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Subtle defects in cognitive flexibility and increased prefrontal-hippocampal network synchronicity in APP^{NL-G-F} mice before plaque deposition

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Abstract

Amyloid- β (A β) 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 A β 42 amyloidosis in the absence of changes in APP neurochemistry. 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. This assessment showed no impairment in locomotor activity or general exploration, whereas subtle deficits in anxiety-related behavior and cognitive flexibility occurred from 6 months. 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 already in 3-month-old APP^{NL-G-F} mice. Notably, these behavioral and rsfMRI changes occurred in APP^{NL-G-F} mice before amyloid plaque deposition.

Introduction

Alzheimer's disease (AD) is characterized by the progressive brain deposition of extracellular 40–42 residue amyloid- β peptide (A β) [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 β_{42} without overexpressing APP have been generated by knock-in (KI) of a humanized A β 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 cognitive defects that relate to clinical AD only at a late age [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-G-F} mice carrying Iberian and Arctic mutations in the A β sequence were compared to APP^{NL} mice carrying only the Swedish mutation to dissociate the effects of aggressive A β 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.

In the present study, we included behavioral assessment and reversal learning similar to 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} in the 10-month-old group. Saito and colleagues observed age-dependent A β 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 μ m) from mice transcardially perfused with PFA. The sections were stained for amyloid plaques using immunofluorescence with an A β primary antibody (6E10, against A β 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 ($w \times d \times h$: $50 \times 50 \times 30$ 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 ($w \times d \times h$: $94 \times 28 \times 30$ cm) divided into three chambers. Mice could circulate between left, right ($29 \times 28 \times 30$ cm) and central chamber ($36 \times 28 \times 30$ cm) via openings ($w \times h$: 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™ Video Tracking System software (Stoelting Co., IL, USA) was used to record and analyse 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 ($\text{Ratio}_{\text{Pref}}$) as $\text{Time}_{\text{S}1}/(\text{Time}_{\text{S}1} + \text{Time}_{\text{empty}})$, and recognition ratio ($\text{Ratio}_{\text{Rec}}$) as $\text{Time}_{\text{S}2}/(\text{Time}_{\text{S}1} + \text{Time}_{\text{S}2})$. 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 \pm 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

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

Antibodies to the N and C termini appeared to bind to both A β species in a similar manner. Using a combination of antibodies, we observed A β amyloidosis in APP^{NL-G-F} mice in an age dependent manner. We observe initial accumulation of soluble A β 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 disappeared at 6 months (Figure 2A middle panel). Interestingly, 10-month-old APP^{NL-G-F} mice (Figure 2A, right panel) showed significantly increased activity overall when we analyzed 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 the total distance moved allows investigating the exploration level. As

depicted in figure 2B (right panel), 6-month-old APPNLGF mice spend 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 a 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 with 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} (crossings: 30 ± 5 , $n=7$) mice 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 enter 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} for the open arm versus the closed arm: a main effect of arm (open vs. 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, the APP^{NL-G-F} display 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, whereas the close arms are significantly less visited by the APP^{NL-G-F} mice ($t = 2.593$; $p = 0.0223$). In wild-type C57BL/6 mice, older mice exhibit a significantly higher percentage of open arm entries compared to animals in the younger age groups [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 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 main effects of arena side ($F_{(1, 15)} = 28.02$; $p < 0.0001$) at 3 months. Figure 4A (left panel) shows that both groups prefer to approach mouse S1 to the empty side, although APP^{NL-G-F} mice to a higher degree than APP^{NL}. At 6 months, we found a similar effect of stranger side

($F_{1,13} = 7.203$; $p= 0.0188$), although this time the preference of APP^{NL-G-F} mice for S1 over the empty side is much smaller than at 3 months.

Surprisingly, 10-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 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 less than the known S1, although the difference is not significant. The fact that 10 months-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 social preference trial, APP^{NL}mice showed preference for S1 over 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 keeps going until the end of the trial (bins 3, 4 and 5), with a clear overall preference for the empty side (Figure 4F). During 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} shows slightly increased preference for S2 over S1 during just the first time bin (Figure 4G), with a strong preference for the familiar mouse (S1) over the novel one (S2) along the next 4 time bins, but RM-ANOVA did not indicate significant effects. The later finding

might be due to the decreased preference towards S1 during the first trial, becoming S1 a novel mouse during the recognition trial. In summary, APP^{NL}-mice showed pronounced sociability and preference for social novelty especially during the first time bins, while such behavior was to some extent less pronounced or not present 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 opacified 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* ($F_{(4, 52)} = 5.514$; $p= 0.0009$). However, during the reversal probe, APP^{NL} mice seemed to remember better the new platform position because they spent more time in the target quadrant than in the other quadrants ($p= 0.007$) compared to APP^{NL-G-F} mice ($p= 0.2$). At 10-11 months of age (Figure 6C), no differences were observed, neither in reversal learning, nor in probe trial performance.

