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Title: PSD-95 in CA1 area regulates spatial choice depending on age

Anna Cały[1]*, Małgorzata A. Śliwińska[1]*, Magdalena Ziółkowska[1], Kacper Łukasiewicz[1], Roberto Pagano[1], Jakub M. Dzik[2], Katarzyna Kalita[3], Tytus Bernaś[4], Michael G. Stewart[5], K. Peter Giese[6] and Kasia Radwanska[1]

[1]Laboratory of Molecular Basis of Behavior, Nencki Institute of Experimental Biology of Polish Academy of Sciences, 3 Pasteur St., Warsaw 02-093, Poland.

[2]Laboratory of Neuroinformatics, Nencki Institute of Experimental Biology of Polish Academy of Sciences, 3 Pasteur St., Warsaw 02-093, Poland.

[3]Laboratory of Neurobiology, BRAINCITY, Nencki Institute of Experimental Biology of Polish Academy of Sciences, 3 Pasteur St., Warsaw 02-093, Poland.

[4]Department of Anatomy and Neurobiology, Virginia Commonwealth University, PO Box 980709, 1101 East Marshall Street, Richmond, VA, 23298, United States.

[5]Department of Life, Health & Chemical Sciences, The Open University, Walton Hall, Milton Keynes MK7 6AA, United Kingdom.

[6]Department of Basic and Clinical Neuroscience, Institute of Psychiatry, Psychology & Neuroscience, King's College London, De Crespigny Park, London, SE5 8AF, United Kingdom.

*these authors contributed equally to this work

Corresponding author: Kasia Radwanska, Ph.D., Laboratory of Molecular Basis of Behavior, the Nencki Institute of Experimental Biology of Polish Academy of Sciences, ul. L. Pasteura 3, Warsaw 02-093, Poland; e-mail: k.radwanska@nencki.edu.pl; phone: +48501736942

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Current affiliation of: MAS is Laboratory of Imaging Tissue Structure and Function, Nencki Institute of Experimental Biology of Polish Academy of Sciences, 3 Pasteur St., Warsaw 02-093, Poland; KŁ is Department of Molecular, Cell and Developmental Biology, University of California Santa Cruz, 1156 High Street, Santa Cruz, CA, 95064, United States.

Abstract

Cognitive processes that require spatial information rely on synaptic plasticity in the dorsal CA1 area (dCA1) of the hippocampus. Since the function of the hippocampus is impaired in aged individuals, it remains unknown how aged animals make spatial choices. Here, we used IntelliCage to study behavioural processes that support spatial choices of aged female mice living in a group. As a proxy of training-induced synaptic plasticity, we analysed the morphology of dendritic spines and expression of a synaptic scaffold protein, PSD-95. We observed that spatial choice training in young adult mice induced correlated shrinkage of dendritic spines and downregulation of PSD-95 in dCA1. Moreover, long-term depletion of PSD-95 by shRNA in dCA1 limited correct choices to a reward corner, while reward preference was intact. In contrast, old mice used behavioural strategies characterised by an increased tendency for perseverative visits and social interactions. This strategy resulted in a robust preference for the reward corner during the spatial choice task. Moreover, training decreased the correlation between PSD-95 expression and the size of dendritic spines. Furthermore, PSD-95 depletion did not impair place choice or reward preference in old mice. Thus, our data indicate that while young mice require PSD-95-dependent synaptic plasticity in dCA1 to make correct spatial choices, old animals observe cage-mates and stick to a preferred corner to seek the reward. This strategy is resistant to the depletion of PSD-95 in the CA1 area. Overall, our study demonstrates that aged mice combine alternative behavioral and molecular strategies to approach and consume rewards in a complex environment.

Significance Statement

It remains poorly understood how ageing affects behavioural and molecular processes that support cognitive functions. It is, however, essential to understand these processes in order to develop therapeutic interventions that support successful cognitive ageing. Our data indicate that while young mice require PSD-95-dependent synaptic plasticity in dCA1 to make correct spatial choices (i.e. choices that require spatial information), old animals observe cage-mates and stick to a preferred corner to seek the reward. This strategy is resistant to the depletion of PSD-95 in the CA1 area. Overall, our study demonstrates that aged mice combine alternative behavioral and molecular strategies to approach and consume rewards in a complex environment. Secondly, the contribution of PSD-95-dependent synaptic functions in spatial choice changes with age.

Introduction

Many cognitive functions deteriorate with age, particularly, hippocampus-dependent cognitive processes that require spatial information (Hedden and Gabrieli, 2004; Kennard and Woodruff-Pak, 2011; Tromp et al., 2015). However, older people and animals are still able to use spatial information to direct their behaviour (Kiryk et al., 2011; Aziz et al., 2019; Kwapis et al., 2019; Śliwińska et al., 2019). Aged individuals often use alternative behavioral strategies for spatial tasks (Bach et al., 1999), require more training trials (Gallagher et al., 1993; Murphy et al., 2004), and the spatial memories are more resistant to updating (Jones et al., 2015; Aziz et al., 2019), altogether, suggesting alternative neuronal mechanisms.

NMDA receptor-dependent Hebbian forms of synaptic plasticity are believed to be fundamental mechanisms underlying hippocampus-dependent cognitive processes (Malenka and Bear, 2004; Kessels and Malinow, 2009). Most studies to date have linked cognitive functions with long-term potentiation (LTP) of synaptic transmission (Bliss and Collingridge, 1993; Morris et al., 2003). However, accumulating data indicate that some cognitive processes may also rely on long-term depression (LTD) of synaptic transmission (Kemp and Manahan-Vaughan, 2007; Connor and Wang, 2016). Accordingly, several studies have linked impairments of LTP and LTD with decreased ability to form hippocampus-dependent memories in aged animals (Bach et al., 1999; Luo et al., 2015; Temido-Ferreira et al., 2020). Meanwhile, alternative synaptic processes, such as the formation of multi-innervated dendritic spines (Aziz et al., 2019) and NMDAR-independent LTD (Lee et al., 2005), have been documented during memory formation in aged animals. How ageing affects behavioural and molecular processes that support cognitive functions remains poorly understood. Understanding these processes is required to develop therapeutic interventions to support successful cognitive ageing.

Accumulating data indicate that the dorsal CA1 area (dCA1) is a critical structure that enables spatial choice, i.e. choice that uses spatial information to suppress inappropriate behavioursy (Bannerman et al., 2012, 2014). The current study aims to identify how ageing affects behavioural strategies and synaptic processes in the dCA1 that support spatial choices. To this end, we used an IntelliCage setup as it provides close-to-ecologic conditions to investigate spatial memory and spatial choice (Kiryk et al., 2011; Śliwińska et al., 2019), non-spatial aspects of reward-driven behaviour (Radwanska and Kaczmarek, 2012), and social interactions (Kiryk et al., 2011; Harda et al., 2018). To study synaptic plasticity induced by the training, we applied *ex vivo* analysis of dendritic spines in the dCA1 of Thy1-GFP(M) transgenic mice (Feng et al., 2000) combined with PSD-95 protein expression analysis. PSD-95 is a key protein at mature glutamatergic synapses (Aoki et al., 2001; Chen et al., 2011). Its expression is regulated during synaptic plasticity (Steiner et al., 2008; Nowacka et al., 2020) and predicts stability of dendritic spines (El-Husseini et al., 2000; Ehrlich et al., 2007; Murmu et al., 2013; Meyer et al., 2014). Knockdown of PSD-95 has been shown to impair NMDAR-dependent LTD (Ehrlich et al., 2007), as well as spatial learning, conditioned taste aversion and simple operant associative learning (Migaud et al., 1998; Elkobi et al., 2008; Fitzgerald et al., 2015). However, it is unknown whether PSD-95-dependent synaptic plasticity is affected in the ageing brain.

Overall, our data indicate that old mice use social observation and perseverative behaviours to make spatial choices. PSD-95 depletion in the dCA1 did not effect this alternative strategy and affected only the spatial choice in young mice. Thus our study shows that aged animals combine alternative behavioural and molecular strategies for spatial choice in a complex environment.

Secondly, the contribution of PSD-95-dependent synaptic functions in spatial choice changes with age.

Methods

Animals

C57BL/6J mice were purchased from the Medical University of Bialystok, Poland. Thy1-GFP(M) mice (Thy1-GFP $^{+/-}$) (Feng et al., 2000) were bred as heterozygotes with the C57BL/6J background in the Animal House of the Nencki Institute of Experimental Biology and genotyped as previously described (Feng et al., 2000). Young, adult mice were 5±1 month-old during the behavioral training and old individuals were 18±2 month-old. Only female mice were used for all experiments, as males are too aggressive for group housing in the IntelliCages (Harda et al., 2018; Śliwińska et al., 2019). All mice were housed with access to food and water *ad libitum* and 12:12 hour dark-light cycle, 23–24°C and 35-45% humidity. The studies were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), Animal Protection Act of Poland and approved by the 1st Local Ethics Committee in Warsaw. All efforts were made to minimize the number of animals used and their suffering.

Place preference and avoidance in IntelliCages

The IntelliCage system (NewBehavior AG, Zürich, Switzerland) (http://www.newbehavior.com/) consists of a large standard rat cage (20.5 cm high, 40 cm × 58 cm at the top, 55 cm × 37.5 cm at the base). In each corner of a cage, a triangular learning chamber is located with two bottles. Only one mouse can enter a corner through a plastic ring (outer ring: 50 mm diameter; inner ring: 30 mm diameter; 20 mm depth into the outer ring) that ends with two holes (13-mm in diameter) that provide access to two bottle nipples. Each visit to the corner, nosepoke at the doors governing access to the bottles, and lick were recorded by the system and ascribed to a particular animal. During experiments, two corners were active. Groups of 8 to 15 mice were trained per IntelliCage.

Under brief isoflurane anesthesia, mice were subcutaneously injected with unique microtransponders (10.9 mm length, 1.6 mm diameter; Datamars, Slim Microchip T-SL), which allow for mice identification in the IntelliCage. Next, animals were given 3 days to recover prior to introduction to the IntelliCage. Experiments consisted of two phases: habituation (7-11 days) and training (2 hours or 1 day). During habituation, animals had access to water in both cage corners. Four days before training, mice had 24 hour access to 5% sucrose solution (in tap water) from the top of the IntelliCage to familiarized with the taste. Baseline preference to the cage corners was measured during the last day of the habituation as % of visits or licks. During place preference training, water was replaced by 5% sucrose (Sigma-Aldrich) in the less visited corner. During place aversion training, water in the preferred corner was replaced by quinine solution (200 μM in tap water, Sigma-Aldrich). The change of corner preference during training (% visits to sucrose/quinine corner) was compared to the preference of the same corner during the last day of habituation (Hab.), and used as an index of spatial choice. The change of lick preference was used as an indext of non-spatial reward memory. Training started at the beginning of the dark phase (12:00 a.m.).

