-F} (right panel, n= 11) and APP^{NL} mice (n= 13) at 10 months. See text for statistics. (B) Overall activity measures in APP^{NL-G-F} (black bars) and APP^{NL} mice (grey bars) in the open field. Left panel: at 3, 6 and 10 months of age, APP^{NL-G-F} mice (n= 14, n= 7, n= 12, respectively) travelled equal distances as APP^{NL} mice (n= 14, n= 8, n=12, respectively); right panel: more anxiety-like behavior at 6 months in APP^{NL-G-F} (n= 7) compared to APP^{NL} mice (n= 7; see text for statistical analysis). No differences at 3 months, nor at 10 months between APP^{NL-G-F} (n= 13, n=11, respectively) and APP^{NL} mice (n= 13, n=11, respectively). Data are means ± SEM.

Figure 3 - **Anxiety and hyperactivity in the elevated plus maze in APP^{NL-G-F} (black bars) and APP^{NL} mice (grey bars).** Left panel: at 3 months, APP^{NL-G-F} mice (n=8) showed less preference for the close arm than APP^{NL} mice (n=7); middle panel: preference for the open arm stronger in 6-month-old APP^{NL-G-F} mice (n= 7) compared to APP^{NL} mice (n= 8), with a significant reduction in

the preference for the close arm; right panel: 10-month-old APP^{NL-G-F} mice (n=11) displayed significantly decreased number of beam breaks in the close arm compared to APP^{NL} mice (n= 10). Data are means ± SEM.

Figure 4 - Social preference in APP^{NL-G-F} and APP^{NL} mice at 3, 6 and 10 months of age. (A) Left panel: at 3 months, both groups showed preference for S1 side over the empty side, but more pronounced preference in APP^{NL-G-F} mice (open bars; n= 9, 7, 10 respectively) than APP^{NL} mice (filled bars; n= 8, 8, 8, respectively). Increased preference for the novel mouse (S2) in both genotypes during the recognition phase; middle panel: 6-month-old APP^{NL-G-F} mice had little preference towards S1 during the social preference trial, while they explore S2 more than S1 in the recognition phase. Time spent with the novel mouse in the second trial was reduced in APP^{NL} mice compared to APP^{NL-G-F}; right panel: at 10 months of age, none of the two genotypes displayed any preference for the novel mouse. In fact, APP^{NL-G-F} mice showed preference for the empty side over the S1 during the first trial. (B) During the sociability phase, both groups displayed similar preference ratio at 3 and 6 months, indicating that APP^{NL} (n=8 and n=8, respectively) and APP^{NL-G-F} mice (n=9 and n=7, respectively) displayed similar preference for S1 versus empty the cage. A tendency towards reduced preference in APP^{NL-G-F} mice (n=10) starting at 10 months compared to APP^{NL} mice (n=8). (C) The recognition ratio increased at 3, 6 and 10 months in APP^{NL} mice (not significant). Time bin analysis of social preference (D & F) and recognition for novelty (E & G) in APP^{NL}(D & E) and APP^{NL-G-F} mice (F & G) at 10 months of age: (D) APP^{NL} mice showed increased exploration of S1 compared to empty cage only for the first two time bins. (E) APP^{NL} mice had a strong preference for S2 during the beginning of the recognition phase (time bins 1 and 2). (F) APP^{NL-G-F} mice showed equal interest for S1 and empty side during the first time bin with a pronounced increased in exploration of the empty side from the second time bin. (G) APP^{NL-G-F} mice displayed a preference for S1 over the novel mouse, exploring S2 only during time bin 1. Data are means ± SEM.

Figure 5 - Morris water maze performance at 3-4, 6-7 and 10-11 months of age in APP^{NL} (grey bars; n= 13, 7 and 12 respectively) and APP^{NL-G-F} mice (black bars; n= 12, 8 and 11 respectively) . TQ= Target quadrant; AD1= adjacent 1; AD2= Adjacent 2; OQ= Opposite quadrant. During 10 days of acquisition, mice were given a probe trial on day 6 (probe 1) and 11 (probe 2) for each time point. At 3-4 months of age, APP^{NL} mice performed at a slower rate than APP^{NL-G-F} during the first days of acquisition learning, reaching similar performance on day 6 (A, left panel), the probe trial showed no differences between the two groups (A, middle panel). During probe 2 on day 11 after acquisition learning, memory retention was increased in APP^{NL} compared to APP^{NL-G-F} mice as shown by significant target preference (A, right panel). At 6-7 months, both groups showed good performance during the acquisition of the task (B, left panel). On the first probe trial, although a mild preference for the target quadrant was present, no significant differences were found (B, middle panel). However, a significant increase of time spent in the target quadrant over the other quadrants was detected in both groups (B, right panel). 10-11 months old-APP^{NL} and APP^{NL-G-F} mice learned the platform location (C, left panel) and showed retention memory during probe 2 (C right panel). However, after 5 days of acquisition learning, on day 6 the first probe did not show any indication of preference for the target quadrant in none of the groups (C middle panel). Total distance swam and time spent in quadrant expressed as means ± SEM. Target quadrant versus opposite quadrant indicated with ##P<0.01, ###P<0.001 (Tukey pairwise).