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, which becomes more manifest only at a relatively advanced age [7,8].

Using a somewhat more detailed and challenging approach, we presently report that APP^{NL-G-F} mice do, in fact, already display some behavioral and cognitive impairment 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.

$\text{APP}^{\text{NL-G-F}}$ mice displayed reduced anxiety-related behavior in the open field as well as in the elevated plus maze from 3 months of age. The former test results were somewhat more variable as 6 months-old $\text{APP}^{\text{NL-G-F}}$ mice spent more time in the center of the open field, whereas 3- and 10-month-old $\text{APP}^{\text{NL-G-F}}$ mice spent equal time exploring the center and the periphery. However, it should be noted that open field activity is indeed reportedly variable, and might be less reliable to measure anxiety [37,38], compared to other anxiety-related tasks [39]. $\text{APP}^{\text{NL-G-F}}$ 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]. $\text{APP}^{\text{NL-G-F}}$ and APP^{NL} mice performed very similarly in our MWM acquisition experiments, showing only impairments in the reversal learning task at 6 months of age. This subtle reversal defect is notable as we will discuss later, but was less obvious at later age, possibly overshadowed by the known age-related decline in this test in wild-type C57BL/6 mice [44]. Studies in other mouse models of A β accumulation have also found impaired reversal learning [33,45–47], but these studies differ from ours in several ways. Several of these reported phenotypes in older animals (e.g., 12 months of age), when the pathophysiology is more advanced compared to the pre-plaque stage in our mice. Also, they used mouse models that overexpress APP, whereas our model exhibits A β 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 even 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 cortex network. It still remains somewhat obscure what this hypersynchronized activity signifies or to which aspect of the pathology it would 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 plaque deposition could be attributed to the neurotoxic effects of soluble A β , rather than A β plaques that 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 in the relatively early age in these mice [49], but these knock-in models do not display any tau pathology or over cell death, suggesting that the changes are entirely due to A β -induced effects. Thus, the observed rsfMRI changes could be an early sign of pathology, but we cannot exclude that the hyperconnected frontal network could also be a neurobehavioral response to compensate for A β -induced dysfunction.

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Figure legends

Figure 1 - A β deposition in APP^{NL-G-F} and APP^{NL} brains. A) Brain sections from 1.5, 2, 3.5 and 6-month-old mice were immunostained using antibody to A β ₄₂. Cortical and hippocampal immunoreactive amyloid plaque load were measured using confocal microscopy revealing accelerated onset of amyloid plaques accumulation already at the age of 3.5 months, although very minor compared to 6 months-old APP^{NL-G-F} mice (n = 7, 10, 5 and 6 mice per indicated time point, respectively). (B) APP^{NL} on the left panel indicating absence of amyloid plaques in the cortex and hippocampus compared to APP^{NL-G-F} 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 testing at 3, 6 and 10 months in APP^{NL-G-F} (grey circles) and APP^{NL} (black circles) mice and activity measurements in APP^{NL-G-F} (black bars) and APP^{NL}(grey bars) in the Open Field setup. A) Left panel: at 3 months of age, APP^{NL-G-F} (n= 14) show increased locomotor activity compared to APP^{NL}(n=14); middle panel: no significant differences between APP^{NL-G-F} (n= 14) and APP^{NL}(n=14) at 6 months; right panel: locomotor hyperactivity in APP^{NL-G-F} (n= 11) compared to APP^{NL}(n= 13) during the whole interval of 23h and more particularly during the night activity peaks. B) 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 than APP^{NL}(n= 14, n= 8, n=12, respectively); right panel: evidence for increased anxiety in OF at 6 months in APP^{NL-G-F} (n= 7) compared to APP^{NL}(n= 7; see text for statistical details). No differences in time spent in the center were detected at 3 months nor at 10 months between APP^{NL-G-F} (n= 13, n=11, respectively) and APP^{NL}(n= 13, n=11, respectively). Data are shown as means \pm SEM.