To measure social interaction in the IntelliCage, we used analysis of immediate visits, i.e. visits of cage-mates with inter-visit interval < 1 s (Dzik, 2018; Dzik et al., 2018; Harda et al., 2018)

(PyMICE software, RRID: nlx_158570). To determine the frequency of patrolling visits, we analyzed % of visits without licks. Perseveration was calculated as the % of visits in a preferred corner on the last day of habituation.

Immunostaining on brain slices

Mice were anesthetized and transcardially perfused with filtered PBS (Sigma-Aldrich) followed by 4% PFA (Sigma-Aldrich) in PBS. Brains were removed and placed overnight in the same fixing solution and transfered to 30% sucrose in PBS for three days. Next, coronal brain sections (40 µm thick) were prepared (Cryostat Leica CM1950, Leica Biosystems Nussloch GmbH, Wetzlar, Germany) and stored at –20 °C in PBSAF [PBS, 15% sucrose (Sigma-Aldrich), 30% ethylene glycol (Sigma-Aldrich), and 0.05% NaN_3 (Sigma-Aldrich)]. The sections were washed with PBS, PBS/0.3%/Triton X-100 (Sigma-Aldrich) followed by 1-hour incubation in a blocking solution (5% normal donkey serum in PBS/0.3% Triton X-100) and overnight incubation with a primary antibody directed against PSD-95 (1:500, MAB1598; Merck-Millipore, RRID:AB_94278). Next, the sections were washed in PBS with 0.3% Triton X-100 and incubated for 90 minutes with the secondary antibody: anti-mouse Alexa Fluor 555 (1:500, A31570, Invitrogen, RRID:AB_2536180). The sections were mounted on glass microscope slides (Thermo Fisher Scientific), air-dried and coverslipped with Fluoromount-G medium with DAPI for fluorescence (00-4959-52, Invitrogen).

Analysis of dendritic spines and PSD-95(+) puncta

The staining was analyzed with the aid of a confocal, laser-scanning microscope. Z-stacks of dendrites in the medial partition of the *stratum radiatum* of dCA1 area were acquired using the Zeiss Spinning Disc microscope (63 × oil objective, NA 1.4, pixel size 0.13 µm × 0.13 µm) (Zeiss, Göttingen, Germany). A series of 18 continuous optical sections (67.72 µm × 67.72 µm), at 0.26 µm intervals, were scanned along the *z*-axis of the tissue section. Six to eight z-stacks of microphotographs were taken per animal, from every sixth section through dCA1 (one dendrite per neuron per image). Z-stacks were flattened to maximal projections and analyzed with Fiji software. The density and area of PSD-95(+) puncta and dendritic spines, as well as their colocalization, were analyzed using ImageJ 1.52n software and measured using the analyze particle tool as previously described (Fedulov et al., 2007). Each dendritic spine was manually outlined and spine area was measured with the measure tool. Custom-written Python scripts were used for ImageJ 1.52n software to analyze the total area of PSD-95(+) puncta per dendritic spine.

Stereotactic intracranial injections

Mice were anaesthetized with isoflurane (5% for induction, 1.5-2.0% after), fixed in the stereotactic frame (51503, Stoelting, Wood Dale, IL, USA), and their body temperatures were maintained using a heating pad. Stereotactic injections were performed bilaterally into the dCA1 region of the hippocampus using coordinates from the Bregma: AP, -2.1mm; ML, ±1.1 mm; DV, -1.3mm according to (Paxinos and Franklin, 2001). 0.5 µl of virus solution was microinjected through a beveled 26 gauge metal needle, attached to a 10 µl microsyringe (SGE010RNS, WPI, USA) connected to a microsyringe pump (UMP3, WPI, Sarasota, USA) and its controller (Micro4, WPI, Sarasota, USA), at a rate 0.1 µl/min. The microsyringe was left in place for an additional 10 minutes following injection to prevent leakage of the vector. Mice were injected with lentiviral vectors (LVs) coding short-hairpin RNA silencing PSD-95 expression (H1-shRNA_PSD95-Ub-GFP (0.5 µl/ site, viral titer 2,52 $x10^8$/µl)) (gift from Dr. Oliver M. *Schlüter* European Neuroscience Institute Göttingen, Germany) (Schlüter et al., 2006) or control vector based on a pSUPER shRNA targeting the *Renilla* luciferase (5'-

CTGACGCGGAATACTTCGA-3') cloned into pTRIP (H1-shRNA_luciferase) (0.5 µl/ site, viral titer 6,52 x10^8/µl) were used. The viruses were prepared by the Laboratory of Animal Models at Nencki Institute of Experimental Biology, Polish Academy of Sciences. After the surgery, animals were given 14 days to recover before training in the IntelliCages. After training, the animals were perfused with 4% PFA in PBS and brain sections from the dorsal hippocampus were immunostained for PSD-95 and imaged with Zeiss Spinning Disc confocal microscope (magnification: 10x) to assess the extent of the viral expression and PSD-95 expression.

Experimental design and statistical analysis

Data acquisition and quantification were performed by an experimenter blind to experimental groups. All statistical analyses were performed using GraphPad Prism 8 Software. The exact sample size (the number of mice, dendrites or spines) of each experiment is provided in figures or figures legends. For data with normal distribution and equal variance, Student's t-test, one-way or two-way analysis of variance (ANOVA), or mixed-effects model with repeated measures and *post hoc* Sidak's or Dunn's tests for multiple comparison, were used. LSD *post hoc* tests were used only for planned comparisons. The Wilcoxon matched-pairs signed rank test was used for data with nonparametric distributions.

Areas of dendritic spines and PSD-95 puncta did not follow normal distributions and were analysed with the Kruskal-Wallis test. Frequency distributions of PSD-95 to the spine area ratio were compared with the Kolmogorov-Smirnov test. Correlations were analysed using Spearman correlation, and the difference between slopes or elevation between linear regression lines was calculated with ANCOVA. All data with normal distribution are presented as the mean ± standard error of the mean (SEM). For samples that did not follow a normal distribution, medians and interquartile range (IQR) are shown. The difference between the experimental groups was considered significant if $P < 0.05$.

Results

Spatial choice training in young and old mice

Young adult (5±1 month-old, n=6) and old (18±2 month-old, n=6) mice were separately housed in two IntelliCages. During habituation, mice had access to two active corners with water. During training, sucrose replaced water in the corner less visited by mice during habituation (**Fig. 1A**) (Harda et al., 2018; Śliwińska et al., 2019). Activity (number of visits to the corners) and liquid consumption (on the last day of habituation and training) were measured to verify that the old mice did not have major behavioural impairments. There was a significant effect of training and no effect of age on mice activity (two-way RM ANOVA, training: $F_{1, 10} = 33.10$, $P < 0.001$; age: $F_{1, 10} = 0.094$, $P = 0.766$) and liquid consumption (training: $F_{1, 10} = 28.12$, $P < 0.001$; age: $F_{1, 10} = 4.054$, $P = 0.072$). As confirmed by Sidak's multiple comparisons *post hoc* tests both young and old animals increased activity (young and old, $P < 0.05$) (**Fig. 1B.i**) but only young mice drunk more during training (young: $P < 0.001$; old: $P = 0.128$) (**Fig. 1B.ii**), compared to the last day of habituation. Moreover, there was no difference in activity between young and old mice during habituation or training. These observations show that young and old mice do not differ in basal and training-induced activity, which indicates no gross motor impairments in aged mice.

During training, a correct spatial choice was measured as a change of visit preference to a reward corner, while a non-spatial reward memory was measured as a change of lick preference. Both young and old mice increased the frequency of visits to a reward (sucrose) corner (mixed-effects model, $\chi^2 = 19.84$, df = 1, $P < 0.001$; effect of training: $F_{3.076, 30.15} = 50.82$, $P < 0.001$) indicating formation of spatial preference (**Fig. 1C.i**). Old mice showed, however, a greater preference for the reward corner than young mice (effect of age: $F_{1, 10} = 12.31$, $P = 0.006$; age × time interaction: $F_{5, 49} = 5.418$, $P < 0.001$), as confirmed by LSD *post hoc* test for planned comparisons ($P < 0.05$) (**Fig. 1C.i**). Moreover, young, and more so the old mice, increased the frequency of licks at the sucrose corner (mixed-effects model, $\chi^2 = 34.90$, df = 1, $P < 0.001$; effect of training: $F_{1.788, 17.16} = 137.1$, $P < 0.001$), indicating non-spatial reward memory as demonstrated in a significant effect of age ($F_{1, 10} = 15.36$, $P < 0.003$) and age × time interaction ($F_{5, 48} = 8.865$, $P < 0.001$) (**Fig. 1C.ii**). Thus old mice showed a greater change in reward corner preference than young mice ($P < 0.001$) (**Fig. 1C.i,ii**).

Overall, our data indicate that during training, both young and old mice made mostly correct spatial choices and formed non-spatial reward memory. Surprisingly, the change in reward corner preference was more robust in the old mice group. The frequency of visits in the IntelliCage corners is determined not only by the choices that require spatial information but also by habitual patrolling cage corners, visits in preferred places, as well as social interactions (Kiryk et al., 2011, 2020; Harda et al., 2018; Śliwińska et al., 2019). Therefore, we tested whether these innate mice behaviours affected place preference during training.

Frequency of patrolling visits (visit without licks) (Kiryk et al., 2020) decreased significantly during training (two-way RM ANOVA, $F_{1, 10} = 56.54$, $P < 0.001$), and there was no age effect ($F_{1, 10} = 3.420$, $P = 0.094$) (**Fig. 1D**). Sidak's *post hoc* tests confirmed no significant difference in the frequency of patrolling visits during habituation and training between young and old mice (both $P > 0.05$). The decrease in the frequency of patrolling visits during training indicates that both young and old mice limited cage exploration and therefore is not the reason for the difference in the place preference scores observed between young and old mice.

Perservation is measured as the frequency of visits and licks in a preferred corner. During habituation, old mice were more perseverative in visits (Student's t-test, $t = 4.194$, df = 10, $P = 0.002$) (**Fig. 1E.i**) and licks ($t = 4.500$, df = 10, $P = 0.001$) compared to the young mice (**Fig. 1E.ii**). Therefore, these perseverative behaviours of the old mice contributed to a stronger reward corner preference during training.