Figure 6 - Water maze reversal learning at 3-4, 6-7 and 10-11 months of age in APP^{NL} (grey bars; n= 13, 7 and 12 respectively) and APP^{NL-G-F} mice (black bars; n= 12, 8 and 11 respectively). TQ= Target quadrant; AD1= adjacent 1; AD2= Adjacent 2; OQ= Opposite quadrant. Total distance swam and time spent in quadrant expressed as means ± SEM. At 3-4 months, both APP^{NL} and APP^{NL-G-F} mice learned the reversed platform location (A, left panel) and

showed good memory retention in the probe test (A, right panel). 6-7 months old-APP^{NL} and APP^{NL-G-F} mice showed similar performance during the acquisition of the new platform location (B, left panel). During the probe test APP^{NL} mice had a significant preference for target quadrant over the other quadrants, whereas APP^{NL-G-F} mice were marginally worse at this (B right panel). At 10-11 months, there was no significant reversal learning curve (C, left panel), but both APP^{NL} and APP^{NL-G-F} mice eventually did display a preference for the new target location (C right panel). Data are means ± SEM. Target quadrant versus opposite quadrants indicated with ##P<0.01, ###P<0.001 (Tukey pairwise).

Figure 7 - Increased functional connectivity at 3 months in APP^{NL-G-F} mice. (A) The functional connectivity (FC) map shows increased synchrony in regions that are functionally connected to the prefrontal cortex. (B) Correlation coefficients of paired regions indicate increased prefrontal connectivity in APP^{NL-G-F} mice (left panel, upper part) compared to APP^{NL} (lower part). This hypersynchrony was no longer present at later ages as shown by mean FC at 7 and 11 months of age (right panel).

Spatial reversal learning defect coincides with hypersynchronous telencephalic BOLD functional connectivity in APP$^{NL-F/NL-F}$ knock-in mice

Disha Shah[1,2,3*], Amira Latif Hernandez[2*], Bart De Strooper[3], Takashi Saito[4], Takaomi Saido[4], Marleen Verhoye[1], Rudi D'Hooge[2], Annemie Van der Linden[1]

[1] Bio-Imaging Lab, University of Antwerp, Belgium

[2] Laboratory of Biological Psychology, KU Leuven, Belgium

[3] VIB Center for the Biology of Disease, KU Leuven, Belgium

[4] Laboratory for Proteolytic Neuroscience, Riken Brain Science Institute, Japan

* Equal contribution

Corresponding authors: Annemie Van der Linden, PhD; Disha Shah

annemie.vanderlinden@uantwerpen.be; disha.shah@uantwerpen.be

Abstract

Amyloid pathology occurs early in Alzheimer's disease (AD), and has therefore been the focus of numerous studies. Transgenic mouse models have been instrumental to study amyloidosis, but observations might have been confounded by APP-overexpression artifacts. The current study investigated early functional defects in an APP knock-in mouse model, which allows assessing the effects of pathological amyloid-beta (Aβ) without interference of APP-artifacts. Female $APP^{NL/NL}$ knock-in mice of 3 and 7 months old were compared to age-matched $APP^{NL-F/NL-F}$ mice with increased Aβ42/40 ratio and initial Aβ-plaque deposition around 6 months of age. Spatial learning was examined using a Morris water maze protocol consisting of acquisition and reversal trials interleaved with reference memory tests. Functional connectivity (FC) of brain networks was assessed using resting-state functional MRI (rsfMRI). The Morris water maze data revealed that 3 months old $APP^{NL-F/NL-F}$ mice were unable to reach the same reference memory proficiency as $APP^{NL/NL}$ mice after reversal training. This cognitive defect in 3-month-old $APP^{NL-F/NL-F}$ mice coincided with hypersynchronous FC of the hippocampal, cingulate, caudate-putamen, and default-mode-like networks. The occurrence of these defects in $APP^{NL-F/NL-F}$ mice demonstrates that cognitive flexibility and synchronicity of telencephalic activity are specifically altered by early Aβ pathology without changes in APP neurochemistry.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, characterized by progressive impairments in learning and memory, and other cognitive dysfunctions[1]. Its pathological hallmarks include the accumulation of extracellular amyloid plaques and intracellular tau tangles, but the mechanisms leading to functional defects and full-blown AD pathology are poorly understood. Available treatment offers symptomatic benefit without halting or reversing disease progression. AD pathology progresses over decades before symptoms develop, at which stage the damage might be too extensive, emphasizing the importance of early diagnosis and intervention. In the interest of establishing early biomarkers and therapy, many studies focused on the pathological changes at initial stages of the disease. According to the amyloid cascade hypothesis, accumulation of amyloid-beta (Aβ) peptide is such an event that impairs brain function early on, and triggers tau pathology and neurodegeneration[2]. Recent studies suggest that early disease signs are not caused by Aβ plaque deposition as such, but rather by pre-plaque levels of soluble Aβ peptides with high Aβ42/40 ratio[3–5].

Transgenic mouse models that overexpress APP have been instrumental to our present knowledge of AD pathogenesis in general, and Aβ-related mechanisms in particular. Much work on transgenic mouse models has, however, been confounded by the possibility that overexpression of APP and APP fragments induces artificial phenotypes. For example, overexpression of wild-type APP can interfere with cellular transport mechanisms, cause loss of synapses, and lead to memory disruption without actual Aβ involvement[6,7]. Knock-in mouse models, which express APP at wild-type levels while overproducing pathogenic Aβ, have been specifically developed to control for these possible confounds[6,7]. We will presently use such a knock-in model to investigate the putative occurrence of functional defects at the pre-plaque stage. High Aβ42/40 ratio prior to plaque deposition has been suggested to cause synaptic and neural network dysfunction leading to cognitive defects in early phases of AD[3–5].

