Experience-Dependent, Rapid Structural Changes in Hippocampal Pyramidal Cell Spines

Morphological changes in dendritic spines may contribute to the fine tuning of neural network connectivity. The relationship between spine morphology and experience-dependent neuronal activity, however, is largely unknown. In the present study, we combined 2 histological analyses to examine this relationship: 1) Measurement of spines of neurons whose morphology was visualized in brain sections of mice expressing membrane-targeted green florescent protein (Thy1-mGFP mice) and 2) Categorization of CA1 neurons by immunohistochemical monitoring of Arc expression as a putative marker of recent neuronal activity. After mice were exposed to a novel, enriched environment for 60 min, neurons that expressed Arc had fewer small spines and more large spines than Arc-negative cells. These differences were not observed when the exploration time was shortened to 15 min. This net-balanced structural change is consistent with both synapse-specific enhancement and suppression. These results provide the first evidence of rapid morphological changes in spines that were preferential to a subset of neurons in association with an animal’s experiences.

Keywords: behavior, hippocampus, immediate-early gene, plasticity, sparse coding, spine dynamics

Introduction

Dendritic spines, tiny protrusions that form the postsynaptic sites of most excitatory synapses (Harris and Stevens 1989), are the basic functional units of neuronal integration. Dendritic spine size positively correlates with the alpha-amino-3-hydroxy-5-methyl-i-isoxazolepropionate (AMPA) current before and after the induction of long-term synaptic plasticity in hippocampal slices (Matsuzaki et al. 2001, 2004), and electrical stimulation that is classically used to induce long-term potentiation and depression leads to spine formation and retraction, respectively (Nagerl et al. 2004). Theoretical studies have suggested that the formation and elimination of spines together constitute a potential mechanism for memory (Stepanyants et al. 2002). Findings from experiments in in vivo models support the notion that the structural plasticity of spines is linked to memory-associated circuit reorganization (Moser et al. 1997; Geinisman et al. 2001). For example, the density of distinct spines in the hippocampus increases 24 h after eye-blink conditioning (Leuner et al. 2003), and in vivo imaging of spines in the whisker barrel model suggests that the change in somatotopic representation induced by whisker-trimming is associated with stabilization of a subset of new spines over a period of days (Holtmaat et al. 2006). Although these studies indicate that structural changes occur within days after stimulation, mice show memory formation for novel objects after only a brief exposure period (Bevins and Besheer 2006). Such rapid structural changes linked to natural neuronal activity during behavior have not been described.

To analyze the effects of experience-evoked activity on spine morphology, we combined 2 histological techniques: 1) neuronal structure was visualized in brain sections of mice expressing membrane-targeted green florescent protein (Thy1-mGFP mice) (Richards et al. 2005); 2) a subset of neurons potentially activated in mice during brief exposure to a novel, enriched environment was detected by monitoring protein expression of the immediate-early gene Arc/Arg3.1 (Link et al. 1995; Lyford et al. 1995) by immunohistochemistry. Although the direct demonstration of an association between Arc signals and cellular activity is still lacking, accumulating evidence suggests that neuronal activity of cells precedes the Arc expression (Lyford et al. 1995; Steward and Worley 2001; Shepherd et al. 2006). Furthermore, the selectivity of the Arc-positive cell population for a particular environment (Guzowski et al. 1999; Ramirez-Amaya et al. 2005) and the inhibition of Arc expression following memory-impairing fornix lesions (Fletcher et al. 2006) suggest that Arc-expressing neurons are involved in neural encoding and memory formation.

Accordingly, we compared the spine morphology of Arc-expressing and nonexpressing neurons to examine how brief exposure to a novel, enriched environment alters the spine structure in hippocampal CA1 pyramidal cells.