Finally, we analysed the frequency of social visits [immediate (interval < 1s) visits after another mouse] (Harda et al., 2018) in young and old mice (**Fig. 1F**). Wilcoxon matched-pairs signed rank tests showed that during training, young animals performed less social visits than during habituation ($P = 0.031$), while the old mice performed more ($P = 0.031$), suggesting that only old mice observed and followed cage-mates during the training. Together, our experiment indicated that both young and old mice made correct spatial choices and formed non-spatial memory. Old mice had a greater change in corner preference during the training as a result of the behavioral strategy to increase perseverative and social visits.

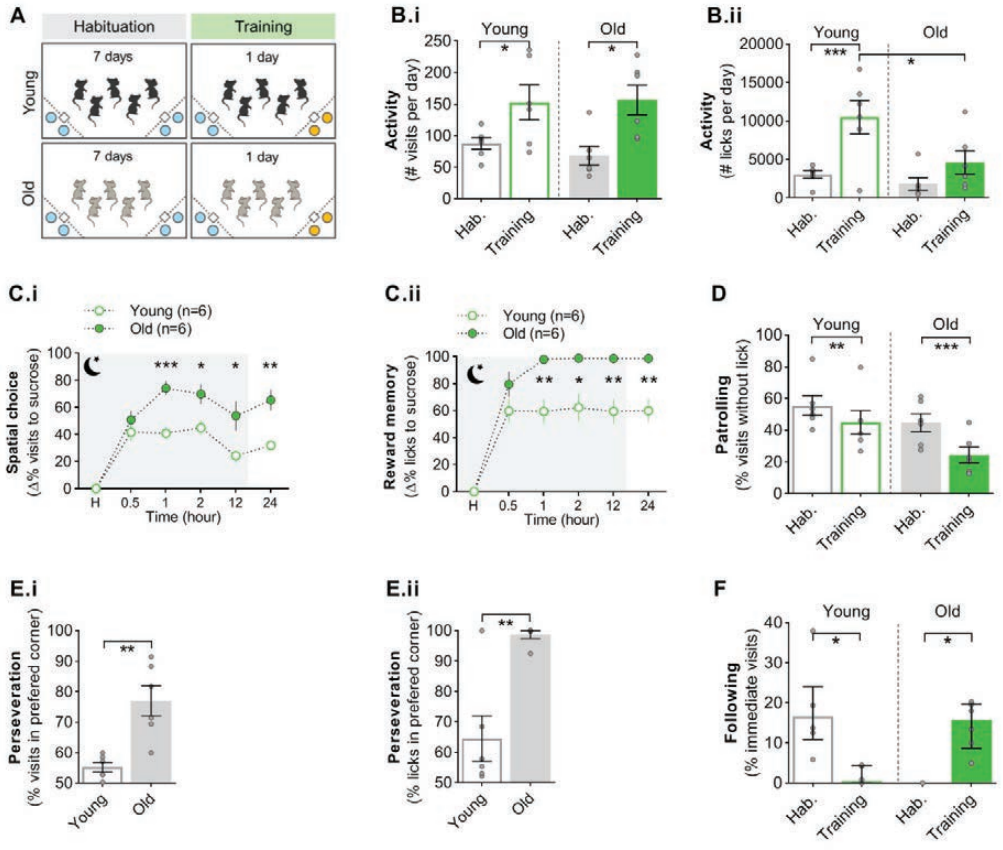


Figure 1. Old mice develop a robust preference for the reward corner as a result of an increased frequency of perseverative and social visits.

(**A**) Experimental timeline. During habituation, mice had access to two active corners with water. During spatial choice training, sucrose was given in the less visited corner during habituation. Young and old animals were housed separately. (**B**) Summary of data showing mice activity during habituation and training. Daily visits (i) and licks of all liquids (ii) are shown. (**C**) Summarized results show an increase in reward corner preference during training. Change of visit (i) and lick preference (ii) from the sucrose corner is shown. A dark phase of a day is indicated as a gray background. (**D**) Summary data of patrolling visits (without licks), (**E**) perseverative visits (i) and licks (ii) to the preferred corner, and (**F**) social visits (following other mice to the corners for inter-visit interval < 1s) during habituation and training. *P < 0.05, **P < 0.01, ***P < 0.001, for *post hoc* tests. Mean ± SEM are shown for (B-E); median ± IQR for (F). H on graphs presents preference of the corner during the last day of the habituation.

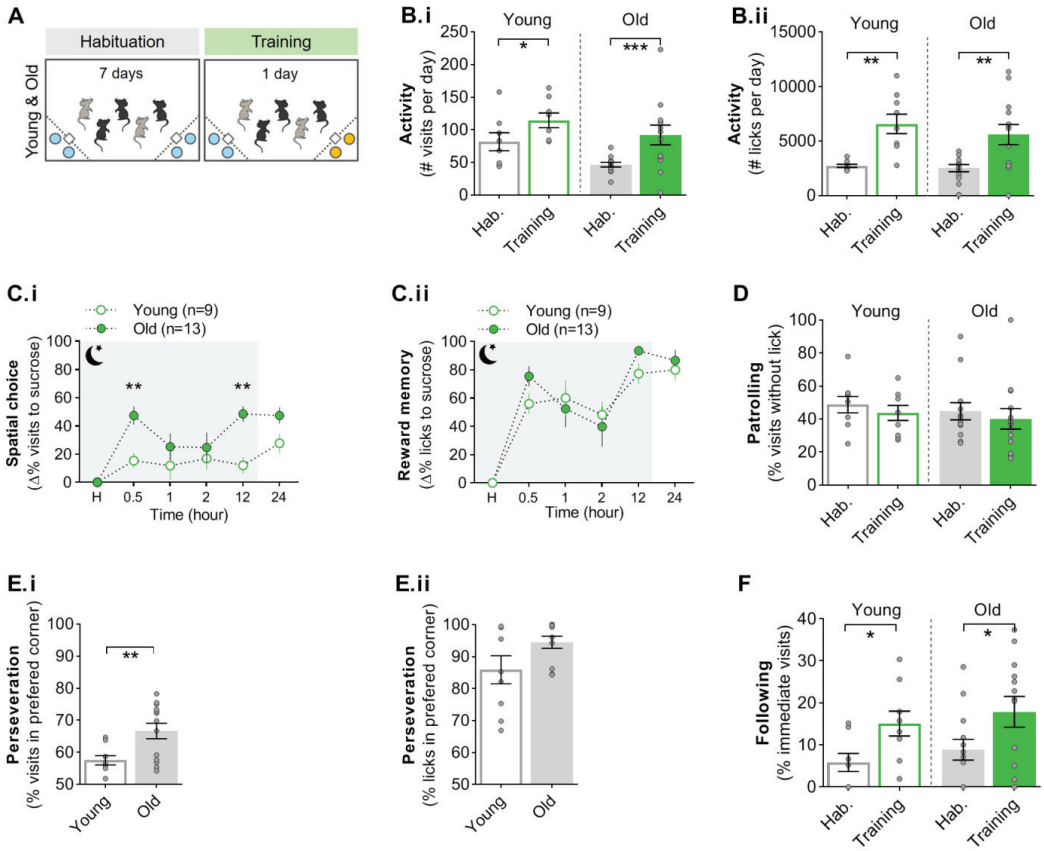
The effect of social interactions on spatial choice

To validate the role of social interactions in spatial choice of old mice, we tested whether spatial choice and non-spatial learning of aged mice are altered by the presence of young cage-mates. To this end, new cohorts of young (n=9) and old mice (n=13) were mixed in the IntelliCages (**Fig. 2A**) and trained together according to the protocol described in Figure 1A. We observed a

298 significant effect of training ($F_{1, 19}$ = 22.17, P < 0.001) but not age (two-way RM ANOVA, F1, 19 =
299 3.643, P = 0.072) on mice activity measured as daily visits (**Fig. 2B.i**). As revealed by Sidak's *post hoc*
300 tests, young and old mice both increased activity during training (P = 0.042 and P < 0.001). There was
301 also a significant effect of training ($F_{1, 20}$ = 40.01, P < 0.001), but no effect of age ($F_{1, 20}$ = 0.496, P =
302 0.489) on liquid consumption (**Fig. 2B.ii**). As confirmed by *post hoc* analysis, both age groups
303 increased consumption during training (P < 0.001 for both). Thus, mixing young and old mice did not
304 affect their activity during training, but slightly increased reward consumption by old mice.

305 The analysis of reward corner preference during training showed a significant effect of time
306 on the change of visit preference (mixed-effects model, χ^2 = 3.342, df = 1, P = 0.068; $F_{3.247, 61.69}$ = 8.7, P
307 < 0.001) indicating correct spatial choices (**Fig. 2C.i**). Moreover, there was a significant effect of age
308 ($F_{1, 20}$ = 12.85, P < 0.002) and interaction between age and time ($F_{5, 95}$ = 2.385, P = 0.044). Sidak's
309 multiple comparisons *post hoc* tests revealed that the difference between young and old animals was
310 significant only at the beginning of training (0.5h, P = 0.005) and at the end of the dark phase (12h, P
311 = 0.001), but not at other time-points. Moreover, the analysis of licks in the reward corner showed a
312 significant effect of training (mixed-effects model, χ^2 = 5.734, df = 1, P = 0.017; $F_{3.064, 53.93}$ = 42.33, P <
313 0.001) indicating non-spatial memory formation (**Fig. 2C.ii**). There was no effect of age ($F_{1, 20}$ = 0.612,
314 P = 0.443) or interaction between age and time ($F_{5, 88}$ = 1.235, P = 0.300) demonstrating that young
315 and old mice behaved similarly in a mixed population.

