Synapsin-I- and synapsin-II-null mice display an increased age-dependent cognitive impairment

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Summary

Synapsin I (SynI) and synapsin II (SynII) are major synaptic vesicle (SV) proteins that function in the regulation of the availability of SVs for release in mature neurons. SynI and SynII show a high level of sequence similarity and share many functions in vivo, although distinct physiological roles for the two proteins have been proposed. Both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice have a normal lifespan, but exhibit a decreased number of SVs and synaptic depression upon high-frequency stimulation. Because of the role of the synapsin proteins in synaptic organization and plasticity, we studied the long-lasting effects of synapsin deletion on the phenotype of *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice during aging. Both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice displayed behavioural defects that emerged during aging and involved emotional memory in both mutants, and spatial memory in *SynII*$^{-/-}$ mice. These abnormalities, which were more pronounced in *SynII*$^{-/-}$ mice, were associated with neuronal loss and gliosis in the cerebral cortex and hippocampus. The data indicate that SynI and SynII have specific and non-redundant functions, and that synaptic dysfunctions associated with synapsin mutations negatively modulate cognitive performances and neuronal survival during senescence.

Key words: Synapsins, Aging, Knock out, Behaviour, Learning and memory, Neurodegeneration

Introduction

Synapsins are a family of synaptic vesicle (SV)-associated phosphoproteins that play a prominent role in synaptic transmission and plasticity (Baldelli et al., 2006; Fdez and Hilfiker, 2006). Three distinct genes, *SynI* (*Syn1*), *SynII* (*Syn2*) and *SynIII* (*Syn3*), give rise in mammals to several differentially spliced isoforms sharing a high level of sequence similarity and whose expression is developmentally regulated. SynI and SynII are the predominant isoforms expressed by mature neurons, where they account for over 6% of the protein complement of SVs (Ferreira et al., 2000; Ferreira and Rapoport, 2002; Huttner et al., 1983; Takamori et al., 2006). SynI and SynII are involved in both synaptogenesis and plasticity of mature synapses (Chin et al., 1995; Ferreira et al., 1994; Ferreira et al., 1995; Ferreira et al., 1996), by controlling SV trafficking at pre- and post-docking levels (Bonanomi et al., 2005; Chi et al., 2001; Chi et al., 2003; Fassio et al., 2006; Hilfiker et al., 1998; Hilfiker et al., 2005; Humeau et al., 2001; Hvalby et al., 2006; Menegon et al., 2006; Sun et al., 2006). By contrast, SynIII is highly expressed at early stages of neuronal development, in which it regulates the proliferation of neural progenitors and axonal differentiation, but its expression declines thereafter (Ferreira et al., 2000; Kao et al., 1998; Kao et al., 2008).

Synapsin-null mice are viable and fertile, have a normal life expectancy, and their brains have a normal size and structure (Li et al., 1995; Rosahl et al., 1993; Rosahl et al., 1995). However, they have an increased seizure propensity (Li et al., 1995) and their central synapses exhibit a decrease in the total number of SVs (Feng et al., 2002; Gitler et al., 2004; Li et al., 1995; Rosahl et al., 1995; Takei et al., 1995). This is accompanied by various impairments in neurotransmitter (NT) release and in short-term plasticity. Recently, a differential impact of synapsin inactivation at excitatory and inhibitory synapses has been shown. At mammalian excitatory synapses, synapsin deletion reduced the size of the reserve pool of SVs (Gitler et al., 2004; Li et al., 1995; Mozhayeva et al., 2002; Ryan et al., 1996; Siksou et al., 2007; Takei et al., 1995) and altered short-term plasticity (Gitler et al., 2004; Hvalby et al., 2006; Kielland et al., 2006; Rosahl et al., 1995; Sun et al., 2006) without affecting the release evoked by isolated action potentials (pre-docking action). By contrast, inhibitory synapses lacking SynI (Baldelli et al., 2007; Terada et al., 1999), SynIII (Feng et al., 2002) or all three synapsins (Gitler et al., 2004) showed a decrease in the amplitude of inhibitory postsynaptic currents, suggesting the involvement of post-docking mechanisms. Moreover, distinct deficits have been reported in mice lacking SynI or SynII, suggesting that each isoform has different roles in short-term synaptic plasticity. Although long-term potentiation in either the CA1 or the CA3 region of the hippocampus is virtually unaffected in both mutant lines, *Syn*I$^{-/-}$ mice exhibit increased paired-pulse facilitation (PPF), and *SynII*$^{-/-}$ mice show decreased post-tetanic potentiation (PPT) and increased synaptic depression in the absence of changes in PPF (Rosahl et al., 1995; Spillane et al., 1995).

Because of the role of synapsins in synaptic organization and plasticity, we studied the long-lasting and differential effects of SynI and SynII depletion by analyzing behaviour and brain morphology

of *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice during aging. Relatively few experimental data have been reported on the behavioural consequences of synapsin deletion, and no data are available on the long-term effects of synapsin deletion during the mouse life span. In larval and adult *Drosophila*, inactivation of the single *synapsin* gene (*Syn*) was associated with complex deficits in associative learning and complex behaviour, including learning and memory paradigms (Godenschwege et al., 2004; Michels et al., 2005; Scherer et al., 2003). *SynII*$^{-/-}$ and *Syn*I/*Syn*II$^{-/-}$ mice, but not *SynI*$^{-/-}$ mice, exhibit impaired contextual conditioning (Silva et al., 1996) and triple-synapsin-knockout mice exhibit impaired motor coordination and defective spatial learning (Gitler et al., 2004).

Our data, performed for the first time in completely backcrossed mouse lines, demonstrate the existence of behavioural deficits emerging during senescence in both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ single-knockout mice, with the emotional memory being progressively affected in both mutant strains, and spatial memory being selectively impaired only in *Syn*II$^{-/-}$ mice. These behavioural abnormalities were associated with neuronal loss and gliosis in specific brain regions, with more-pronounced effects in the *SynII*$^{-/-}$ strain. This study suggests that the presence of mutations in the synapsin genes, by producing chronic alterations in synaptic transmission and plasticity, can modulate cognitive performance and neuronal survival during senescence.

Results

SynII$^{-/-}$ mice display an age-dependent impairment in spatial learning

To uncover the presence of impairments in spatial learning and memory, age-matched mice of the various genotypes were tested for their ability to find a hidden platform in the Morris water maze at 3-5 (young), 12-14 (adult) or 24-30 (aged) months of age. In the group of young mice (Fig. 1A), the performance of both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ strains did not significantly differ from the wild-type (WT) group, in agreement with previous data performed on *SynI*$^{-/-}$ mice (Silva et al., 1996). At 24 hours after the last training session, mice were subjected to the transfer test, which is independent of swimming speed. The hidden platform was removed and the percentage of time spent by the mouse in the former platform quadrant was considered as an index of spatial memory. No significant differences were found in the percentage of time spent in the platform quadrant between the young groups of the three genotypes (Fig. 1B), whereas *SynI*$^{-/-}$ mice exhibited a slight increase in the overall distance swum in comparison with WT mice.