In the current study, we compared $APP^{NL\text{-}F/NL\text{-}F}$ knock-in mice with high Aβ42/40 ratio to $APP^{NL/NL}$ mice at two time points that reflect early pathological stages, i.e. before and at the initial stage of plaque deposition[8]. Different aspects of spatial learning and memory were assessed using an extended protocol in the Morris water maze task to model declarative-like memory functions and response-

flexibility or working memory[9] . Functional connectivity (FC) between telencephalic regions was studied using non-invasive resting-state functional Magnetic Resonance Imaging (rsfMRI), which uses low frequency (0.01-0.1Hz) fluctuations in blood-oxygenation-level-dependent (BOLD) signals to measure fluctuations of neuronal activity. FC is defined as the temporal correlation of BOLD fluctuations between spatially distinct brain regions[10,11]. Considering that rsfMRI has been applied extensively in AD patients, it allows relatively easy translation to the clinic[12]. Previous studies have demonstrated the usefulness of rsfMRI to assess the functionality of brain networks in AD-related pharmacological models and transgenic mouse models[13–16].

2. Results

2.1. Spatial learning in the Morris water maze

At 3 months of age (**Figure 1**), repeated measures (RM) two-way ANOVA showed no statistical difference between the learning curves of $APP^{NL-F/NL-F}$ and $APP^{NL/NL}$ mice during acquisition (RM-ANOVA, 'genotype x time' interaction $F_{9,135}=0.446$, $p=0.907$, genotype effect $F_{1,15}=0.094$, $p=0.763$) or reversal trials (RM-ANOVA, 'genotype x time' interaction $F_{4,60}=1.399$, $p=0.245$, genotype effect $F_{1,15}=1.244*10^{-5}$, $p=0.997$). RM-ANOVA indicated that all animals learnt the location of the platform during acquisition (time effect $F_{9,135}=29.86$, $p<0.0001$) and reversal learning (time effect $F_{4,60}=23.36$, $p<0.0001$). There was no significant difference in velocity between groups during the acquisition ('genotype x time' interaction $F_{9,135}=1.416$, $p=0.187$, genotype effect $F_{1,15}=0.066$, $p=0.800$) or reversal phase ('genotype x time' interaction $F_{4,60}=1.567$, $p=0.194$, genotype effect $F_{1,15}=0.611$, $p=0.446$) (**Figure S1**). Probe trials were conducted on days 6 (acquisition probe 1) and 11 (acquisition probe 2) to assess spatial reference memory. The probe trials of the acquisition phase showed a significant preference for the target quadrant compared to the other quadrants for both $APP^{NL/NL}$ and $APP^{NL-F/NL-F}$ mice (one-way ANOVA, probe 1 $APP^{NL/NL}$ $p=0.037$; probe 1 $APP^{NL-F/NL-F}$ $p=0.01$; probe 2 $APP^{NL/NL}$ $p=0.0007$; probe 2 $APP^{NL-F/NL-F}$, $p<0.0001$), but no group differences were observed (two-way ANOVA, probe 1 "genotype x time in quadrant" interaction effect, $F_{3,60}=0.1184$, $p=0.948$; probe 2 interaction effect, $F_{3,60}=0.956$, $p=0.419$). A third probe trial was conducted on day 16 (i.e., after 5 days of reversal training). During this trial, $APP^{NL/NL}$ mice did show a preference for the new target quadrant (one-way ANOVA $p<0.0001$), whereas $APP^{NL-F/NL-F}$ mice did not (one-way ANOVA $p=0.234$). A significant group

difference in preference for the new target quadrant was observed during the probe test of the reversal phase (two-way ANOVA, 'genotype x time in quadrant' interaction effect, $F_{3,60}$=6.123, p=0.001, post-hoc Sidak test preference for new target quadrant p=0.0064).

At 7 months of age (**Figure 1**), there was no statistical difference in the learning curve of the acquisition phase (RM-ANOVA 'genotype x time' interaction $F_{9,135}$=0.523, p=0.854 ;genotype effect $F_{1,1986}$.817, p=0.186;) or reversal phase (RM-ANOVA, 'genotype x time' interaction $F_{4,60}$=0.638, p=0.637, genotype effect $F_{1,15}$=3.224, p=0.095) between $APP^{NL-F/NL-F}$ and $APP^{NL/NL}$ mice. RM-ANOVA indicated that all animals learnt the location of the platform during acquisition (time effect $F_{9,135}$=9.266, p<0.0001) and reversal learning (time effect $F_{4,60}$=3.486, p=0.0123). There was no significant difference in velocity between groups during the acquisition phase (RM-ANOVA, 'genotype x time' interaction $F_{9,135}$=1.393, p=0.199, genotype effect $F_{1,15}$=0.006, p=0.935) or reversal phase ('genotype x time' interaction $F_{4,60}$=0.562, p=0.691, genotype effect $F_{1,15}$=2.698, p=0.121) (**Figure S1**). The first probe trial of the acquisition phase showed no significant preference for the target quadrant compared to the other quadrants for both $APP^{NL/NL}$ (one-way ANOVA p=0.09) and $APP^{NL-F/NL-F}$ mice (one-way ANOVA p=0.1344) and no group differences were observed (two-way ANOVA, 'genotype x time in quadrant' interaction effect, $F_{3,60}$=1.098, p=0.360). The second probe trial of the acquisition phase showed a significant preference for the target quadrant in both $APP^{NL/NL}$ (one-way ANOVA p=0.0003) and $APP^{NL-F/NL-F}$ mice (one-way ANOVA p=0.0009), but no group differences were observed (two-way ANOVA, genotype x time in quadrant' interaction effect, $F_{3,60}$=0.818, p=0.483). The third probe trial (i.e., after 5 days of reversal training) demonstrated that the $APP^{NL/NL}$ control mice did show a slight preference for the new target quadrant (one-way ANOVA p=0.01), whereas the $APP^{NL-F/NL-F}$ mice did not (one-way ANOVA, p=0.224). However, no group difference in preference for the new target quadrant was observed during the reversal probe test (two-way ANOVA, 'genotype x time in quadrant' interaction effect, $F_{3,60}$=1.754, p=0.165).