316 We further asked whether the frequency of patrolling, perseverative and social visits were
317 changed in a mixed population, as compared to separate populations. We found no significant effect
318 of age (two-way RM ANOVA, $F_{1, 20}$ = 0.269, P = 0.610) and training ($F_{1, 20}$ = 1.943, P = 0.179) on the
319 frequency of patrolling visits (**Fig. 2D**). Thus, unlike separate populations, the frequency of patrolling
320 visits in the mixed population remained high during training. Moreover, there was a significant effect
321 of age on the frequency of perseverative visits (t = 2.891, df = 20, P = 0.009), and only close to
322 significant on perseverative licks (t = 2.013, df = 20, P = 0.057) (**Fig. 2E**). Interestingly, during the
323 mixed population habituation, the frequency of perseverative visits in old mice was lower than in the
324 separate populations (67% vs 77%) (student's t-test, t = 2.891, df = 20, P = 0.009). The frequency of
325 perseverative licks in young mice was higher in the mixed population as compared to separate (86 vs
326 65%) (t = 2.013, df = 20, P = 0.058). Finally, we observed a significant effect of training (mixed-effects
327 model, χ^2 = 4.645, df = 1, P = 0.031; $F_{1, 19}$ = 15.68, P < 0.001), but no effect of age ($F_{1, 20}$ = 0.641, P =
328 0.433) or interaction between these factors ($F_{1, 19}$ = 0.006, P = 0.941), on the frequency of social visits
329 (**Fig. 2F**). As confirmed by Sidak's *post hoc* test, there was no significant difference in the frequency of
330 social visits between young and old mice during habituation or training. Both young (P = 0.036) and
331 old mice (P = 0.011) increased the frequency of social visits during training as compared to
332 habituation. Altogether, our data confirm that a high tendency for perseverative visits and social
333 interactions may contribute to the robust place and reward preference in aged mice. Moreover, the
334 frequency of such behaviors is affected by cage-mates.



335

336 **Figure 2. Perseverative spatial choice of old mice is affected by social interactions with young**
337 **animals.**

338 (**A**) Experimental timeline. During habituation, mice had access to two active corners with water.
339 During place preference training, sucrose was given in the less visited corner during habituation.
340 Young and old animals were kept together throughout the experiment. (**B**) Summary of data showing
341 mice activity during habituation and training. Daily visits (i) and licks of all liquids (ii) are shown for a
342 group of old and young mice living in one cage. (**C**) Summarized results showing an increase in
343 reward corner preference during training. Data for visits (i) and licks (ii) from the sucrose corner are
344 shown. The dark phase of the day is indicated as a gray background. Summary of data showing the
345 frequency of (**D**) patrolling visits (without licks), (**E**) perseverative visits (i) and licks (ii) (to a preferred
346 corner), and (**F**) social visits (visits in the corners following another mouse with inter-visit interval <
347 1s) during habituation and training. *P < 0.05, **P < 0.01, ***P < 0.001, for *post-hoc* tests. Mean ±
348 SEM are shown.

349

350 **Ageing affects training-induced plasticity of dendritic spines**

351 Spatial choice is belived to rely on synaptic plasticity in dCA1 (Bannerman et al., 2012, 2014).
352 Therefore, we analysed training-induced changes in dendritic spines of young and old mice, as a
353 proxy of training-induced synaptic plasticity (Graziane et al., 2016). Denritic spines were analysed
354 after 2 hours of training. We chose the 2h time point for the analysis of dendritic spines as our

previous experiments showed significant dendritic spines changes at this time point after hippocampus-dependent training (Radwanska et al., 2011). Here, we used young (5-month old) and old (18-month old) Thy1-GFP(M) mice (Feng et al., 2000), which express GFP in a sparsely distributed population of glutamatergic neurons (**Fig. 3A**). The animals were trained in separate cages to find sucrose in one cage corner, and they were sacrificed after 2 hours of training. Control groups were sacrificed immediately after habituation (**Fig. 3B**). We observed a significant effect of training (mixed-effects model, $\chi^2 = 0.8299$, df = 1, P = 0.362; training: $F_{3, 25} = 52.93$, P < 0.001) on the frequency of reward corner visits, indicating that both young and old mice developed a preference to the sucrose corner (**Fig. 3C**). There was no effect of age ($F_{1, 9} = 0.9315$, P = 0.36) or interaction between age and time ($F_{3, 25} = 1.601$, P = 0.214) on the reward corner preference. However, the LSD *post hoc* test for planned comparisons found that old mice had a higher preference for the reward corner than young animals at the end of training (2h) (P < 0.05), confirming a more robust place preference.

Dendritic spines were analysed in the *stratum radiatum* of the dorsal CA1 area (dCA1) (**Fig. 3A, D**), as this region is involved in cognitive processes that require spatial choice (Bannerman et al., 2012, 2014). We observed a significant effect of training (two-way ANOVA, $F_{1, 14} = 8.126$, P = 0.013), but not age ($F_{1, 14} = 0.0103$, P = 0.921) or age and training interaction ($F_{1, 14} = 1.901$, P = 0.19), on dendritic spine density (**Fig. 3E**). Tukey's multiple comparisons *post hoc* tests revealed that there was no difference in dendritic spine density in any of the groups (young, old, control or trained). A trend for dendritic spine density to increase after 2 hours of training was observed in the old mice, though not significant (P = 0.054). The analysis of dendritic spines areas showed a significant experimental group effect (Kruskal-Wallis test, H = 42.21, P < 0.001) (**Fig. 3F**). *Post hoc* multiple comparisons Dunn's test confirmed that young control mice had larger spines than the old control mice (0.248 vs 0.214 μm^2) (P < 0.001). Moreover, the spines area median in young mice shifted to a smaller value after training (0.248 vs 0.225 μm^2) (P < 0.001), and in contrast, to a larger value in old mice (0.214 vs 0.227 μm^2) (P < 0.001). These observations were confirmed when frequency distributions of spines areas were analysed (**Fig. 3G-I**). There was an increased frequency of small spines in old mice, as compared to young controls (Kolmogorov-Smirnov test, D = 0.1216, P < 0.001), and in young mice after training, as compared to controls (D = 0.074, P < 0.001) (**Fig. 3H**); while in old mice, the frequency of small spines decreased after training compared to controls (D = 0.079, P = 0.003) (**Fig. 3I**). Overall, our data show that spatial training in the IntelliCages resulted in shrinkage of dendritic spines in the dCA1 of young mice. In contrast, in old mice, new spines were generated, the median size of spines was increased, suggesting an alternative form of synaptic plasticity. To further validate this hypothesis, we decided to analyse the expression of the postsynaptic protein, PSD-95, that controls AMPAR localization at the synapse (Bats et al., 2007; Opazo et al., 2012).

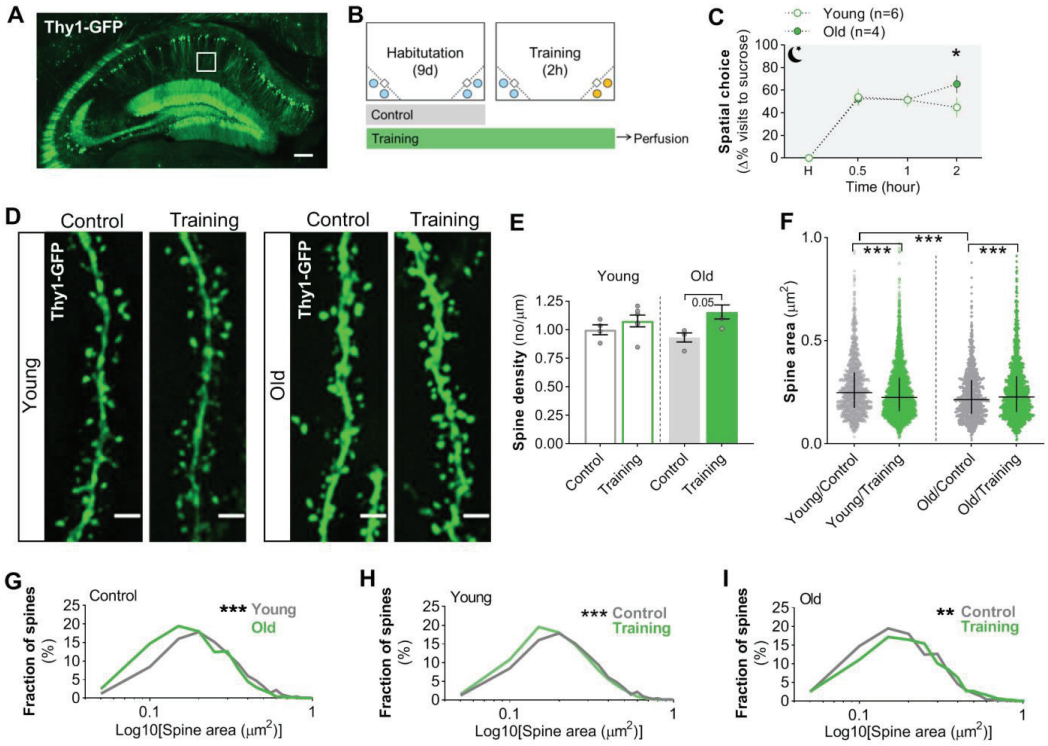


Figure 3. **Training induces CA1 dendritic spines shrinkage in young mice and enlargement in old mice.**

(**A**) Representative image of the dorsal hippocampus of Thy1-GFP(M) mouse. A white rectangle indicates the area of interest (*stratum radiatum* of the dorsal CA1 area). Scale bar: 200 μm (**B**) Experimental timeline. Mice were sacrificed immediately after habituation or 2 hours of training. (**C**) Summarized results show an increase in reward corner preference during training. Data for visits to the sucrose corner is shown. (**D**) Representative high-resolution images of dendritic fragments of control and trained young and old Thy1-GFP(M) mice. Scale bars: 2 μm. (**E**) Summary of results showing a linear dendritic spine density. Data for individual mice are shown (Young/Control, mice/ dendrites = 4/ 20; Young/Training, mice/ dendrites = 6/ 38, Old/Control, mice/ dendrites = 4/ 22; Old/ Training, mice/ dendrites = 5/ 21). (**F**) Summarized results show the area of dendritic spines in young and old animals. Graph shows data for individual spines (Young/Control, mice/ spines = 4/ 1112; Young/ Training, mice/ spines = 6/ 2455, Old/Control, mice/ spines = 4/ 972; Old/ Training, mice/ spines = 5/ 1158). (**G-I**) Frequency distribution of spine areas in young and old, control and trained mice. *P < 0.05, **P < 0.01, ***P < 0.001. Mean ± SEM are shown for (C,E); median ± IQR for (F). Axes are log10-transformed on (G-I) to show differences between the groups. H on graph presents preference of the corner during the last day of the habituation.

The effect of ageing on the correlation between levels of synaptic protein, PSD-95, and dendritic spine size

PSD-95 is a crucial scaffolding protein at mature glutamatergic synapses (Aoki et al., 2001). It promotes the survival of dendritic spines (El-Husseini et al., 2000; Murmu et al., 2013; Meyer et al., 2014) and supports the stability of hippocampus-dependent memories (Fitzgerald et al., 2015). As old mice developed a more robust preference of the reward corner than young animals, we tested if PSD-95 protein expression is regulated differently in young and old mice during spatial choice training.