By contrast, when the performances of the three adult groups were compared, *SynII*$^{-/-}$ mice spent more time to locate the hidden platform as compared with WT or *SynI*$^{-/-}$ mice (Fig. 1C; $P<0.01$ vs WT and $P<0.05$ vs *SynI*$^{-/-}$). This phenotype was also observed in the old group of *SynII*$^{-/-}$ mice (Fig. 1E; $P<0.05$ vs both WT and *SynI*$^{-/-}$). It is interesting to note that both adult and old *SynI*$^{-/-}$ groups showed a performance that did not significantly differ from that of WT mice, indicating that spatial memory became affected during aging only in the absence of SynII.

Indeed, in the transfer test, adult *SynII*$^{-/-}$ mice spent less time swimming in the former platform quadrant (19.6±2.5% of the total duration) as compared with the time spent by WT and *SynI*$^{-/-}$ mice (32.8±3.1% and 37.5±3.4%, respectively) (Fig. 1D; $P<0.05$ *SynII*$^{-/-}$ vs WT and $P<0.01$ *SynII*$^{-/-}$ vs *SynI*$^{-/-}$). Similar results were obtained in this test for the old *SynII*$^{-/-}$ group (21.5±1.4% as compared with 32.9±3.1% for WT and 36.0±4.1% for *SynI*$^{-/-}$) (Fig. 1F, $P<0.05$ *SynII*$^{-/-}$ vs WT and $P<0.01$ *SynII*$^{-/-}$ vs *SynI*$^{-/-}$). In both cases, *SynII*$^{-/-}$ mice showed a significant decrease in the overall distance swum during the transfer test. Although a decreased swimming speed might have contributed to the delay in the learning curve observed in *SynII*$^{-/-}$ mice, the clear deficit present in the transfer test, which is independent of swimming speed, indicates that these mutant mice develop a spatial-learning and memory impairment during aging.

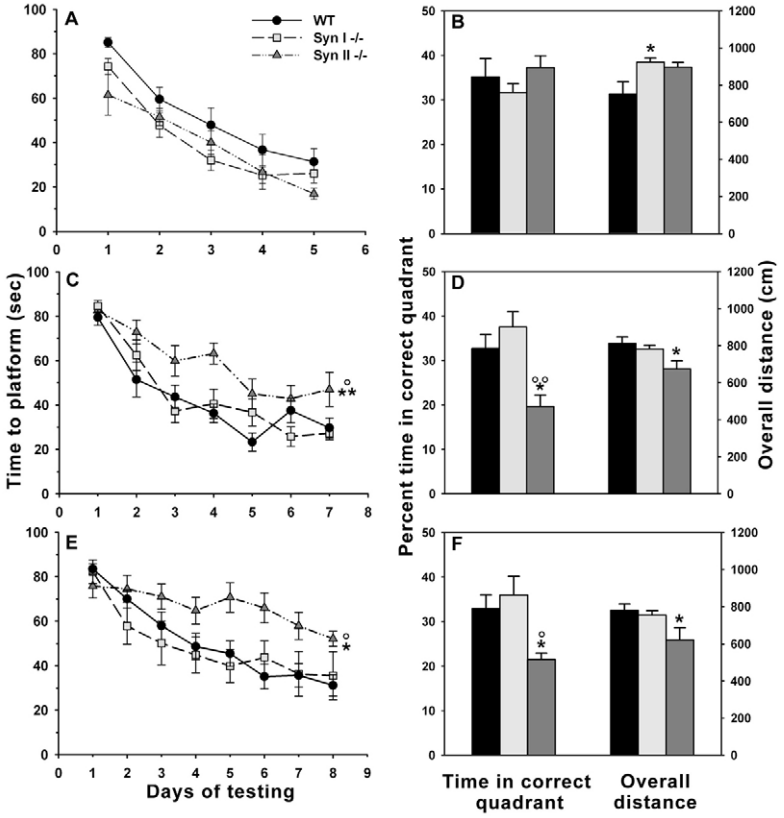

Fig. 1. *SynII*$^{-/-}$ mice display impaired spatial learning during aging. The Morris-water-maze performance of WT, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice was analyzed in young (A,B), adult (C,D) and aged (E,F) mice (eight or nine mice per group). (A,C,E) Learning curves for training to find the hidden platform in the Morris water maze are shown. The latencies to find the platform in the four daily sessions, performed over a period of up to 8 days, were averaged to obtain one value/animal/day and the data are shown as means ± s.e.m. Statistical analysis of the overall learning curves was performed by using one-way ANOVA for repeated measures. Young: $F(4,31)_{Time} = 35.28$, $P<0.001$; $F(2,31)_{Groups} = 2.41$, not significant. Adult: $F(6,34)_{Time} = 41.36$, $P<0.001$; $F(2,34)_{Groups} = 5.79$, $P<0.01$. Aged: $F(7,27)_{Time} = 19.71$, $P<0.001$; $F(2,27)_{Groups} = 5.30$, $P\sim0.01$. This was followed by Bonferroni's multiple comparison test (*$P<0.05$ and **$P<0.01$ for either mutant strain vs WT; °$P<0.05$ for *SynI*$^{-/-}$ vs *SynII*$^{-/-}$). (B,D,F) Illustrate the results of the transfer test, performed on the day immediately after the last training session, in which the platform was removed and the animals were allowed to swim for 1 minute. The percentage of time spent by each mouse in the correct quadrant (the quadrant holding the hidden platform during the previous training sessions) and the overall distance covered during the test are shown on the left and right *y*-axes, respectively, as means ± s.e.m. Statistical analysis of the transfer test was performed by using one-way ANOVA [young: $F(2,33) = 0.58$, not significant; adult: $F(2,36) = 6.71$, $P<0.005$; aged: $F(2,29) = 4.32$, $P<0.05$] followed by the Bonferroni's post-hoc test (*$P<0.05$ for either mutant strain vs WT; °$P<0.05$ and °°$P<0.01$ for *SynI*$^{-/-}$ vs *SynII*$^{-/-}$).

Object-recognition memory is progressively affected in both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice during aging

We then tested the age-dependent performance of *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice in the novel-object recognition test, which is based on the mouse tendency to explore more novel objects than already explored ones (see Materials and Methods). Thus, increased exploration of a novel object, compared with an object previously presented, means that the animal remembers that the old object was already explored. At 3 hours after the presentation of the old object, no significant differences in the exploration time were observed between mutant and WT mice in all three groups of age. However, after 24 hours significant age-related differences in performance were observed between the three groups. At 3 months (Fig. 2A), no significant differences were detected in the performance of WT, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice, suggesting that mutations in the synapsin genes do not affect this form of long-term memory in young mice. By contrast, memory of object features became altered during aging in both mutant strains. At 12-14 months of age, a significant deficit compared with WT was observed in both the *SynI*$^{-/-}$ and *SynII*$^{-/-}$ groups (Fig. 2B), which spent only 51.9±2.4% and 52.3±3.8% of the time exploring the new object, whereas WT mice spent 63.6±2.7% of the time with the novel object (P<0.05, *SynI*$^{-/-}$ or *SynII*$^{-/-}$ vs WT). Mutant 24-month-old mice of both strains presented a more severe deficit in the object-recognition task (Fig. 2C) and the time spent exploring the new object by aged *SynI*$^{-/-}$ or *SynII*$^{-/-}$ mice (47.0±2.2% and 45.0±4.1%, respectively) was further decreased as compared with age-matched WT controls (66.0±2.3%; P<0.01 vs *SynI*$^{-/-}$ or *SynII*$^{-/-}$), indicating a progressive degradation of memories of object features during aging in both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice.