Figure 3 - Anxiety and hyperactivity measured with Elevated Plus Maze test in APP^{NL-G-F} (black bars) and APP^{NL}(grey bars). Left panel: at 3 months, APP^{NL-G-F} (n=8) mice showed less

preference for the close arm since the number of beam breaks indicate that they circulate significantly more than the APP^{NL}(n=7); middle panel: preference for the open arm become stronger at 6 months in APP^{NL-G-F} (n= 7) compared to APP^{NL}(n= 8), with a significant reduction in the preference for the close arm; right panel: despite not preferring the open arm (as seen in previous ages), 10 months-old-APP^{NL-G-F} (n=11) presented significant decreased in the number of beam breaks in the close arm compared to APP^{NL}(n= 10). Data are shown as means ± SEM.

Figure 4 - Social preference in APP^{NL-G-F} and APP^{NL}at 3, 6 and 10 months of age. A) Left panel: at 3 months, during social preference test both groups show preference for S1 side over Empty side, but APP^{NL-G-F} (open bars; n= 9, 7, 10 respectively) show more pronounced preference than APP^{NL}(filled bars; n= 8, 8, 8, respectively). An increased preference for the novel mouse (S2) is present in both genotypes during the recognition phase; middle panel: 6 months old-APP^{NL-G-F} had little preference towards S1 during the social preference trial, while they explore more S2 than S1 in the recognition phase. Time spent with the novel mouse in the second trial was reduced in APP^{NL}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 none of the two phases. In fact, APP^{NL-G-F} mice show only increased 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} (n=9 and n=7, respectively) display similar preference for the S1 versus empty the cage. However, a tendency towards less preference is found in APP^{NL-G-F} (n=10) starting at 10 months compared to APP^{NL}(n=8). C) The recognition ratio is increased at 3, 6 and 10 months in APP NL, although these differences do not reach statistical significance. Time segment analysis of social preference (D & F) and recognition for novelty (E & G) in APP^{NL}(D & E) and APP^{NL-G-F} (F & G) at 10 months of age: D) APP^{NL}mice show increased exploration of S1 compared to empty cage only for the first two time bins of test time. E) APP^{NL}mice had a strong preference towards S2 during the beginning of recognition phase (time

bins 1 and 2), with significant interaction of time bin and stranger side according to RM-ANOVA. F) APP^{NL-G-F} mice show 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 during social preference test. G) APP^{NL-G-F} mice had overall a preference for S1 over the novel mouse, exploring S2 only during time bin 1. Data are shown as means ± SEM.

Figure 5 - Morris water maze performance at 3-4, 6-7 and 10-11 months of age in APP^{NL}(black bars; n= 12, 8 and 11 respectively) and APP^{NL-G-F} mice (grey bars; n= 13, 7 and 12 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 perform 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), day in which a 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} 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 show retention memory during probe 2 (C right panel). However, after 5 days of acquisition learning, on day 6 the first probe does 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 are expressed as means ± SEM. Target quadrant versus opposite quadrant is indicated with ##P<0.01, ###P<0.001 (Tukey pairwise).

Figure 6 - After significant target preference during acquisition learning (previous figure), platform was placed in the opposite quadrant (now called TQ) and five days reversal learning were introduced at 3-4, 6-7 and 10-11 months of age in APP^{NL-G-F} (black bars; n= 12, 8 and 11 respectively) and APP^{NL-G-F} mice (grey bars; n= 13, 7 and 12 respectively). TQ= Target quadrant; AD1= adjacent 1; AD2= Adjacent 2; OQ= Opposite quadrant. Total distance swam and time spent in quadrant are expressed as means ± SEM. At 3-4 months, APP^{NL}and APP^{NL-G-F} learned the reversed platform location with a significant effect of days on both groups (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), whereas during the probe test only control APP^{NL}mice had a significant preference for target quadrant over the other quadrants (B right panel). At 10-11 months, although there was no learning during the reversal learning phase in none of the groups tested (C left panel), APP^{NL}and APP^{NL-G-F} displayed a preference for the new target location than for the rest of quadrants (C right panel). Data are shown as means ± SEM. Target quadrant versus the opposite quadrants is 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) Functional connectivity map shows increased neural synchrony in regions that are functionally connected to the prefrontal cortex. B) Left panel: correlation coefficient of paired of regions show slightly increase in functional connectivity in the APP^{NL-G-F} mice (upper part) compared to APP^{NL}(lower part) in the Prefrontal cortex network; right panel: this hypersynchrony is no longer present at later ages as shown by the mean FC strength at 7 and 11 months of age.