To track co-localisation of PSD-95 protein with dendritic spines, we performed immunostaining on the brain sections of young and old Thy1-GFP(M) mice using specific antibodies (**Fig. 4A**) to identify PSD-95(+) and PSD-95(-) spines (**Fig. 4B**). There was no significant effect of age (three-way ANOVA, $F_{1, 29} = 0.01$, $P = 0.92$) or training ($F_{1, 29} = 0.728$, $P = 0.401$), but PSD-95 expression ($F_{1, 29} = 63.32$, $P < 0.001$) significantly affected dendritic spines density (**Fig. 4C**). Moreover, there was a significant interaction between training, age and PSD-95 ($F_{1, 29} = 5.912$, $P = 0.021$). LSD *post-hoc* tests (for planned comparisons only) confirmed that there were more PSD-95(-) spines in the old control ($P = 0.001$) and trained ($P < 0.001$) mice, as compared to young control and trained animals, respectively. Training did not affect the density of PSD-95(+) and PSD-95(-) spines in young mice, but significantly increased PSD-95(-) spine density in old mice ($P = 0.01$) (compared to the old controls). Old trained mice had more PSD-95(-) than PSD-95(+) spines ($P = 0.001$), and old mice had overall less PSD-95(+) spines than young mice.

Next, we determined the levels of PSD-95 protein per dendritic spine. There was a significant experimental group effect on the area of PSD-95 puncta (Kruskal-Wallis test, $H = 75.64$, $P < 0.001$) (**Fig. 4D**). PSD-95 puncta in young control mice were larger, as compared to the old control mice (0.027 vs 0.013 μm^2) (Dunn's *post hoc* tests, $P < 0.001$), further indicating a downregulation of PSD-95 expression in aged animals. Moreover, the median area of PSD-95 puncta was smaller in the trained animals, as compared to the age-paired control groups (Young: 0.027 vs 0.015 μm^2, $P < 0.001$; Old: 0.013 vs 0.010 μm^2, $P < 0.01$), indicating that the training downregulated PSD-95 expression.

Training-induced enlargement of dendritic spines and shrinkage of PSD-95 puncta in old mice suggest de-correlation of these parameters. To test whether such change also occurs at the level of individual spines, we compared the correlation strength of PSD-95 area and spine area (Spearman correlation, r) (**Fig. 4E-G**). The parameters strongly correlated in all experimental groups (Spearman correlation, $P < 0.001$ for all groups), indicating that large dendritic spines have higher expression of PSD-95 protein than small spines. Furthermore, the regression lines describing the correlations in young and old control groups showed a significant slope difference (ANCOVA, $F_{1, 822} = 21.82$, $P < 0.001$), indicating that dendritic spines in young mice had larger PSD-95 areas compared to those of the same size in old mice (**Fig. 4E**). This difference was size-dependent and most pronounced for large dendritic spines. A similar shift of the regression lines was observed in young mice after training, as compared to young control mice (slopes: $F_{1, 1517} = 26.30$, $P < 0.001$) (**Fig. 4F**). There was no difference in the slopes of the regression lines of old mice after training compared to the old control mice ($F_{1, 536} = 2.783$, $P = 0.096$) (**Fig. 4G**). The drop in elevation of the regression line after training in the old mice ($F_{1, 537} = 7.385$, $P = 0.007$), indicate that dendritic spines of the same size had smaller PSD-95 puncta, but this shift did not depend on the size of the dendritic spine. Moreover, we observed a significant effect of age (two-way ANOVA, $F_{1, 13} = 15.38$, $P = 0.002$) and training ($F_{1, 13} =$

5.983, P = 0.029), but no interaction between these factors ($F_{1, 13}$ = 0.009, P = 0.925) on the strength of PSD-95 and dendritic spine area correlation (Spearman correlation, r) (**Fig. 4H**). The LSD *post hoc* tests for planned comparisons confirmed that the correlation of PSD-95 area and dendritic spines area was weaker for both old control (P = 0.016) and trained (P = 0.016) mice, as compared to young mice (**Fig. 4H**). Furthermore, coefficient of variation (CV) analysis of PSD-95 and dendritic spines areas found a significant effect of age (two-way ANOVA, $F_{1, 15}$ = 9.275, P = 0.008), minor effect of training ($F_{1, 15}$ = 4.374, P = 0.054) and no interaction between these factors ($F_{1, 15}$ = 0.546, P = 0.471) (**Fig. 4I**). The LSD *post hoc* tests for planned comparisons found significantly higher CV in old trained mice as compared to young trained mice (P = 0.011), indicating a higher variability of PSD-95 areas in old animals. Overall, our data indicate that training in young mice induced dendritic spine shrinkage accompanied by downregulation of PSD-95 expression. Training in old mice induced dendritic spines enlargement and PSD-95 puncta shrinkage, presumably, de-correlating these parameters.

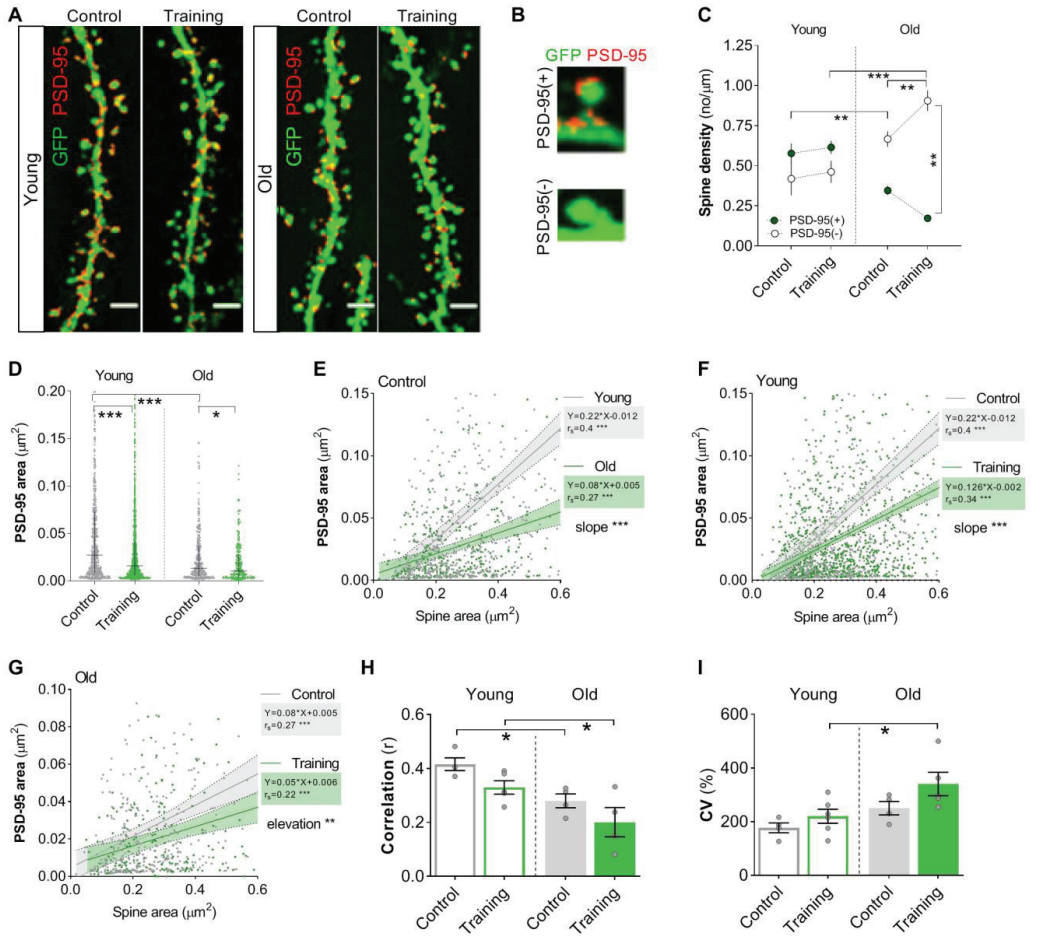


Figure 4. Training downregulates PSD-95 expression in young and old mice and decreases the correlation between areas of PSD-95 puncta and dendritic spines in old mice.

(**A**) Representative images of PSD-95 immunostaining colocalized with dendritic fragments of Thy1-GFP(M) mice. Scale bars: 2 µm. (**B**) Examples of dendritic spines: positive [PSD-95(+)] and negative [PSD-95(-)] for PSD-95. (**C**) Summary of results showing linear density of spines with and without PSD-

95. Graph shows data for animals (Young/Control, mice/ dendrites = 4/ 20; Young/ Training, mice/ dendrites = 6/ 38, Old/Control, mice/ dendrites = 4/ 22; Old/ Training, mice/ dendrites = 4/ 21). (**D**) Summarized results showing area of PSD-95 puncta. Graph shows data for total PSD-95 area per individual spines (Young/Control, mice/ spines = 4/ 497; Young/Learning = 6/ 1024, Old/Control = 4/ 329; Old/Learning = 4/ 214). (**E-G**) Correlation of PSD-95 area and dendritic spine area in young and old, control and trained mice. (**H**) Calculation of Spearman's rank correlation coefficient of dendritic spine area and PSD-95 area (Young/Control n = 4; Young/ Training n = 5; Old/Control n = 4; Old/ Training n = 4). (**I**) Coefficient of variation determined as a ratio between size of PSD-95 and spine areas. *P < 0.05, **P < 0.01, ***P < 0.001. Means ± SEM are shown for (C,H,I). Medians ± IQR are presented for (D). Linear regression lines together with their 95% confidence bands are shown for (E-G).

Spatial choice and non-spatial training induce different patterns of synaptic plasticity

We next studied whether synaptic changes observed during training in young mice were related to spatial or non-spatial aspects of training. To this end, young adult mice were trained in non-spatial sucrose training (sucrose in both active corners), or spatial aversive training (quinine in one corner, water in the other). The control mice were sacrificed after habituation (**Fig. 5A**). There was a significant effect of training on visits and licks (mixed-effects model, visits: $F_{1, 6} = 9.139$, P = 0.023; licks: $F_{1, 6} = 9.356$, P = 0.022), but no effect of time (visits: $F_{1.363, 8.180} = 3.325$, P = 0.098; licks: $F_{1.294, 6.037} = 1.167$, P = 0.342), or interaction between these factors (visits: $F_{3, 18} = 1.45$, P = 0.262; licks: $F_{3, 14} = 3.085$, P = 0.062), on corner preference (**Fig. 5B.i and ii**). Mice in the sucrose group did not show spatial preference after 2 hours of training (LSD *post hoc* tests for planned comparisons, P > 0.05 for visits and licks at 2h vs H). In contrast, mice in the quinine group significantly decreased preference to the quinine corner (P < 0.05 for visits and licks at 2h vs H), indicating the formation of spatial and non-spatial aversive memories.