2.2. Functional connectivity within brain networks

Figure 2 shows the neurologically relevant ICA components, each of which consist of voxels that show highly correlated BOLD time courses, and therefore form resting-state networks. The following networks were identified: the hippocampal network, default-mode-like (DMN) network, the

frontal/cingulate network, the cingulate/thalamus network, the caudate putamen network, the nucleus accumbens/hypothalamus network, the sensorimotor network and the piriform network.

Table 1 specifies the brain regions observed in each of these ICA components. Those brain regions were used to compute FC-matrices (**Figure 3**). At 3 months of age there was an overall hypersynchrony of BOLD FC in $APP^{NL-F/NL-F}$ mice compared to $APP^{NL/NL}$ mice (**Figure 3A**), as is shown by the T-values representing the difference between groups (**Figure 3C**). BOLD FC within brain networks was significantly increased in $APP^{NL-F/NL-F}$ mice in the hippocampal (two-way ANOVA, p=0.01) and frontal/cingulate networks (two-way ANOVA, p=0.03) (**Figure 3D**). At 7 months there is an overall hyposynchrony of BOLD FC in $APP^{NL-F/NL-F}$ mice compared to $APP^{NL/NL}$ mice (**Figure 3B**), as is shown by the T-values representing the difference between groups (**Figure 3C**). However, the decrease of BOLD FC within brain networks observed in $APP^{NL-F/NL-F}$ vs. $APP^{NL/NL}$ mice did not reach statistical significance when correcting for multiple comparisons (**Figure 3E**).

2.3. Connectivity between brain networks

Besides analyzing BOLD FC *within* networks, FC *between* brain networks resulting from ICA was additionally assessed. At 3 months of age, significant hypersynchrony of BOLD FC was observed in $APP^{NL-F/NL-F}$ vs. $APP^{NL/NL}$ mice (two sample T-test of zFC-matrices) between the hippocampus-caudate putamen (p=0.01), hippocampus-nucleus accumbens (p=0.02), hippocampus-sensorimotor (p=0.01), DMN like-frontal/thalamus (p=0.03), DMN like-caudate putamen (p=0.03), DMN like-sensorimotor (p=0.01), cingulate/frontal-sensorimotor (p=0.03), frontal/thalamus-caudate putamen (p=0.02), caudate putamen-nucleus accumbens (p=0.01) and caudate putamen-sensorimotor (p=0.01) (**Figure 4**). At 7 months of age, significant hyposynchrony of BOLD FC was observed in $APP^{NL-F/NL-F}$ vs. $APP^{NL/NL}$ mice (two sample T-test of zFC-matrices) between the hippocampus-caudate putamen (p=0.008) and DMN like- caudate putamen (p=0.03) (**Figure 4**).

Compared to $APP^{NL/NL}$ mice, $APP^{NL-F/NL-F}$ mice showed deficits of cognitive flexibility observed during reversal learning in the MWM task. These type of cognitive functions depend on the functionality of the hippocampus and its connection to the retrosplenial areas and frontal cortex. Additionally, the analyses of FC within and between brain networks showed a significant impairment of hippocampal FC in 3 months old $APP^{NL-F/NL-F}$ mice. Therefore, to have a more detailed view of FC of the hippocampus with other brain regions, FC-maps of the right hippocampus were computed for each

group (**Figure 5**). The hippocampal FC map shows that at 3 months of age the $APP^{NL-F/NL-F}$ mice demonstrated hypersynchronous BOLD FC in the hippocampus bilaterally (two-way ANOVA, p=0.007) compared to $APP^{NL/NL}$ mice. Additionally, hypersynchronous BOLD FC between the hippocampus and the frontal cortex (two-way ANOVA, p=0.001) and between the hippocampus and the retrosplenial cortex (two-way ANOVA, p=0.001) was also observed in $APP^{NL-F/NL-F}$ vs. $APP^{NL/NL}$ mice. At 7 months of age, $APP^{NL-F/NL-F}$ mice showed no significant hyposynchrony of BOLD FC between the hippocampus bilaterally (two-way ANOVA, p=0.111) or between the hippocampus and retrosplenial cortex (two-way ANOVA, p=0.118) compared to $APP^{NL/NL}$ mice. Notably, $APP^{NL-F/NL-F}$ mice showed significant hyposynchrony of BOLD FC between hippocampus and frontal cortex (two-way ANOVA, p=0.02).

Moreover, the analyses of FC between brain networks showed a significant impairment of FC between the striatal (caudate putamen) and DMN-like network, and between the caudate putamen and hippocampus, in $APP^{NL-F/NL-F}$ mice at 3 months, but also at 7 months of age. These findings were confirmed when analyzing the FC maps of the right caudate putamen for each group (**Figure 6**). The caudate putamen FC map shows hypersynchrony of BOLD FC between the caudate putamen and cingulate region, which is a major node of the DMN-like network (two-way ANOVA, p=0.001), as well as between the caudate putamen and hippocampus (two-way ANOVA, p=0.001) at 3 months of age in the $APP^{NL-F/NL-F}$ vs. $APP^{NL/NL}$ mice. At 7 months of age, $APP^{NL-F/NL-F}$ mice showed a significant hyposynchrony of BOLD FC between caudate putamen and cingulate regions (two-way ANOVA, p=0.02), as well as between the caudate putamen and hippocampus (two-way ANOVA, p=0.02) compared to $APP^{NL/NL}$ mice.