Emotional memory is progressively affected during aging in both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice

In order to study emotional memory, we tested the various experimental groups of mutant and WT mice in the fear-conditioning test. In this memory paradigm, 24 hours after conditioning with foot shock accompanied by a tone (see Materials and Methods) the mice are put again into the conditioning cage for 5 minutes (contextual test), then in a different cage for 3 minutes (altered context text) and finally, in the latter different cage, exposed to the same tone to which they had been exposed during the conditioning procedure, and observed for 3 minutes (cued test). By measuring the mouse freezing time (caused by the memory of the shock) in the three tests, it is possible to obtain an index of the strength of the association that occurred between the context and the shock (contextual test) or the tone and the shock (cued test), whereas the altered context test is a control for non-specific reactivity of the animals. The networks involved in this task include hippocampus (contextual test) and amygdala (cued test). As shown in Fig. 3A, the young groups of animals of both mutant strains presented no differences as compared with control mice in both contextual and cued tests. All mice of the three strains spent around 45% (contextual test) and 35% (cued test) of the testing intervals freezing, suggesting that the strong association between the shock and the environment or the tone was independent of SynI or SynII at this age. Interestingly, we found that the ability to associate the shock with the context and with the tone was progressively impaired during aging in both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice. As shown in Fig. 3B, the strength of association between the shock and the environment was decreased in adult *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice as compared with the age-matched WT controls: mutant mice showed a significantly decreased percentage of time spent freezing with respect to controls (*SynI*$^{-/-}$, 28.8±6.7%; *SynII*$^{-/-}$, 15.3±2.7%; WT, 47.8±4.9%; P<0.01 *SynI*$^{-/-}$ vs WT and P<0.001 *SynII*$^{-/-}$ vs WT). In the case of *SynI*$^{-/-}$ mice, a significant deficit was also present in the cued test (P<0.01 vs WT).

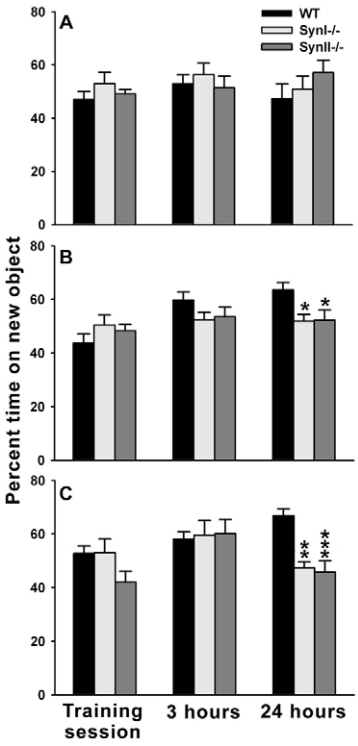

Fig. 2. Memory for objects is impaired during aging in both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice. The object-recognition performance of WT, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice was analyzed in young (A), adult (B) and aged (C) mice (eight to ten mice per group). The two test sessions were carried out 3 and 24 hours after the presentation of the first pairs of objects and the percentage of time spent on the new object was plotted as means ± s.e.m. Statistical analysis was performed by using one-way ANOVA. Young: $F(2,27)_{\text{Time 0hrs}} = 0.91$, not significant; $F(2,27)_{\text{Time 3hrs}} = 0.41$, not significant; $F(2,27)_{\text{Time 24hrs}} = 0.94$, not significant. Adult: $F(2,35)_{\text{Time 0hrs}} = 1.15$, not significant; $F(2,35)_{\text{Time 3hrs}} = 1.88$, not significant; $F(2,35)_{\text{Time 24hrs}} = 5.38$, P<0.01. Aged: $F(2,30)_{\text{Time 0hrs}} = 2.79$, not significant; $F(2,30)_{\text{Time 3hrs}} = 0.94$, not significant; $F(2,30)_{\text{Time 24hrs}} = 16.93$, P<0.001. This was followed by the Bonferroni's multiple comparison test (*P<0.05, **P<0.01 and ***P<0.001 for either *SynI*$^{-/-}$ or *SynII*$^{-/-}$ vs WT).

Aged *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice had a severe deficit in both the contextual and cued tests with respect to age-matched controls (Fig. 3C). In the contextual test, the percentage of time spent freezing of aged *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice was only 27.7±5.8% and 18.7±4.9%, respectively, as compared with the higher amount of time observed for WT mice (43.1±2.3%; P<0.05 and P<0.001 vs WT for *SynI*$^{-/-}$ and *SynII*$^{-/-}$, respectively). In the cued test, the controls showed a percentage of freezing time of 42.0±2.4%, whereas for *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice it was only 13.0±1.7% and 19.4±1.5%, respectively (P<0.001 vs WT for both genotypes). Taken together, these results suggest that the shock-tone and shock-environment associations at the basis of emotional memory become strongly impaired during aging in the *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice.

SynI$^{-/-}$ and *SynII*$^{-/-}$ mice have normal short-term memory and anxiety, but show increased locomotor activity

The above-described experiments show that *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice have several age-related impairments in long-term-memory

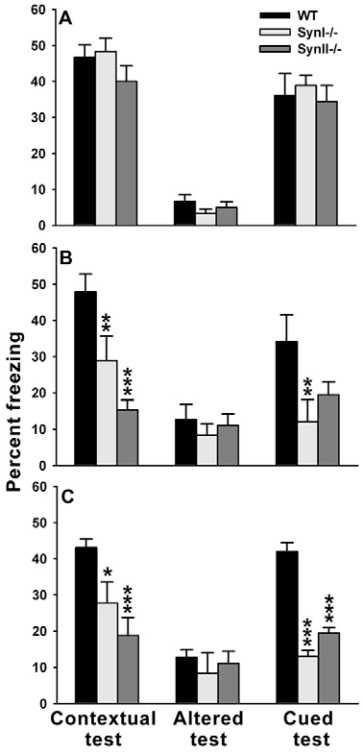

Fig. 3. Emotional memory is impaired during aging in both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice. Conditioned fear displayed by WT, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice was evaluated in young (A), adult (B) and aged (C) mice (eight to ten mice per group). The test session was carried out 24 hours after conditioning and the percentage of time spent freezing is shown as means ± s.e.m. Statistical analysis was carried out by using the one-way ANOVA followed by the Bonferroni's multiple comparison test. Contextual test: $F(2,27)_{Young} = 1.33$, not significant; $F(2,33)_{Adult} = 15.53$, $P<0.001$; $F(2,26)_{Aged} = 12.29$, $P<0.001$. Cued test: $F(2,27)_{Young} = 0.24$, not significant; $F(2,33)_{Adult} = 6.44$, $P<0.005$; $F(2,26)_{Aged} = 44.22$, $P<0.001$. Bonferroni's multiple comparison test vs WT: *$P<0.05$; **$P<0.01$; ***$P<0.001$.