The analysis of dendritic spines showed that there was a significant effect of training on spine density in the three experimental groups (one-way ANOVA, $F_{2, 9} = 2.343$, P < 0.001) (**Fig. 5C.i-ii**). Spine density was increased in the sucrose group, as compared to the control (P = 0.015), and there was no significant difference between the quinine and control groups (P > 0.05). We also observed a decrease in the spine area median in the quinine group compared to the control group (0.198 μm^2 vs 0.207 μm^2), while an increase in the sucrose group (0.207 vs 0.213 μm^2) (Kruskal-Wallis test with Dunn's *post hoc* tests, U = 15.03, P < 0.001) (**Fig. 5C.iii**).

Furthermore, an increased frequency of PSD-95(+) spines was found in the sucrose group compared to the controls (P < 0.05), and no change in spine frequency in the quinine group (two-way RM ANOVA with Tukey's multiple comparisons test; effect of training: $F_{2, 9} = 15.27$, P = 0.001; PSD-95: $F_{1, 9} = 333.7$, P < 0.001; interaction: $F_{2, 9} = 3.114$, P = 0.094) (**Fig. 5D.i-ii**). The quinine group also had decreased PSD-95 puncta area per dendritic spine (0.041 μm^2) compared to the controls (0.050 μm^2) (P < 0.001), while the sucrose group had increased PSD-95 puncta area (0.060 μm^2) (P < 0.001) (**Fig. 5D.iii**) (Kruskal-Wallis test with Dunn's *post hoc* tests, U = 50.89, P = 0.001).

There was a strong correlation between the PSD-95 area and the dendritic spine area in all experimental groups (Spearman correlation, P < 0.001 for all groups) (**Fig. 5E**). The regression line describing the correlation in the quinine group was shifted downward and differed in slope, compared to the controls (ANCOVA, $F_{1, 2752} = 11.53$, P < 0.001), indicating that the quinine group spines had relatively smaller PSDs than in the control, and this difference was most pronounced in

large spines. In the sucrose group, the regression line was shifted upwards, as compared to the controls, and differed in elevation (ANCOVA, $F_{1, 2924} = 7.345$, $P = 0.007$) (**Fig. 5E**) indicating that the PSDs were enlarged. We did not observe any effect of training on the correlation strength (one-way ANOVA, $F_{2, 9} = 0.704$, $P = 0.52$) (**Fig. 5F**) or CV (one-way ANOVA, $F_{2, 9} = 1.118$, $P = 0.368$) (**Fig. 5G**) of PSD-95 puncta and dendritic spine area. Overall, our experiments show that the pattern of synaptic changes observed in spatial training groups (sucrose and quinine) were similar and characterised by the downregulation of synaptic PSD-95 protein and shrinkage of dendritic spines. In non-spatial sucrose training, dendritic spines were enlarged, and the expression of PSD-95 increased. In all experimental groups, the changes in the areas of dendritic spines and PSD-95 correlated. Thus, our data suggest the role of PSD-95 protein in the dCA1 for both spatial choice and non-spatial memory in young mice.

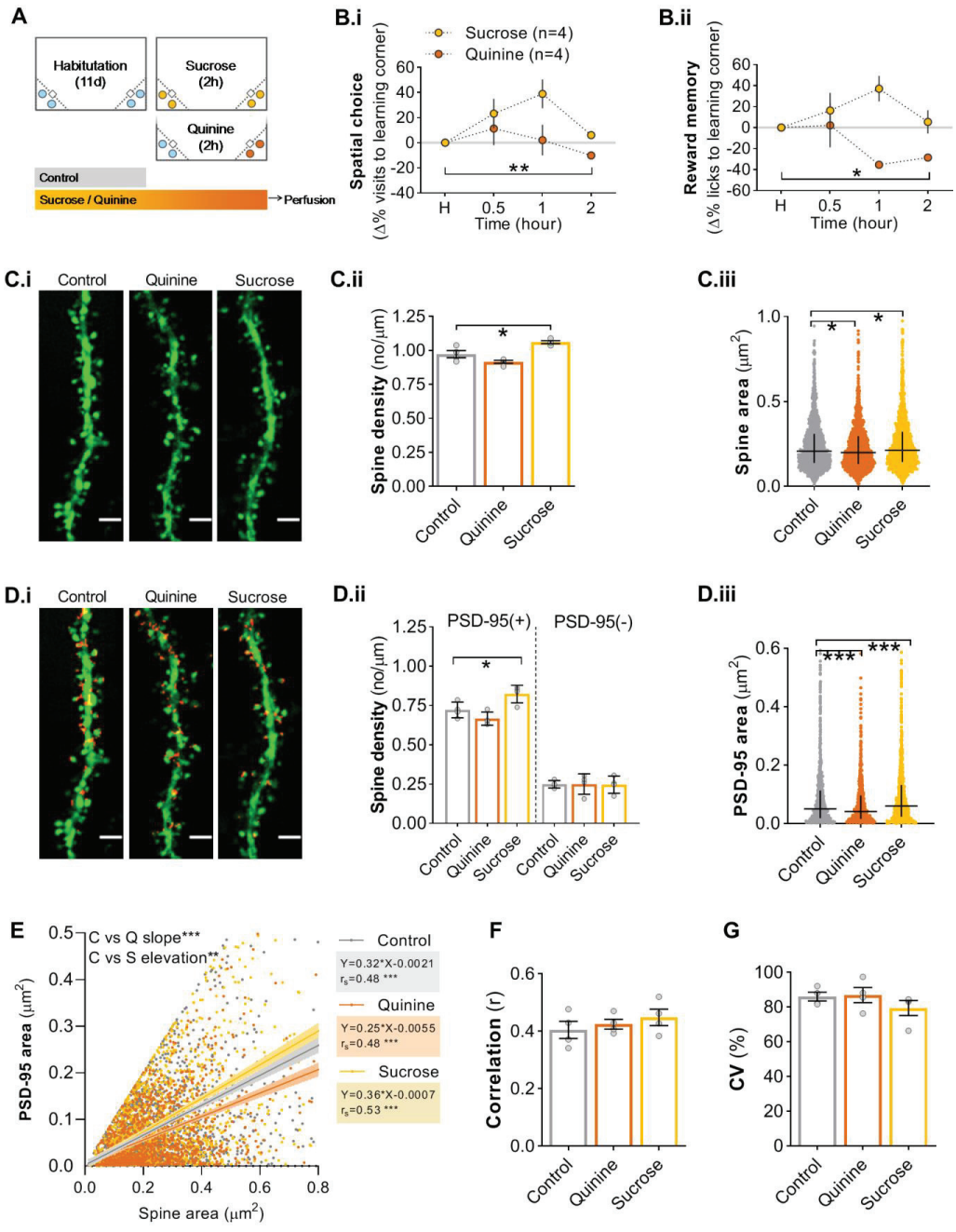


Figure 5. In young mice, aversive spatial choice training induces dendritic spines shrinkage, while appetitive non-spatial training results in dendritic spines enlargement.

(**A**) Experimental timeline and IntelliCage setups during non-spatial reward (sucrose in both corners) or spatial aversive training (quinine in one corner). The control group (water in both corners) was sacrificed after habituation. (**B**) Summary of data showing that mice avoided the corner with quinine, measured as visits (i) and licks (ii), and did not change corner preference when sucrose was in both corners. (**C**) Analysis of dendritic spines. (**i**) Representative microphotographs of dendritic fragments

of the young Thy1-GFP(M) mice drinking water (control group), quinine or sucrose. Scale bars: 2 µm. Summary of data showing (**ii**) spine density (mean values for animals; Control, mice/ dendrites = 4/ 31, Quinine = 4/ 28, Sucrose = 4/ 27) and (**iii**) dendritic spines area (individual spine data; Control, mice/ spines = 4/ 2105; Quinine, mice/ spines = 4/ 1653; Sucrose, mice/ spines = 4/ 1171). (**D**) Analysis of dendritic spines with PSD-95 puncta. (**i**) Representative microphotographs of dendritic fragments (Thy1-GFP(M)) with PSD-95 immunostaining (Scale bars: 2 µm) and summary of data showing (**ii**) density of PSD-95-(+) and PSD-95(-) spines (individual mice data; Control, mice/ dendrites = 4/ 31; Quinine, mice/ dendrites = 4/ 28; Sucrose, mice/ dendrites = 4/ 27) as well as (**iii**) area of PSD-95 puncta (individual spine data; Control, mice/ spines = 4/ 2105, Quinine = 4/ 1653, Sucrose = 4/ 1171). (**E**) Correlation of PSD-95 and dendritic spine areas in control, quinine and sucrose mice. Linear regression lines, together with their 95% confidence bands are shown. (**F**) Calculation of Spearman's rank correlation coefficient of the dendritic spine area and PSD-95 area. (**G**) Coefficient of variation calculated for individual mice (Control n = 4; Quinine n = 4; Sucrose n = 4). *P < 0.05, **P < 0.01 ***P < 0.001; Mean ± SEM are shown for (B.i,ii, C.ii, D.ii, F, G), median ± IQR for (C.iii, D.iii). H on graph presents preference of the corner during the last day of the habituation.

Role of dCA1 PSD-95 in spatial choice of young and old mice

To test the function of PSD-95 protein in dCA1 in spatial choice we used lentiviruses (LV) encoding short hairpin RNA (shRNA) targeted to PSD-95 mRNA (LV: H1-shRNA_PSD-95-Ub-GFP) (shPSD95) (Schlüter et al., 2006). For control, a lentivirus coding shRNA designed for *Renilla* luciferase (LV:H1-shRNA_luciferase) (shLuc) was used. Young (5-month old) and old (20-month old) C57BL/6J mice had LVs; shPSD95 or shLuc, stereotactically injected into dCA1 before the training in the IntelliCages (**Fig. 6A**). The post-training analysis of the hippocampal sections confirmed that shPSD95 expression was limited to dCA1 (Bregma > -2.5 mm) (**Fig. 6B-C**) and it effectively downregulated PSD-95 protein levels in young (Student's t-test, t = 4.243, df = 6, P = 0.005) and old mice (t = 2.292, df = 9, P = 0.048) (**Fig. 6D**). Importantly, shPSD95 did not impair basal mice activity and liquid consumption (**Fig. 6E and J**), indicating no gross behavioural impairments.