3. Discussion

The current study aimed at investigating functional changes associated with early Aβ pathology in an APP knock-in mouse model, which allows assessing these effects without confounds by APP or APP fragments. $APP^{NL-F/NL-F}$ mice were constructed to display pathologically increased Aβ42/40 ratio compared to $APP^{NL/NL}$ mice[8]. In the present report, we examined these mice well before (3 months) and at the initial stages of Aβ plaque deposition (7 months) (**Figure S2**). Similar to brain pathology in AD patients, plaques in the cortex of $APP^{NL-F/NL-F}$ mice consist mainly of the Aβ42 species[8]. $APP^{NL-F/NL-F}$ mice (but not $APP^{NL/NL}$ mice) display initial Aβ plaques around 6 months of age,

concurrent with accumulation of microglia and astrocytes, whereas synaptic loss was reported to occur not before 9-12 months of age[8].

Behavior defects were previously shown to occur very late in these mice. Using $APP^{NL/NL}$ mice as controls, impaired avoidance behavior and compulsivity were observed in $APP^{NL-F/NL-F}$ mice at 8-12 months, and deficits in place preference learning between 13-17 months[17]. $APP^{NL-F/NL-F}$ mice also showed deficits in Y-maze alteration at 18 months of age[8]. In accordance, we failed to observe differences in 3- and 7-month-old $APP^{NL/NL}$ and $APP^{NL-F/NL-F}$ mice during the acquisition phase of MWM learning. However, we did find indications of impaired spatial reversal learning in 3-month-old $APP^{NL-F/NL-F}$ mice. After a series of reversal learning trials, during which again no major impairment was observed, $APP^{NL-F/NL-F}$ mice failed to show similar reference memory proficiency in the reversal probe trial compared to $APP^{NL/NL}$ mice. Apparently, general learning abilities were not affected in these mice, but they failed to acquire and/or remember the novel platform position as effectively as $APP^{NL/NL}$ mice. At 7 months of age, on the other hand, again no differences were observed during acquisition and reversal trails between $APP^{NL-F/NL-F}$ and $APP^{NL/NL}$ mice. During the reversal trials $APP^{NL-F/NL-F}$ mice seem to travel less distance than $APP^{NL/NL}$ mice, suggesting improved performance. However, this difference was not statistically significant and the reversal probe trial showed that 7-month-old $APP^{NL-F/NL-F}$ mice were actually slightly less accurate in searching for the platform than $APP^{NL/NL}$ mice of that age. We also observed that $APP^{NL/NL}$ mice failed to acquire the same spatial proficiency after reversal training as they did at 3 months of age, which might have been due to age-related deteriorations in cognitive flexibility. Indeed, $APP^{NL/NL}$ mice reportedly show age-dependent defects of learning abilities which could overshadow group differences[17,18] . As a probable result, the difference between $APP^{NL-F/NL-F}$ and $APP^{NL/NL}$ mice in the reversal probe trial was much less pronounced than that at 3 months.

Thus, impaired cognitive flexibility appears to be the earliest behavioral or cognitive change in this mouse model. The reversal defect in 3-month-old $APP^{NL-F/NL-F}$ mice is especially interesting since it occurred at an age when these mice hardly have Aβ plaques or other signs of major neuropathology. Notably, our rsfMRI data revealed hypersynchrony between telencephalic neural networks at this specific age, in particular within hippocampus and between hippocampus and prefrontal cortex. Reversal learning requires animals to update a previously learnt associative spatial map. Deficits in

reversal learning indicate decreased adaptability of acquired information to changing environmental demands (i.e., cognitive flexibility). It has been well established that different aspects of spatial learning in the MWM task depend on telencephalic regions, most prominently hippocampus[9,19,20]. However, reversal learning abilities appear to depend specifically on a network of reciprocal connections and subsequent crosstalk between hippocampus, prefrontal cortex and striatum[9,21,22].

RsfMRI is a non-invasive tool that allows assessing spatiotemporal dynamics between the characteristics of functional brain networks and pathological changes. RsfMRI has been applied in the $APP^{NL-F/NL-F}$ and $APP^{NL/NL}$ mice at 3 and 7 months of age to establish whether their increased Aβ42/40 ratio and concomitant early Aβ pathology are associated with deficits in brain function. At 3 months of age, $APP^{NL-F/NL-F}$ mice showed hypersynchrony of BOLD FC *within* the hippocampus and frontal/cingulate networks compared to $APP^{NL/NL}$ mice. Deficits of the hippocampus could affect learning and memory abilities, but the specific early involvement of frontal brain regions could be an additional early indicator of impairments in the ability to elaborate new rules, or cognitive flexibility, which were observed in 3 months old $APP^{NL-F/NL-F}$ mice. In line with these data, previous reports show that 5XFAD mice demonstrate early cognitive deficits related to frontal brain regions that occur before hippocampal-dependent learning impairments[23]. Additionally, 3 months old $APP^{NL-F/NL-F}$ mice demonstrate hypersynchronized FC *between* functional brain networks compared to $APP^{NL/NL}$ mice, more specifically involving the hippocampal, cingulate-frontal, frontal-thalamic, default-mode like, striatal (caudate putamen), and sensorimotor networks. BOLD FC was specifically increased between hippocampus and prefrontal cortex, hippocampus and retrosplenial cortex, striatum and hippocampus, and striatum and cingulate cortex. These are connections that are reportedly involved in cognitive flexibility[9]. More importantly, these data indicate changes in brain function at an early stage of pathology in $APP^{NL-F/NL-F}$ mice, occurring before Aβ deposition.