paradigms. To ascertain whether mutant mice were also impaired in short-term memory, we analyzed the performance of synapsin mutants of different ages and their matched WT controls in a test of spontaneous alternation in a Y maze in which the percentage of alternation was considered as a measure of short-term memory and the total number of entries in the maze arm as a measure of locomotion. Using this test, no significant differences in the percentage of alternation were detected among the three experimental groups at the three ages analyzed (not shown). However, a significant increase in the total number of arm entries with respect to WT controls was present in adult and aged *SynI*$^{-/-}$ mice and at all ages in *SynII*$^{-/-}$ mice (Table 1). Thus, whereas short-term memory does not appear to be affected, locomotion is significantly increased in both mutant strains at all ages, except for young *SynI*$^{-/-}$ mice.

To complete the behavioural screening of *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice, we measured anxiety-related behaviours using the elevated-plus-maze and the open-field tests. Also in these tasks, no significant differences between mutant mice and matched WT controls were found in any age group (not shown), suggesting that the baseline anxiety level is not greatly affected by synapsin mutations.

Table 1. Locomotion in a Y maze

	Total number of arm entries		
	Young	Adult	Aged
WT	25.0±1.2	22.3±2.4	25.1±1.7
SynI$^{-/-}$	29.8±1.6^{NS}	34.0±2.9*	37.1±3.7**
SynII$^{-/-}$	31.1±1.8*	34.8±3.0**	34.1±2.0*

The test was repeated on three consecutive days and the figures shown in the table are the averages of the three sessions. Data are shown as means ± s.e.m. Statistical analysis was performed by using one-way ANOVA [young: $F(2,25) = 4.47$, $P=0.022$; adult: $F(2,33) = 7.59$, $P=0.002$; aged: $F(2,29) = 7.59$, $P=0.002$], followed by Bonferroni's post-hoc test for multiple comparisons (*$P<0.05$, **$P<0.01$ either mutant vs WT; NS, not significant).

Aged *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice display increased neuronal loss in the neocortex and hippocampus

Although the gross anatomy and general brain morphology of young *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice was previously reported to be normal (Rosahl et al., 1995), the possibility exists of long-term effects of the lack of synapsins as a consequence of sustained synaptic deficits. In view of the impairments found in the behavioural tests, we decided to analyze in more detail the brain morphology of synapsin mutants in a few selected brain areas, such as the hippocampus and the neocortex. To assess the presence of overt abnormalities in the gross brain morphology, we first performed a magnetic resonance imaging (MRI) analysis on adult and aged WT, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice ($n=3$ for each genotype). No significant differences were observed in synapsin mutants with respect to WT mice as far as the size and general structure of the forebrain, other brain areas and the size of the lateral ventricles were concerned (not shown).

Neuronal hypotrophy and loss is moderate during physiological aging of both humans and rodents (Coleman and Flood, 1987). This process can be greatly enhanced during neurodegenerative diseases. In the latter case, typical signs of neuronal pathology can be observed, such as cellular dispersion in the granular or pyramidal cell layer of the hippocampus. We therefore used morphometry and immunohistochemistry to assess the degree of brain atrophy and neuronal loss in *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice and their age-matched controls during aging.

Quantitative measurements of the size and thickness of the hippocampal subregions (CA1, CA3 and the dentate gyrus) as well as of various neocortical areas did not show significant differences between the synapsin mutants and WT mice at all ages analyzed (not shown), suggesting that severe tissue atrophy and/or cellular dispersion did not occur in *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice.

Neuronal density was determined in the CA1 subfield of the hippocampus as well as in the S1 trunk region of the neocortex [bregma –1.58 mm according to Paxinos and Franklin (Paxinos and Franklin, 2001)] using Nissl staining and NeuN (a neuronal-specific nuclear protein) immunohistochemistry. Neither staining method revealed a difference in neuronal cell number between young mutant and WT mice in the CA1 subfield of the hippocampus (Fig. 4) or in the neocortex (Fig. 5), consistent with previous observations (Rosahl et al., 1995). By contrast, a significant decrease in the number of NeuN-positive neuronal nuclei was detected in the pyramidal layer of the CA1 subfield of old *SynI*$^{-/-}$ mice (–11.4±2%, $P<0.05$ vs WT) and *SynII*$^{-/-}$ mice (–13.2±2.54%; $P< 0.001$ vs WT) as compared with their matched WT controls (Fig. 4). The analysis of the mean neuronal nuclear diameter did not reveal any significant difference between

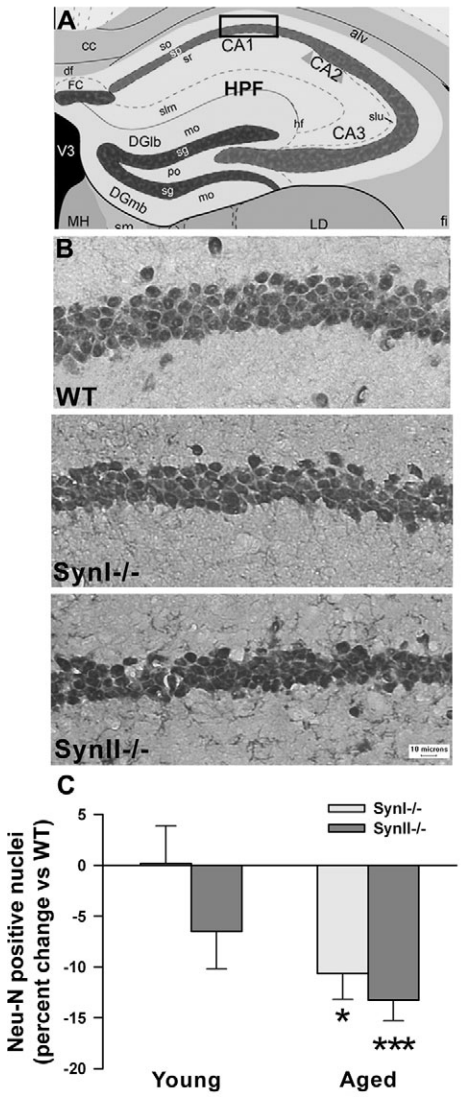

Fig. 4. Aged *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice have an increased neuronal loss in the CA1 region of the hippocampus. (A) Schematic drawing of a coronal section of mouse brain at bregma level –1.55 mm (modifed from the Allen Reference Atlas, http://www.brain-map.org/welcome.do). The boxed area corresponds to the region of the hippocampal field CA1 that was sampled in young (C) and aged (B,C) WT, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice (11-12 mice per group). (B) Immunostaining with anti-NeuN antibody of 10-μm cryostat sections of the CA1 region of aged WT, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ brains. Scale bar: 10 μm. (C) The counts of NeuN-stained nuclei in the sampled area of young and aged mice, plotted as mean percent changes ± s.e.m. with respect to age-matched WT mice, show a loss of pyramidal neurons in the CA1 hippocampal field of aged mutant mice. The counts of NeuN-stained nuclei/sampled area in WT mice were 97.0±2.9 and 102.9±2.0 for young and aged mice, respectively. Statistical analysis was performed by means of one-way ANOVA [$F(2,32)=10.97$, $P<0.001$] followed by Bonferroni's multiple comparison test (*$P<0.05$, ***$P<0.001$ vs WT).