The activity of young mice with shLuc and shPSD95 (**Fig. 6E**) was significantly affected by the training (two-way RM ANOVA, visits: $F_{1, 16} = 15.59$, P = 0.001; licks: $F_{1, 16} = 31.6$, P < 0.001), but not by shRNA expression (visits: $F_{1, 16} = 0.099$, P = 0.757; licks: $F_{1, 16} = 0.022$, P = 0.883). There was also no significant interaction between training and shRNA (visits: $F_{1, 16} = 1.211$, P = 0.287; licks: $F_{1, 16} = 0.505$, P = 0.488). The Sidak's *post hoc* tests confirmed that training increased the frequency of visits and licks in the shLuc group (P < 0.01). However, in shPSD95 group, training increased daily number of licks (P < 0.01), but not visits (P > 0.05). Thus, depletion of PSD-95 prevented training-induced rise in young mice activity.

During trainnig, there was also a significant effect of time on a sucrose corner preference of young mice (mixed-effects model for visits, $\chi^2 = 14.30$, df = 1, P < 0.001; time: $F_{5, 77} = 15.59$, P < 0.001; licks frequency, $\chi^2 = 31.62$, df = 1, P < 0.001; time: $F_{3.031, 46.68} = 40.37$, P < 0.001) (**Fig. 6F**). This indicates that young mice in both experimental groups formed spatial and non-spatial memory. However, we also noticed a significant effect of shRNA targeting PSD-95 ($F_{1, 16} = 12.13$, P = 0.003), and interaction of time and shRNA ($F_{5, 77} = 5.82$, P < 0.001), on the change of visit preference. Mice with depleted PSD-95 levels showed a lower preference for the sucrose corner during the initial phase of the training than the controls (P < 0.001, for 1 and 2h) (**Fig. 6F.i**), indicating impairment of spatial choice. The licks preference analysis revealed that there was no significant effect of silencing PSD-95 gene expression ($F_{1, 16} = 0.118$, P = 0.736) or interaction of shRNA and time ($F_{5, 77} = 0.989$, P = 0.43),

indicating that non-spatial memory was preserved in young mice despite the downregulation of PSD-95 (**Fig. 6F.ii**).

Since corner patrolling, perseverative visits and social interaction may affect mice performance in spatial choice test, we analysed the effect of shPSD95 on these behaviours. The analysis of the patrolling visits revealed a significant effect of training (two-way RM ANOVA, $F_{1, 11} = 21.68$, $P < 0.001$), but no effect of shPSD95 ($F_{1, 11} = 0.75$, $P = 0.405$) or interaction of shRNA and training ($F_{1, 11} = 3.156$, $P = 0.103$), on their frequency (**Fig. 6G**). The Sidak's *post hoc* tests showed, however, that the training decreased the frequency of patrolling visits only in the shLuc group ($P < 0.01$), but not in the shPSD95 group ($P > 0.05$). Thus, depletion of PSD-95 prevented training-induced decrease in cage patrolling. There was no effect of shRNA on the frequency of perseverative visits (Student's t-test, $t = 0.956$, $df = 11$, $P = 0.36$) and licks ($t = 0.737$, $df = 11$, $P = 0.477$) during habituation (**Fig. 6H.i and ii**). Finally, there was no effect of shRNA ($F_{1, 11} = 0.199$, $P = 0.664$), but a significant effect of training (two-way RM ANOVA, $F_{1, 11} = 57.77$, $P < 0.001$), on the frequency of social visits (**Fig. 6I**). As confirmed by the *post hoc* tests, the frequency of social visits was increased during training in both experimental groups ($P < 0.001$ for both). Thus, unlike the trained separately young mice (**Figure 1**), the trained together young mice expressing shLuc and shPSD95 increased frequency of social interactions during training. This observation confirms that social context (old mice or mice with depleted PSD-95 in dCA1) affects social behaviour of young animals.

Similarly to the young animals, activity of aged mice with shLuc and shPSD95 was significantly affected by the training (two-way RM ANOVA, effect of time, visits: $F_{1, 13} = 7.844$, $P = 0.015$; licks: $F_{1, 12} = 27.10$, $P < 0.001$) (**Fig. 6J**), but there was no effect of shRNA (visits: $F_{1, 13} = 1.553$, $P = 0.235$; licks: $F_{1, 12} = 2.457$, $P = 0.143$) or interaction between time and shRNA (visits: $F_{1, 13} = 1.946$, $P = 0.184$; licks: $F_{1, 12} = 0.669$, $P = 0.429$). The Sidak's *post hoc* tests indicated that, while in shLuc group both visits and licks were more frequent during training as compared to the habituation ($P < 0.01$), in shPSD95 group training increased only number of licks, but not visits (licks: $P < 0.01$; visits: $P > 0.05$). Thus, depletion of PSD-95 prevented training-induced rise in aged mice activity.

Moreover, we found a significant effect of time ($F_{5, 55} = 52.59$, $P < 0.001$), but no effect of shRNA ($F_{1, 12} = 0.77$, $P = 0.40$) or interaction between shRNA and time ($F_{5, 55} = 1.912$, $P = 0.107$), on visit preference in old mice (**Fig. 6K.i**). We also observed a significant effect of time (mixed-effects model, $\chi^2 = 21.56$, $df = 1$, $P < 0.001$; training: $F_{1.977, 21.35} = 137.4$, $P < 0.001$), but no effect of shRNA ($F_{1, 12} = 2.93$, $P = 0.113$) or interaction of shRNA and time ($F_{5, 54} = 1.362$, $P = 0.253$), on licks preference (**Fig. 6K.ii**). The absence of significant differences in reward corner preference during training between shLuc and shPSD95 groups was also confirmed by Sidak's *post hoc* tests. Thus, depletion of PSD-95 levels affected neither spatial choice nor non-spatial memory in old mice.

The analysis of the patrolling visits revealed that there also was no effect of time ($F_{1, 12} = 2.608$, $P = 0.132$) or shRNA ($F_{1, 12} = 0.457$, $P = 0.512$), but a significant time and shRNA interaction on their frequency ($F_{1, 12} = 7.427$, $P = 0.018$) (**Fig. 6L**). The Sidak's *post hoc* tests confirmed that the frequency of the patrolling visits was decreased during training in the shLuc group, but not in the shPSD95, indicating that depletion of PSD-95 impaired training-induced decrease of cage patrolling. No significant difference was observed between shPSD95 and shLuc groups in the frequency of perseverative visits (Student's t-test, $t = 1.727$, $df = 13$, $P = 0.108$) and licks ($t = 2.036$, $df = 13$, $P = 0.063$) (**Fig. 6M.i, ii**). Finally, we observed a significant effect of training on the frequency of social visits both in shLuc (Wilcoxon matched-pairs signed rank test, $P = 0.031$) and shPSD95 group ($P =$

0.031) (**Fig. 6N**). Overall, our data indicates that dCA1-targeted downregulation of PSD-95 impaired spatial choice in young mice, but it had no significant effect on the aged animals' spatial choices. Interestingly, depletion of PSD-95 also prevented training-induced increase of mice activity and decrease of the patrolling visits independent of mice age. However, since these parameters did not differ between the experimental groups during training (shLuc vs shPSD95), the impairments could not explain poor spatial choices in the young shPSD95 group.

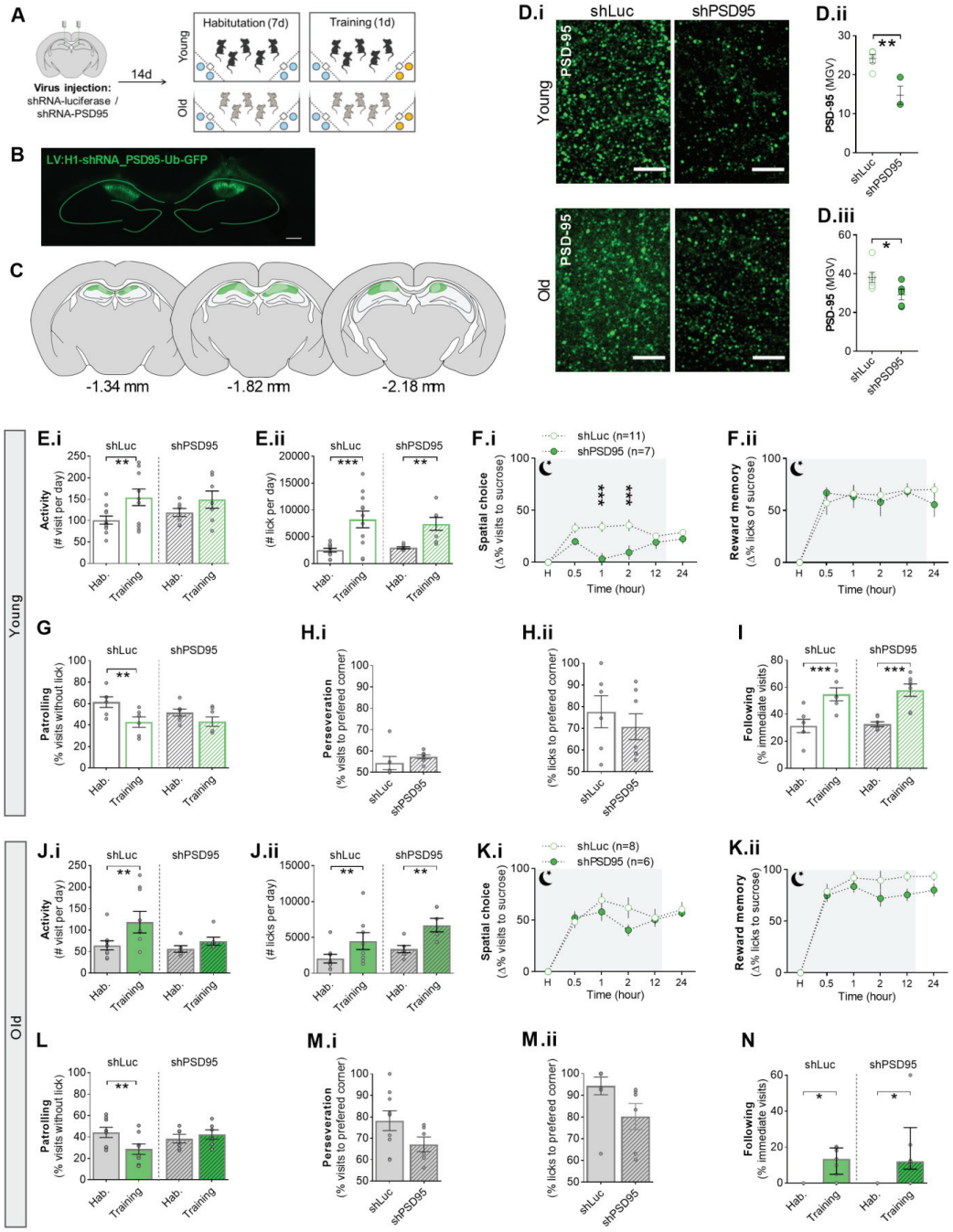


Figure 6. PSD-95 protein in dCA1 is required for spatial choice in young, but not old mice.