In contrast, $APP^{NL-F/NL-F}$ mice showed telencephalic hyposynchrony of BOLD FC at 7 months of age. This change was less extensive than the hypersynchrony observed at 3 months of age, but still involved the hippocampal, striatal and default mode-like networks. Hyposynchronous BOLD FC was observed between hippocampus and prefrontal cortex, striatum and hippocampus, and striatum and cingulate cortex. This dynamic pattern of hypersynchrony before Aβ deposition and subsequent hyposynchrony at later age is consistent with other studies in transgenic APP mouse models. We

previously reported that TG2576 (APPK670/671L Swedish) and PDAPP (APP V717F Indiana) transgenic mice display early hypersynchrony of BOLD FC in hippocampus and frontal cortex, respectively[14]. This early hypersynchrony was associated with increased levels of pre-plaque stage Aβ, and in TG2576 mice, this altered BOLD FC (and synaptic deficits) could be prevented by an anti-Aβ antibody. At later stages of Aβ deposition, both TG2576 and PDAPP transgenic mouse models displayed hyposynchronous telencephalic BOLD FC[14], which was also reported in APP/PS1 transgenic mice at advanced stages of Aβ pathology[16]. Notably, this phasic effect on brain BOLD FC appears to be translationable to clinical AD as early hypersynchrony was reported in children carrying the PSEN1 mutation[24]. Moreover, late stage hyposynchronous telencephalic BOLD FC has been observed consistently at more advanced stages of AD pathology[12].

Apparently, telencephalic networks in $APP^{NL\text{-}F/NL\text{-}F}$ mice progress from a hypersynchronous state to hyposynchrony between 3 and 7 months of age. It has been shown that hypersynchrony of BOLD FC in transgenic mouse models is associated with increased ratio of excitatory/inhibitory functioning[4,14,25], probably caused by the damaging effects of pathologically increased Aβ42/40 ratio, which is increased from 3 months of age onwards in the $APP^{NLF/NLF}$ mice (**Figure S2A**). This hyper-to-hyposynchrony shift at the functional network level could be caused by progressive damage induced by this hyperexcitability and the complex neurotoxic effects of Aβ42 (note that between 3-7 months Aβ deposition is still low) (**Figure S2B**)[8]. More severe cognitive deficits reported in this model at advanced ages[17] could have resulted from progressive synaptic and neural network defects.

The reversal defects we observed in $APP^{NL\text{-}F/NL\text{-}F}$ mice could be considered to be relatively mild compared to the extensive telencephalic hypersynchrony of BOLD FC in these mice. However, it has been well established that during the preclinical phase of AD, brain network dysfunctions also occur in absence of overt cognitive symptoms[26]. Thus, FC MRI could be a useful tool to determine early stage pre-symptomatic changes in functional brain networks.

4. Material and methods

4.1. Animals

Female $APP^{NL\text{-}F/NL\text{-}F}$ knock-in mice (APP KM670/671N Swedish, APP I716F Iberian) were compared to age-matched $APP^{NL/NL}$ knock-in mice (APP KM670/671N Swedish) at 3 months ($APP^{NL\text{-}F/NL\text{-}F}$ N=9, $APP^{NL/NL}$ N=13) and 7 months of age ($APP^{NL\text{-}F/NL\text{-}F}$ N=9, $APP^{NL/NL}$ N=10). APP knock-in mice[8] were

derived from the Riken Institute colony (Laboratory for Proteolytic Neuroscience, PI: Dr. Takaomi Saido, Riken Brain Science Institute, Japan). $APP^{NLF/NLF}$ mice show a progressive increase of Aβ42/Aβ40 ratio at 3 months of age compared to wild-type and $APP^{NL/NL}$ mice (**Figure S2A**), and the first Aβ plaques deposit around 6 months of age (**Figure S2B**). $APP^{NL/NL}$ mice do not develop Aβ plaques during their entire lifespan and are considered an appropriate negative control for $APP^{NL\text{-}F/NL\text{-}F}$ mice as the levels of APP, APP intracellular domain (AICD) and C-terminal fragment β (CTF-β) are equivalent in both models, thus facilitating interpretation of the effects of increased Aβ42/Aβ40 ratio caused by the Iberian mutation in the $APP^{NL\text{-}F/NL\text{-}F}$ mice (**Figure S2**). All procedures were performed in strict accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The protocols were approved by the Committee on Animal Care and Use at KU Leuven, Belgium (permit number: P073/2013) and all efforts were made to minimize animal suffering. All mice were first subjected to rsfMRI imaging, after which the behavior tasks were performed, to avoid variation in the FC data caused by functional or structural reorganization elicited during learning procedures.

4.2. Resting-state functional MRI

MRI procedures

For the MRI handling procedures all mice were anesthetized with 2.5% isoflurane (IsoFlo, Abbott, Illinois, USA), which was administered in a mixture of 70% nitrogen (400 cc/min) and 30% oxygen (200 cc/min). During the rsfMRI imaging procedures, a combination of medetomidine (Domitor, Pfizer, Karlsruhe, Germany) and isoflurane was used to sedate the animals[14]. After positioning of the animal in the scanner, medetomidine was administered subcutaneously as a bolus injection (0.3 mg/kg), after which the isoflurane level was immediately decreased to 1%. Ten minutes before the rsfMRI acquisition, isoflurane was decreased to 0.5%. RsfMRI scans were consistently acquired 40 min after the bolus injection, during which the isoflurane level was kept at 0.5%. After the imaging procedures, the effects of medetomidine were counteracted by subcutaneously injecting 0.1mg/kg atipamezole (Antisedan, Pfizer, Karlsruhe, Germany). The physiological status of all animals was monitored throughout the imaging procedure. A pressure sensitive pad (MR-compatible Small Animal Monitoring and Gating system, SA Instruments, Inc.) was used to monitor breathing rate and a rectal thermistor

with feedback controlled warm air circuitry (MR-compatible Small Animal Heating System, SA Instruments, Inc.) was used to maintain body temperature at 37.0 ± 0.5°C.