the three genotypes. A significant decrease in neuronal cell number was also observed in the somatosensory cortex of old mutant mice with respect to old WT mice (Fig. 5). In the case of the neocortex, the decrease in neuronal cells was higher for *SynII*$^{-/-}$ (–20.1±1.75; $P<0.001$ vs WT) than for *SynI*$^{-/-}$ (–13.9±2.05%; $P<0.01$ *SynI*$^{-/-}$ vs WT).

Aged *SynII*$^{-/-}$ mice display an increased astrogliosis in the hippocampus

Neuronal loss is often accompanied by astrogliosis, which is considered to be a marker of pathological processes in the senescent brain. In humans, a prominent marker of normal and pathological aging is the accumulation of senile plaques formed by amyloid that are surrounded by astrogliosis and microgliosis (Selkoe, 1994). In rodents, there is no evidence of amyloid deposition during physiological aging (Meyer-Luehmann et al., 2006), but gliosis has been proposed as a rodent homologue of senile plaque formation (McMillian et al., 1994). We therefore looked for changes in astroglial markers in brain sections of young and old synapsin mutants and WT controls by semi-quantitative glial fibrillary acidic protein (GFAP) immunohistochemistry. Although no changes in GFAP immunostaining were found in young mice (not shown), a significant increase of GFAP immunostaining in the CA1 field of the hippocampus was found in aged *SynII*$^{-/-}$ mice versus age-matched controls (+24.5±5%; $P<0.01$ vs WT) (Fig. 6). Although neuronal loss was comparable in *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice, the increase in GFAP immunoreactivity in *SynI*$^{-/-}$ mice (+5.7±4.5%) was much smaller than that observed in *SynII*$^{-/-}$ mice and not significant.

Discussion

Physiological brain aging is characterized by a decreased density of synaptic contacts and by neuronal degeneration, which are responsible for the age-dependent decline of cognitive functions, and sensory and motor performance. Neural redundancy and plastic remodelling of brain networks, also secondary to mental and physical training, might partially mask these underlying processes, so that brain activity in healthy elderly people can be maintained with affective and intellectual tasks that are compatible with the everyday life. In this study, we focused our attention on mice lacking the synapsins, which are important synaptic proteins with a recognized role in NT release and synaptic plasticity. Mutations in the synapsin genes give rise to several abnormalities, including a dispersion of SVs in the nerve terminal, and alterations in synaptic transmission and short-term plasticity. These changes, although compatible with a normal life expectancy, might interfere with the process of physiological aging by inducing a chronic decrease in the information transfer within the brain. The numerous data reported thus far on synapsin-null mutants were obtained in young animals and nothing was known about the long-lasting effects of synapsin mutations during aging.

To explore this topic, we undertook a longitudinal-behavioural and morphological study of *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice through youth, adulthood and senescence. Our data demonstrate that both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice display behavioural defects that, virtually absent during youth, significantly emerge during aging with respect to age-matched WT controls. The age-dependent impairment in cognitive functions was, to a certain extent, genotype specific: various forms of long-term memory, such as emotional memory and object recognition, were affected to a similar extent in both mutant strains, whereas spatial learning was only affected in old *SynII*$^{-/-}$ mice. The absence of a behavioural phenotype in young mutant mice is in partial agreement with previous data performed in incompletely backcrossed *SynI*$^{-/-}$ and *SynII*$^{-/-}$ strains (Silva et al., 1996). Consistent with our data, these authors found normal performance of *SynI*$^{-/-}$ mice, but they found an impaired contextual fear conditioning in *SynII*$^{-/-}$ mice. In contrast to the data observed in young mutant mice, a significant impairment in both cued and

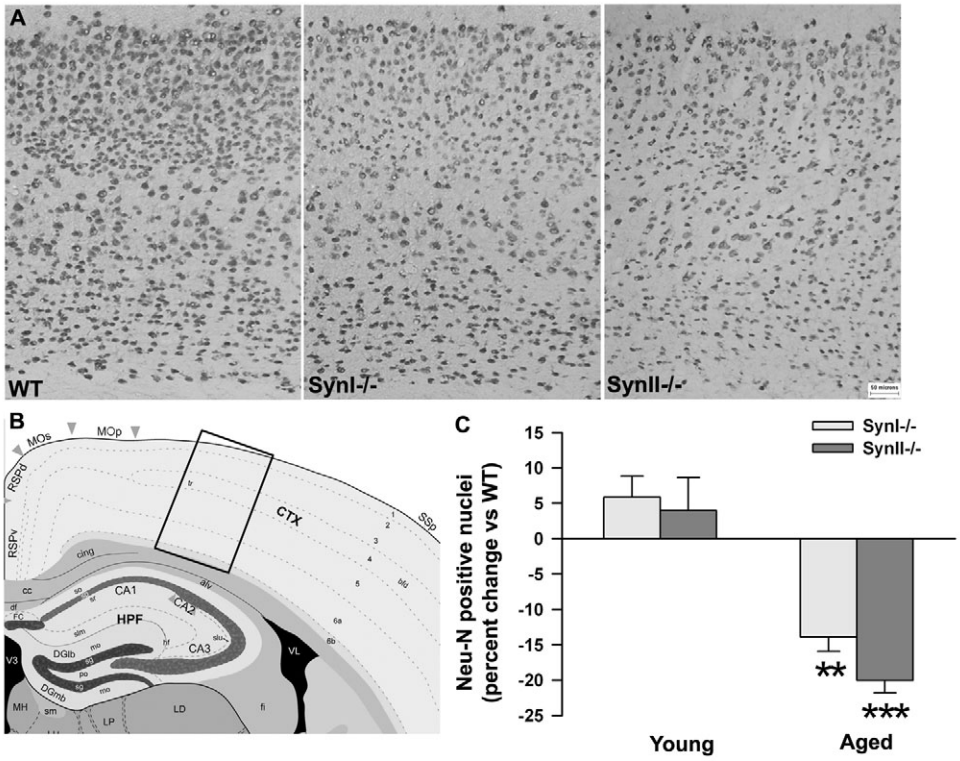

Fig. 5. Aged *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice have an increased neuronal loss in the neocortex. (A) Immunostaining with anti-NeuN antibody of 10-µm cryostat sections of the somatosensory cortex of aged WT, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ brains (eight mice per group). Scale bar: 50 µm. (B) Schematic drawing of a coronal section of mouse brain at bregma level –1.55 mm (modified from the Allen Reference Atlas, http://www.brain-map.org/welcome.do), showing the sampled region of the somatosensory cortex that was analyzed (boxed area). (C) The number of NeuN-stained nuclei counted in the sampled area of young and aged mice, plotted as mean percent changes ± s.e.m. with respect to age-matched WT mice, showed a significant neuronal loss in aged mutant mice. The counts of NeuN-stained nuclei/sampled area in WT mice were 946.7±36.5 and 907.6±37.5 for young and aged mice, respectively. Statistical analysis was performed by means of one-way ANOVA [$F(2,20) = 12.94$, $P<0.001$] followed by Bonferroni's multiple comparison test (**$P<0.01$, ***$P<0.001$ vs WT).