(**A**) Experimental timeline. After injection of shPSD95 or shLuc into dCA1, mice were trained in the IntelliCage. First, the animals were habituated to the cage, with water in both corners. Next, during spatial training, a bottle with sucrose was placed in one cage corner. (**B**) Representative confocal scan of the dorsal hippocampus with local overexpression of shRNA targeted to PSD-95. Scale bar: 100 µm. (**C**) The map of shPSD95 transfection. Maximal and minimal transfections are presented. (**D**) (i) Representative microphotographs of PSD-95 immunostaining. Scale bars: 5 µm. (ii) Summary of data

showing that local expression of shPSD95, downregulated PSD-95 expression in the CA1 area of young mice. (iii) Summary of data showing that shRNA for PSD-95, downregulated PSD-95 levels in the CA1 of old mice. (**E**) Summary of data showing young mice activity during habituation and training. Daily visits (i) and licks of all liquids (ii) are shown. (**F**) Summarized data showing that downregulation of PSD-95 expression in the CA1 area decreased place preference in young animals (i), but did not affect sucrose preference (ii). (**G-I**) Summary of frequency data of (**G**) patrolling visits (without licks), (**H**) perseverative visits (i) and licks (ii) (to preferred corner), and (**I**) social visits (following another mouse to the corner with inter-visit interval < 1s) during habituation and training. (**J**) Summary of data showing the old mice activity during habituation and training. Daily visits (i) and licks of all liquids (ii) are shown. (**K**) Summarized data for old mice, showing the effect of local downregulation of PSD-95 expression in the CA1 area by shRNA on place preference (i), and sucrose preference (ii). (**L-N**) Summary of data showing the frequency of (**L**) patrolling visits (without licks), (**M**) perseverative visits (i) and licks (ii) (to preferred corner), and (**N**) social visits during habituation and training. *P < 0.05, **P < 0.01, ***P < 0.001. Mean ± SEM is shown on graphs for (D-M); median ± IQR for (N). H on graphs presents preference of the corner during the last day of the habituation.

Discussion

Accumulating data indicate that synaptic plasticity in dCA1 is essential for spatial choice, rather than spatial memory (Bannerman et al., 2012, 2014). Here, mice living in a group were trained to make spatial choices between two visually identical cage corners. We demonstrate that aversive and appetitive spatial choice training in young mice induced similar synaptic alterations in the dCA1 that were characterised by dendritic spines shrinkage and downregulation of PSD-95 protein levels. Moreover, spatial choice in young mice is impaired by local downregulation of PSD-95 in the dCA1. Thus, our data support the role of PSD-95-dependent synaptic plasticity in dCA1 in spatial choice of young animals. Surprisingly, dCA1-targeted depletion of PSD-95 does not affect spatial choice in aged mice, suggesting that neuronal bases of spatial choice change with ageing.

Old mice are impaired in hippocampus-dependent tasks, including spatial choice in the radial maze or Barnes maze (Ammassari-Teule et al., 1994; Bach et al., 1999). However, old animals can use alternative non-spatial behavioural strategies to solve tasks that require the hippocampus. For example, young mice use spatial strategy in the Barnes maze, while old animals tend to use a non-spatial serial search strategy (Bach et al., 1999). Similarly, in our study old mice used alternative behavioural strategies to find a reward corner in the IntelliCage. They relied on social observations and perseverative behaviour (visiting and drinking only from one corner even during habituation), strategies commonly used also by ealderly people (Ridderinkhof et al., 2002). Previously, it was shown that social interactions improved place learning of old APP (amyloid precursor protein) mutant mice living in a group (Kiryk et al., 2011). Here, we demonstrate for the first time that both healthy old and young mice use social information during spatial choice training and, accordingly, mice behavior depends on social context, e.g. it is altered in the company of mice that use alternative strategy due to their age or functional impairments of dCA1. Interestingly, perseverative behavior is observed also in animals with hippocampal lesions and mutations (Brookes et al., 1983; Benice et al., 2008). The animals with impaired hippocampi tend to choose strategies that relay on other brain regions, e.g. striatum (Brookes et al., 1983; Benice et al., 2008). In agreement with this hypothesis, local depletion of PSD-95 protein in the CA1 area of aged mice did not affect place preference, suggesting that aged mice use dCA1-independent strategy during spatial choice task. It is, however, possible that spatial choice in old mice involves dCA1, but not PSD-95.

Interestingly, except for the effect of PSD-95 depletion in dCA1 on spatial choice in young mice, this local molecular manipulation prevented training-induced raise in mice activity and decrease in frequency of patrolling visits, both in young and old mice. These effects may result from the interaction between dCA1 and nucleus accumbens (NAc), an interface between the limbic and the motor systems. As recently shown, NAc integrates dCA1 information about reward-predicting environment that can be used to guide appetitive behaviour and arousal (Trouche et al., 2019). Thus, some functions of dCA1 that require PSD-95 seem to be preserved in old mice.

Most studies to date link cognitive processes with LTP of synaptic transmission (Bliss and Collingridge, 1993; Morris et al., 2003), reflected on a structural level as growth and addition of new dendritic spines (harboring glutamatergic synapses) (Restivo et al., 2009; Radwanska et al., 2011; Mahmmoud et al., 2015; Aziz et al., 2019), long-lasting growth of postsynaptic densities (PSDs) (excitatory synapses) (Śliwińska et al., 2019), and recruitment of AMPA receptors into existing spines (Matsuo et al., 2008). However, accumulating data indicate that spatial choice may also rely on LTD of synaptic transmission (Kemp and Manahan-Vaughan, 2007; Connor and Wang, 2016) that is

characterized by shrinkage of dendritic spines and PSDs or spine elimination (Nägerl et al., 2004; Zhou et al., 2004). Although structural correlates of learning-induced LTD has been rarely documented *in vivo*, spatial choice in the Barnes maze has been found to induce LTD in the CA1 area and interventions that prevent LTD, disrupted formation and updating of spatial choices (Kemp and Manahan-Vaughan, 2004; Griffiths et al., 2008; Goh and Manahan-Vaughan, 2013).

Here, we demonstrate that spatial choice training in young mice induced dendritic spines shrinkage in the *stratum radiatum* of dCA1. Moreover, the size of PSD-95 clusters in spines was also decreased, indicating downregulation of PSD-95 expression. A similar pattern of synaptic changes was observed after aversive spatial training, while non-spatial reward training resulted in dendritic spine enlargement and increased area of PSD-95 puncta. Since PSD-95 protein controls the localization of AMPARs at the synapse (Bats et al., 2007; Opazo et al., 2012) and AMPAR currents (Beique et al., 2006; Schlüter et al., 2006; Opazo et al., 2012), then these morphological and molecular changes observed in mice that underwent spatial training point to at least temporal weakening of synapses in the CA1 circuit. Still, it has to be established whether spatial choice training in the close-to-ethologic condition induces LTD in dCA1, in a similar way as spatial choice in the Barnes maze (Kemp and Manahan-Vaughan, 2004; Griffiths et al., 2008; Goh and Manahan-Vaughan, 2013). In support of this assumption shPSD95, that impaired spatial choice of young mice in our experiments, not only downregulates PSD-95 levels and prevents maturation of dendritic spines but also occludes LTD (Ehrlich et al., 2007; Xu et al., 2008). The shrinkage of dCA1 synapses during spatial choice may reflect the role of the hippocampus in behavioural inhibition to prevent wrong choices (Bannerman et al., 2012, 2014). However, understanding how synaptic shrinkage enables selection of the rewarded corner requires further studies.

Training-induced changes in dendritic spines further support an alternative neuronal strategy for spatial choice in aged mice. In old mice, spatial choice training increased the density and size of dendritic spines, while the expression of PSD-95 per spine decreased. Thus, the pattern of synaptic changes did not resemble the synaptic weakening observed during spatial choice training in the young mice. Instead, it bears components of non-spatial reward training (increased density and growth of dendritic spines) as well as spatial training (downregulation of PSD-95). Moreover, we observed that old mice have smaller dendritic spines, fewer spines with PSD-95, decreased size of PSD-95 clusters per spine, and de-correlation of PSD-95 levels with dendritic spine size, as compared to young mice. The shrinkage of dendritic spines and decreased levels of synaptic proteins is in agreement with earlier studies in aged animals (Ganeshina et al., 2004; Counts et al., 2014; Rogers et al., 2017; Śliwińska et al., 2019). Since small dendritic spines with low levels of PSD-95 are less stable than large spines (Cane et al., 2014), our observation also supports the notion that dendritic spines in aged mice are less stable (Mostany et al., 2013). Ours is the first study to show the de-correlation of PSD-95 expression and dendritic spine size in old animals. This phenomenon seems to be specific for the aged brain as the correlation between these two parameters was not affected by spatial choice or non-spatial reward training in young mice. Previously, the uncoupling of PSD and spine volumes was observed after glutamate uncaging in cell culture. However, it was transient (< 7 min) and presumably caused by a slower PSD growth compared to the spine (Bosch et al., 2014; Meyer et al., 2014). Moreover, NMDAR-dependent LTP in organotypic hippocampal slices was shown to increase the correlation between PSD size and dendritic spine volume (Borczyk et al., 2019). Precise reasons for dendritic spine volume and PSD-95 expression correlation remain unknown. However, since PSD-95 anchors AMPA and NMDA receptors (Sheng and Kim, 2011; Chen et al., 2015), PSD-95 levels may

affect Ca^{2+} signalling. Computational models predict that Ca^{2+} influx and spine size both crucially determine long-term synaptic stability, synaptic strength and distribution of dendritic spine sizes (O'Donnel et al., 2011). Accordingly, all these processes may be altered with age, resulting in poor specificity and stability of cognitive maps in the dCA1 of old animals (Barnes et al., 1997; Attardo et al., 2018) and switch to a PSD-95-independent strategy during training.

Overall, our data show that spatial choice in young animals involves PSD-95-dependent processes in the dCA1. Surprisingly in aged animals, although PSD-95 in the dCA1 is depleted, spatial choice remains intact. To obtain reward in a complex environment, old mice use an alternative behavioural strategy that relies on social observation and perseverative visits to the reward corner.

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