MRI procedures were performed on a 9.4T Biospec MRI system (Bruker BioSpin, Germany) with the Paravision 5.1 software (www.bruker.com). Images were acquired using a standard Bruker cross coil set-up with a quadrature volume coil and a quadrature surface coil for mice. Three orthogonal multi-slice Turbo RARE T2-weighted images were acquired to render slice-positioning uniform (repetition time 2000 ms, echo time 33 ms, 16 slices of 0.4 mm). Field maps were acquired for each animal to assess field homogeneity, followed by local shimming, which corrects for the measured inhomogeneity in a rectangular VOI within the brain. Resting-state signals were measured using a T2*-weighted single shot EPI sequence (repetition time 2000 ms, echo time 15 ms, 16 slices of 0.4 mm with a gap of 0.1 mm, 300 repetitions). The field-of-view was (20 x 20) mm^2 and matrix size (128 x 64), resulting in voxel dimensions of (0.156 x 0.312 x 0.5) mm^3.

MRI data pre-processing

Pre-processing of the rsfMRI data, including realignment, normalization and smoothing, was performed using SPM8 software (Statistical Parametric Mapping, http://www.fil.ion.ucl.ac.uk). First, all images within each session were realigned to the first image. This was done using a least-squares approach and a 6-parameter (rigid body) spatial transformation. For the rsfMRI data analyses, motion parameters resulting from the realignment were included as covariates to correct for possible movement that occurred during the scanning procedure. Second, all datasets were normalized to a study specific EPI template and co-registered to an anatomical T2-weighted template. The normalization steps consisted of a global 12-parameter affine transformation followed by the estimation of the nonlinear deformations. Finally, in plane smoothing was done using a Gaussian kernel with full width at half maximum of twice the voxel size (0.31 x 0.62) mm^2. All rsfMRI data were filtered between 0.01-0.25 Hz using the REST toolbox (REST1.7, http://resting-fmri.sourceforge.net).

MRI data analysis

RsfMRI data were first analyzed with group independent component analysis (ICA) to determine which brain networks can be discerned using the GIFT-toolbox (Group ICA of fMRI toolbox version 2.0a: http://icatb.sourceforge.net/). First the data of each individual animal was concatenated. Then

group ICA was performed using the Infomax algorithm, followed by back reconstruction of the data to single-subject independent components and time courses. ICA was performed using a pre-set of 15 components, which was shown to be appropriate to identify networks in mice[27,28]. Masks containing the individual brain regions resulting from the ICA analyses were defined using MRicron software (MRicron version 6.6, 2013, http://www.mccauslandcenter.sc.edu/mricro/) and used for region-of-interest (ROI) correlation analyses, where pairwise correlation coefficients between each pair of ROIs were calculated and z-transformed using an in-house program developed in MATLAB (MATLAB R2013a, The MathWorks Inc. Natick, MA, USA). Mean z-transformed FC matrices were calculated for each group. For inter-network analyses, homologous ICA components were grouped and the resulting brain networks were then used for inter-network correlation analyses. Statistical analyses of the rsfMRI data included two-sample T-tests and two-way ANOVA with Sidak correction for multiple comparisons ($p<0.05$).

Additionally, seed-based analyses were performed by computing individual z-transformed FC-maps of the right hippocampus and right caudate putamen using REST toolbox, resulting in FC-maps for each of these seed regions for each group. FC between the seed-region and other regions on the FC-map were calculated by defining a mask containing the ROIs derived from the mean statistical FC-maps, and then calculating the z-values from these ROIs for each individual subject using REST-toolbox. Statistical analyses of the FC-maps included a one-sample T-test ($p<0.001$, uncorrected, threshold 10 voxels) for within group analyses, and included a two-way ANOVA with Sidak correction for multiple comparisons ($p<0.05$) for between group analyses of specific functional connections i.e. hippocampus bilateral, hippocampus-frontal, hippocampus-retrosplenial, caudate putamen-cingulate and caudate putamen-hippocampus.

4.3. Spatial learning in the Morris water maze

The Morris water maze test was performed to assess spatial memory that relies on distal cues to locate a submerged platform (15 cm diameter) in an open circular swimming arena (150 cm diameter) filled with opaque water (non-toxic white paint, 26 ± 1 °C), as previously described[9]. Analyses included 10 days of acquisition training, where each daily session consisted of 4 swimming trials (15 min interval between trials) starting randomly from 4 starting positions. Swimming tracks were

recorded using video hardware and Ethovision software (Noldus, The Netherlands). Mice that failed to find the platform within 120 s were guided to it and remained there for 15 s before being returned to their cages. Reference memory performance is measured as preference for the platform area when the platform is absent, and was tested during probe trials (100 s) after 5 and 10 acquisition sessions and after 5 reversal sessions, i.e. on days 6, 11, and 16 respectively. After 10 days of acquisition training, when the mice have established a robust preference for the platform location, reversal training was performed during 5 days, during which the location of the platform was changed, requiring relearning and cognitive flexibility. Analyses included calculating path length (i.e. distance traveled by the mouse before finding the platform), % time spent in the target quadrant during the 15 training sessions, and % time spent in each quadrant during the probe trials. Statistical analyses included one-way and two-way repeated measures ANOVA with Sidak correction for multiple comparisons ($p<0.05$).

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Author contribution statement: DS and ALH contributed to experimental set-up, data acquisition, data analyses and manuscript preparation. BDS, TS and TS provided the mouse models. RDH oversaw all data acquisition and analyses and reporting of behavioural data. MV and AVDL oversaw all data acquisition and analyses and reporting of MRI data.