contextual conditioning and object-recognition memory was found in adult and aged *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice, whereas only *SynII*$^{-/-}$ mice showed an age-related impairment in the Morris maze test. It is tempting to speculate that the substantial absence of a behavioural and histological phenotype in young mutants could be contributed by the concomitant expression of SynIII and to its recently reported compensatory effect on adult neurogenesis (Kao et al., 2008). If this is the case, the emergence of the behavioural and histological impairments would parallel the age-dependent decrease in SynIII expression (Ferreira et al., 2000). Interestingly, triple-synapsin-mutant mice tested in the radial maze (Gitler et al., 2004) displayed an impaired spatial learning during youth, suggesting a cumulative effect of the synapsin mutations.

Neuronal hypotrophy and loss is moderate during physiological aging of both humans and rodents (Coleman and Flood, 1987), but is more pronounced in cases of pathological aging, such as that seen in Alzheimer disease. Moreover, recent studies performed on WT C57/BL6J mice showed no significant age-related decline in the number of dentate-gyrus granule cells and CA1 pyramidal cells, or in the number of synaptophysin-positive synaptic boutons in the molecular layer of the dentate gyrus and the CA1 region of hippocampus (Calhoun et al., 1998). Similarly, no major changes in astrocyte activation and/or proliferation were found during aging (Long et al., 1998). These results demonstrate that the C57/BL6J strain is not susceptible to major age-related deficits in the hippocampus and therefore represents an ideal background in which to study the genotype dependency of brain aging. Consistent with these findings, we did not find major differences in behavioural performance, neuronal number or astrocyte proliferation in the CA1 subfield of the hippocampus or in the neocortex of WT mice during aging. By contrast, the impairment in the behavioural performance emerging in aged synapsin mutant mice was accompanied by moderate, but significant, histological alterations, such as neuronal loss in both *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice and astrogliosis in *SynII*$^{-/-}$ mice only, consistent with the more-severe age-dependent behavioural phenotype of the latter strain.

Although SynI and SynII are highly homologous and share several structural domains, the phenotypic effects of their absence are partially different, suggesting non-overlapping functions in synaptic physiology. Although SynI and SynII mutant strains show similar age-dependent impairments emerging after 12 months of age in fear conditioning and novel-object recognition, only *SynII*$^{-/-}$ mice were impaired in the Morris water maze. Whereas the latter test requires the integrity of the hippocampus, the implication of hippocampal circuits for the novel-object recognition test is more controversial (Dere et al., 2007). Thus, the specific impairment in the Morris-maze test suggests that a more intense hippocampal defect occurs in *SynII*$^{-/-}$ mice during aging. This more pronounced deficit is also strengthened by the significant astrogliosis found only in *SynII*$^{-/-}$ mice, despite a neuronal loss comparable to that of *SynI*$^{-/-}$ mice. These data add to the phenotypic differences observed in hippocampal short-term-plasticity paradigms between *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice, in which only the latter strain exhibited impaired hippocampal post-tetanic potentiation, and enhanced synaptic depression after repetitive stimulation (Rosahl et al., 1995). Because several lines of evidence suggest that these paradigms of short-lived plasticity are implicated in memory processes (Kushner et al., 2005; Silva et al., 1996), the chronically impaired plasticity of *SynII*$^{-/-}$ mice could play a role in the onset of learning deficits, as well as in the histological changes that appear during aging in these mice.

The partially different phenotype of *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice could in principle be attributable either to distinct presynaptic functions of the two gene products or to a differential distribution of the synapsin isoforms in distinct neuronal populations, which are differently involved in learning and memory processes. Because of the different mosaic of structural domains and

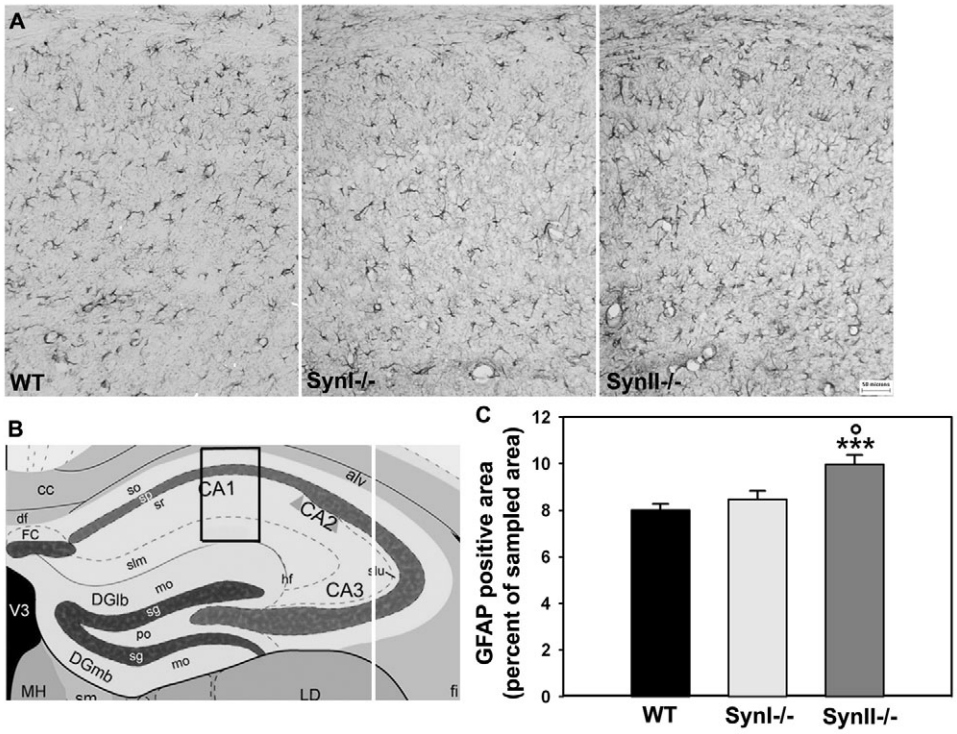

Fig. 6. Aged *SynII*$^{-/-}$ but not *SynI*$^{-/-}$, mice display increased astrogliosis in the CA1 region of the hippocampus. (A) Immunostaining with anti-GFAP antibodies of 10-µm cryostat sections of the CA1 hippocampal field of aged WT, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ brains (9-12 mice per group). Scale bar: 50 µm. (B) Schematic drawing of a coronal section of mouse brain at bregma level –1.55 mm (modifed from the Allen Reference Atlas, http://www.brain-map.org/welcome.do), showing the sampled region of the hippocampal field CA1 (boxed area). (C) The GFAP immunopositive field area was measured and is expressed as a percentage of the total sampled area. The data, plotted as means ± s.e.m., showed a selective increase in the GFAP-immunoreactive area (+24.5±5%) in *SynII*$^{-/-}$ mice with respect to age-matched WT or *SynI*$^{-/-}$ mice. Statistical analysis was performed by means of one-way ANOVA [$F(2,31) = 9.65$, $P<0.001$] followed by Bonferroni's multiple comparison test (***$P<0.001$ *SynII*$^{-/-}$ vs WT mice; °$P<0.05$ *SynII*$^{-/-}$ vs *SynI*$^{-/-}$ mice).

developmental expression patterns of the two proteins (Ferreira and Rapoport, 2002; Südhof et al., 1989), SynI and SynII display distinct biochemical properties. SynII associates more strongly with SVs than does SynI and displays a much more potent actin-binding activity (Nielander et al., 1997), and it is likely to play a more structural role in synaptic physiology than SynI. Synapsins are known to form homo- and heterodimers on the SV surface (Hosaka and Südhof, 1999), and the participation of the various synapsin isoforms in these dimers might confer distinct properties to the dimers.

In addition, SynI and SynII play distinct roles in neuronal development, the former being predominantly involved in the late stages of development and in synapse formation, and the latter being necessary in the early stages of axon formation (Ferreira et al., 1998; Ferreira and Rapoport, 2002). When injected into *Xenopus* embryonic motor neurons, both SynI and SynII accelerated synaptic maturation, but SynII was much more potent than SynI in promoting the functional development of neuromuscular synapses (Lu et al., 1992; Schaeffer et al., 1994).

Despite the widespread expression of synapsins in both excitatory and inhibitory terminals, it has been demonstrated that the different synapsin isoforms are not equally expressed at all synapses, and a differential distribution exists between glutamatergic and GABAergic neurons, as well as among specific neuronal systems (Bragina et al., 2007; Kielland et al., 2006; Südhof et al., 1989). For example, lack of SynI and SynII does not affect retinogeniculate terminals, which do not express these isoforms (Kielland et al., 2006), and the absence of all three isoforms affects glutamatergic, GABAergic and dopaminergic neurons to a very different extent (Gitler et al., 2004; Kile et al., 2007). Thus, a differential expression of SynI and SynII in hippocampal and/or neocortical neurons could account for the behavioural and histological differences observed in our study.

Synapsin-null mice also show increased seizure propensity and experience seizures with a frequency that is proportional to the number of mutant alleles (Rosahl et al., 1995). Recently, a form of familial X-linked epilepsy was reported to be associated with a nonsense mutation in the human *SYN1* gene (Garcia et al., 2004). In the family that was studied, the epileptic phenotype was variably associated with, and in a few cases replaced by, learning difficulties and behavioural disturbances. Although the latter observations provide strong support to our findings, the age-related behavioural and histological alterations in synapsin mutant mice could, in addition to the genotype, be caused by neuronal deficits secondary to epileptic seizures. However, a careful analysis of seizure frequency and learning deficits supports the hypothesis of a primary effect of the genotype in the generation of the age-dependent phenotype in synapsin mutant mice. First, *SynI*$^{-/-}$ and *SynII*$^{-/-}$ mice display a similar seizure propensity but quite different phenotypes as far as synaptic plasticity, Morris-maze performance and hippocampal astrogliosis are concerned (Rosahl et al., 1995; Silva et al., 1996) (this paper). Second, the short-term-plasticity impairments observed in the hippocampus of synapsin mutants are more severe in *SynII*$^{-/-}$ mice than in *SynI*$^{-/-}$ mice and precede the appearance of the epileptic phenotype (Rosahl et al., 1995). Third, behavioural abnormalities are found in the members of the four-generation family bearing the *SYN1* mutation also in the absence of epilepsy (Garcia et al., 2004). In addition to the behavioural pathology associated with syndromic epilepsy generated by *SYN1* mutation, numerous reports found associations between mutations in the human *SYN2* gene or SynII expression levels and human schizophrenia (Chen et al., 2004a; Chen et al., 2004b; Grebb and Greengard, 1990; Imai et al., 2001; Mirnics et al., 2000; Saviouk et al., 2007; Vawter et al., 2002) or animals models of schizophrenia (Dyck et al., 2007; Iwazaki et al., 2007). These associations might explain the more-severe behavioural traits displayed by *SynII*$^{-/-}$ mice

during aging and further implicate synapsin mutations in the pathogenesis of neuropsychiatric diseases.

In conclusion, the present data confirm that the synapsin proteins play important roles in regulating synaptic transmission and plasticity throughout life. The deficits in learning and memory that appear during the course of aging in single-knockout synapsin mice indicate that a low degree of neural redundancy exists in synapsin mutants. Thus, it is conceivable that the long-lasting synaptic impairments brought about by the absence of either SynI or SynII could generate functional and structural alterations in the cortico-hippocampal networks, leading to an enhanced and progressive worsening of the behavioural performance in the elderly.

Materials and Methods

Experimental animals

Mice were housed in groups under constant temperature and humidity conditions, and a standard 12-hour light/dark cycle with ad libitum access to standard diet and water. *SynI*- and *SynII*-null mice have been previously generated (Li et al., 1995) and were backcrossed to a C57/BL6J background through at least ten generations. Control animals of identical genetic background were purchased from Charles River Laboratories (Calco, Italy). WT, $SynI^{-/-}$ and $SynII^{-/-}$ mice were grown in the same animal facility under identical conditions. Each strain was analyzed at three different ages [young (3- to 5-months old), adult (12- to 14-months old) and aged (24- to 30-months old)] and each experimental group was composed of 70% males and 30% females. All animal experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local Ethical Committee and by the National Ministry of Health. The same animals were sequentially subjected to the battery of behavioural tests described below, to in vivo anatomical analysis by MRI as previously described (Sironi et al., 2003), and finally to histology and immunohistochemistry experiments.

Behaviour

Morris water maze

The Morris-maze task was modified from Morris (Morris, 1989; Zanardi et al., 2007; Zoli et al., 1999). To assess spatial learning, animals were placed in a circular pool with a diameter of 125 cm filled with milky water at 19°C and allowed to swim for 90 seconds or until they found the location of a hidden circular platform with an 11 cm diameter. Mice were trained with four trials per day (starting from a different quadrant for each trial), with an inter-trial interval of 15 minutes and for a maximum of 8 days. Escape latency was recorded and averaged across the four trials for each day. On the day after the last training session, the platform was removed and the animals were allowed to swim for 60 seconds (transfer test). The time spent in each quadrant as well as the overall distance covered by each mouse were measured.