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385 **Tables**

386 **Table 1: Brain regions in the ICA components**

ICA component/brain network	Brain regions	Abbreviation
Hippocampus	subiculum dorsal hippocampus ventral hippocampus	Sub dHC vHC
DMN-like	Prefrontal cortex Cingulate cortex Retrosplenial cortex Hippocampus Thalamus Parietal association cortex	PLC Cg Resp HC T PaA
Frontal/cingulate	Prefrontal cortex Cingulate cortex	PLC Cg
Cingulate/thalamus	Cingulate cortex Thalamus	Cg T
Caudate putamen	Caudate putamen	Cpu
Nucleus accumbens/hypothalamus	Nucleus accumbens Anterior hypothalamus	NA HT
Sensorimotor	Somatosensory cortex Motor cortex	SS MC

Piriform	Piriform cortex	Pir

Figure legends

<u>Figure 1:</u> Spatial learning in the Morris water maze. A-B show learning curves of the acquisition (10 days) and reversal phase (5 days) as distance moved (cm) for $APP^{NL/NL}$ and $APP^{NL-F/NL-F}$ mice at 3 months (**A**) and 7 months (**B**) of age. Timing of the probe trials are indicated in red i.e. on day 6 (acquisition probe 1), day 11 (acquisition probe 2) and day 16 (reversal probe 3). **C-E** show the results of the probe trials as % time spent in each quadrant for $APP^{NL/NL}$ and $APP^{NL-F/NL-F}$ mice at 3 and 7 months of age during the acquisition (**C-D**) and reversal phase (**E**). *p<0.05, **p<0.01, ***p<0.001, corrected for multiple comparisons, one-way ANOVA=black, two-way ANOVA=grey.

<u>Figure 2:</u> ICA components in $APP^{NL/NL}$ mice. This figure shows which neurologically relevant ICA components were observed in $APP^{NL/NL}$ mice. Four slices of the ICA components are shown on an anatomical T2-weighted MRI image and overlaid with the Franklin and Paxinos anatomical mouse brain atlas[29] with indication of the stereotactic coordinates a=interaural, b=bregma. The color scale represents the z-score i.e. the strength of FC within each ICA component. Homologous ICA networks are shown on the same image (left ICA component in red scale, right ICA component in regreen scale). A) hippocampus network, B) Default-mode like (DMN-like) network, C) frontal/cingulate network, D) cingulate/thalamus network, E) caudate putamen network, F) nucleus accumbens/hypothalamus network, G) sensorimotor network, H) piriform network.

<u>Figure 3:</u> BOLD FC within brain networks. A-B) zFC-matrices of 3 months **(A)** and 7 months **(B)** old $APP^{NL/NL}$ (lower half of the matrix) and $APP^{NL-F/NL-F}$ mice (upper half of the matrix). Color scale represents the z-score i.e. strength of FC between each pair of brain regions. **C)** T-values

representing the statistical group difference (two-sample T-test) between $APP^{NL/NL}$ and $APP^{NL-F/NL-F}$ mice at 3 months (lower half of the matrix) and 7 months of age (upper half of the matrix). Color scale represents the T-values. **D-E)** graph shows FC within each brain network in 3 months **(D)** and 7 months **(E)** old $APP^{NL/NL}$ and $APP^{NL-F/NL-F}$ mice. *p<0.05, **p<0.01, ***p<0.001, two-way-ANOVA. Abbreviations are listed in **Table 1**, L=left hemisphere, R=right hemisphere.

Figure 4: BOLD FC between networks. A-B) zFC-matrices of 3 months **(A)** and 7 months **(B)** old $APP^{NL/NL}$ (lower half of the matrix) and $APP^{NL-F/NL-F}$ mice (upper half of the matrix). Color scale represents the z-score i.e. strength of FC between each pair of brain networks. **C-D)** T-values representing the statistical group difference (lower half of the matrix) and binary matrix with statistically significant differences (upper half of the matrix) between $APP^{NL/NL}$ and $APP^{NL-F/NL-F}$ mice at 3 months (**C**) and 7 months of age (**D**). Color scale represents the T-values (twog-sample T-test). Abbreviations are listed in **Table 1**.

Figure 5: FC-map of the hippocampus. Statistical zFC-maps of the right hippocampus are shown for 3 and 7 months old $APP^{NL/NL}$ and $APP^{NL-F/NL-F}$ mice. Five slices of the zFC-maps are shown an anatomical T2-weighted MRI image and overlaid with the Franklin and Paxinos anatomical mouse brain atlas[29] with indication of the stereotactic coordinates a=interaural, b=bregma. Color scale represents the T-value (one-sample T-test), i.e. strength of FC of the right hippocampus with all other voxels in the brain. Graphs show strength of FC as z-scores for FC of the hippocampus bilaterally, FC between the hippocampus and frontal cortex and between the hippocampus and retrosplenial cortex. *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA, corrected for multiple comparisons.

Figure 6: FC-map of the caudate putamen. Statistical zFC-maps of the right caudate putamen are shown for 3 and 7 months old $APP^{NL/NL}$ and $APP^{NL-F/NL-F}$ mice. Five slices of the zFC-maps are shown an anatomical T2-weighted MRI image and overlaid with the Franklin and Paxinos anatomical mouse brain atlas[29] with indication of the stereotactic coordinates a=interaural, b=bregma. Color scale represents the T-value (one-sample T-test), i.e. strength of FC of the right caudate putamen with all

other voxels in the brain. Graphs show strength of FC as z-scores for FC between the caudate putamen and cingulate regions, and between the caudate putamen and hippocampus. *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA, corrected for multiple comparisons.

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