Novel-object recognition test

Object recognition was assessed as described (Vaucher et al., 2002) with slight modifications (Zanardi et al., 2007). Briefly, the animals were tested in a clear plastic cage with sloping side-walls to prevent shadows from overhead illumination (36×22 cm, 1 cm sawdust on floor). Habituation consisted of a 5-minute period in the empty cage the day before the test. On the first day of testing, mice were exposed for 5 minutes to one pair of objects selected from a set of four objects that differed in shape, surface, colour, contrast and texture. The four objects were previously shown to induce a comparable time of exploration in C57/BL6 mice. At 3 hours after the initial exposure, mice were re-exposed for 5 minutes to one of the original objects and a member of the second pair of objects. At 24 hours after the initial exposure, mice were allowed to explore the other member of the original sample object pair, and the remaining member of the second pair, for 5 minutes. In all tests, the time spent exploring each object was recorded. This parameter was not affected by the various levels of locomotion observed in WT and mutant mice. A mouse was considered to be engaging in exploratory behaviour if the animal touched the object with its forepaw or nose or sniffed at the object within a distance of 1.5 cm. Testing was performed in blind, with the experimenter unaware of the genotype group. After each exposure, the objects and the cage were wiped with 70% ethanol to eliminate odorant cues.

Fear-conditioning test

Training took place in a conditioning chamber (23×22×24 cm) with grey Perspex walls and ceiling. Scrambled shock was delivered by a shock source to a grid floor made of stainless-steel bars (2 mm in diameter, spaced 0.5 cm apart). This chamber was housed in an ice box (60×40×33 cm) with a clear Perspex window (60×33 cm) cut into the front of the chest. A speaker was mounted on the back wall, through which a computer-generated tone (1000 Hz) was delivered. A fan, mounted on the back wall of the chest, provided ventilation as well as background masking noise. The chamber was cleaned with 70% ethanol before conditioning and before the contextual test, and again with 70% isopropyl alcohol before the altered context and cued tests. Fear-conditioning procedures were based on published methods (Paylor et al., 1994). Mice were transferred to the conditioning chamber and, after an initial acclimatization period of 2 minutes, were presented with three pairings of the tone with foot shock (0.5 mA, 2 seconds). The tone was presented for 30 seconds and the shock was administered during the last 2 seconds of the tone. Pairings were separated by 2 minutes and mice were removed from the chamber 30 seconds after the last shock. Approximately 24 hours after conditioning, mice were tested for contextual conditioning. Mice were placed into the conditioning chamber for 5 minutes, and freezing behaviour was scored. After all mice were scored in the contextual test, subjects were transferred to an altered context and then tested for freezing to the tone. The altered context consisted of a 20×20×20 cm clear plastic cage that was covered with a filter lid. A novel odour was added to the context by placing a few drops of orange extract (McCormick) mixed with water into a cup that was placed outside the cage. Mice were scored for freezing in the altered context for 3 minutes (altered context test) and then for 3 minutes after presentation of the tone (cued conditioning test). Freezing was scored using a time sampling procedure in which, every 10 seconds, a determination was made whether or not a mouse was freezing. Freezing was defined as the absence of movement except for respiration for a minimum of 1 second (Fanselow, 1984). Scoring began 15 seconds after the mouse was placed into the chamber. Mice were scored for 30 intervals during the context test, 18 intervals during the altered context test and 18 intervals during the cued test.

Y-maze test

Spontaneous alternation performance was assessed using a symmetrical Y-maze as described (Sarter et al., 1988). The maze was constructed of black plastic. Each arm was 22×10×7 cm (length×height×width) and the three arms were connected through a symmetrical three-way central corridor. Arms were randomly designated as A, B or C. Mice were allowed to roam freely through the maze during an 8-minute trial and the series of arm entries was recorded. Alternation was defined as entries into all three arms consecutively (i.e., ABC, ACB, CAB, etc.). The maximum number of alternations was, therefore, the total number of arm entries minus two, and the percentage of alternation was calculated as: (actual number of alternations/maximum number of alternations) × 100.

Other behavioural tests

Anxiety was evaluated using the elevated plus maze and the open-field tests, which were carried out as previously described (Pellow et al., 1985; Poggioli et al., 1992).

Immunohistochemistry

At the end of behavioural testing and of MRI analysis performed on a few selected animals, mice were anaesthetized with cloralium hydrate (400 mg/kg) and transcardially perfused with 0.9% NaCl followed by a solution of 4% paraformaldehyde and 0.168% picric acid. Brains were rapidly removed, post-fixed for 4 hours in the same solution, cryoprotected with 10% and then 30% sucrose overnight (ON), included in OCT (Bio-Optica, Milan, Italy) and stored at –80°C. Frozen coronal sections (10 μm thick) were cut using a cryostat (Leica Microsystems, Wetzlar, Germany) at bregma –1.58 mm (Paxinos and Franklin, 2001) and mounted on Superfrost Plus slides (Bio-Optica). After incubation at room temperature (RT) for 15 minutes in a solution of 0.6% H_2O_2/10% CH_3OH in 0.01 M phosphate-buffered saline, pH 7.4 (PBS 1×) to block endogenous peroxidase, sections were briefly rinsed in PBS 1× and incubated for 1 hour in PBS 1× containing 10% bovine serum/0.1% Tween-20 (blocking solution). Sections were then incubated ON at 4°C with either anti-neuronal-nuclei (NeuN) monoclonal antibody (Chemicon, Billerica, MA; antibody dilution 1:1000) or anti-GFAP polyclonal antibody (Dako, Glostrup, Denmark; antibody dilution 1:500) diluted in blocking solution. Sections were then washed three times in PBS 1× and incubated for 2 hours at RT with either biotinylated goat anti-mouse or biotinylated goat anti-rabbit IgG (dilution 1:200; Vector Laboratories, Peterborough, UK) diluted in blocking solution. Subsequently, sections were rinsed in PBS 1× and incubated with avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories) for 30 minutes. The ABC reaction was visualized by diaminobenzidine (DAB) substrate kit for peroxidase (Vector Laboratories) and was halted by several washings in PBS 1× before the sections were dehydrated in graded ethanols and coverslipped with Eukitt (Bio-Optica, Milano, Italy).

Image analysis

Morphometric (measurement of the thickness of hippocampal fields and cortical regions and/or sections) as well as stereological (count of profile number) analysis of the sections were performed by an image analyzer (Image Pro Plus software version 5.1, Media Cibernetics, Bethesda, MD). Cell counts in the somatosensory cortex (S1, trunk region) and CA1 pyramidal layer of hippocampus were performed on NeuN-stained nuclei. Astrocytes were identified by using GFAP immunohistochemistry. Several sections for each mouse of different age-matched genotypes were processed in parallel to allow a semi-quantitative analysis. Microdensitometry of GFAP immunoreactivity at the level of the hippocampus was evaluated by measuring the immunopositive area, expressed as a percentage of the sampled area, as previously described (Zoli et al., 1992). Both neuronal count and GFAP microdensitometry were performed by two researchers blind to genotype on five sections for each mouse and the mean value for each mouse was used for statistical analysis.

Statistical analysis

Data are presented as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed by repeated-measures or one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons using the statistical package SPSS (SPSS, Chicago, IL).

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