Materials and Methods

Novel, Enriched Environment Exposure Procedures

Experiments were performed according to the guide for the care and use of laboratory animals of the University of Tokyo. Male Thy1-mGFP mice (line 2,1, gift from Drs V. de Paola and P. Caroni; De Paola et al. 2003) which express membrane-targeted green florescent protein (mGFP) in a small number of CA1 neurons, were housed 4 littermates per cage in a vivarium with controlled temperature and humidity (23 ± 1°C, 50 ± 10%) and free access to food and water on a 12-h light-dark cycle. All mice were handled daily for 5 days and were not exposed to a novel environment for at least 7 days before the mice were exposed to the novel, enriched environment at 8-11 weeks of age. Half of the Thy1-mGFP mice were placed in a plastic cage (37D x 21W x 15H cm, Fig. S1A) that was larger than their home cage (HC) in a novel room for 15 min (N15) or 60 min (N60), whereas their age-matched littermates remained in their home cages (HC group). There were 2 sets of HC mice, one for each of the N15 and N60 groups. Five novel objects and 4 small unfamiliar food pellets were placed in the cage in which 4 distinct markings were displayed on the walls. No apparent eating behavior was observed in the novel cage. N15 mice were retained in their HGs for 45 min after exposure to the environment. For the experiment described in Figure S3, mice were injected with saline or scopalamine hydrobromide (2 mg/kg, i.p., Wako, Osaka, Japan) 20 min prior to exposure to the environment for 60 min. Immediately after the 60-min session, all mice...
were anesthetized by inhalation of diethylether and perfused transcardially with chilled phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The dissected brains were postfixed for at least 2 h at 4°C, immersed first in 20% and then in 30% sucrose in 0.1 M PB (4°C, >72 h in total), frozen, and coronally sectioned (ca. Bregma −1.3 to −2.0 mm) at a thickness of 40 μm. Pair-wise brain sections of mice from the HC and N15 (or N60) groups were mounted on the glass slides and processed for immunohistochemistry in the same solutions.

**Immunohistochemistry Procedures**

Slide-mounted sections were incubated in 0.2% Triton X-100 for 30 min and treated in 1% H2O2 diluted in PBS for 15 min. After blocking with 2% normal goat serum for 1 h, the slides were incubated in anti-Arc antibody (rabbit, 1:8000; Lyford et al. 1995) for 48 h at 4°C, followed by anti-rabbit biotinylated secondary antibody (1:100, Vector Laboratories, Burlingame, CA), sometimes in combination with NeuroTrace 435/455 blue-fluorescent Nissl stain (1:50, Molecular Probes, Eugene, OR), for 60 min at room temperature. Immunolabeling was amplified by incubating with avidin-biotin complex (1:100, Vector Laboratories) for 60 min. The staining was visualized using the Cy-3 TSA fluorescence system (1:20, PerkinElmer Life Sciences, Boston, MA). All binding procedures were followed by 3 PBS washes.

**Confocal Microscopy**

Images of the hippocampal CA1 region were captured with a confocal microscope (MRC-1000, Zeiss, Oberkochen, Germany) equipped with 488-nm argon and 543 helium/neon lasers. First, to classify mGFP-positive pyramidal cells as Arc(+) or Arc(-), image stacks (1.0 μm thickness × 21 planes) of Arc and mGFP from the pyramidal cell layer were collected using a 60× objective (NA 1.2, water immersion). Laser power and gain parameters for Arc images were set such that pixel intensities were not saturated and were kept constant for all sections on the same slide. Image stacks (0.5 μm × 21 planes) of basal dendrites of mGFP-positive pyramidal cells were then collected with 3×digital zoom (0.067 μm/pixel). To capture triple-colored images including Nissl staining, a Zeiss LSM510 confocal microscope equipped with a blue diode laser and a 20× objective (NA 0.5) was used.

**Image Analyses**

Morphological parameters (length, head size, and spine density per unit length of dendrite) of spines in basal dendrites that were included in the images except on the top and bottom planes of the stacks were measured. Raw mGFP images (Fig. S1B) were processed by median filtration and deconvolution (MetaMorph, Molecular Devices, Downington, PA). This processing approach produced smaller standard deviation values (raw, 81 ± 47; processed, 49 ± 25 nm; P < 0.05 by Student’s t-test) of repetitive (5 times) measurements of spine length. Head size was determined as the maximum width of a spine head perpendicular to spine length. These measurement results substantially correlated with those measured with the methods proposed by other groups (Bloodgood and Sabatini 2005; Holtmaat et al. 2005), as shown in Figure S2. The analyzable parts of the dendrites were limited to those connected to their soma within the 40-μm-thick sections. Thus, to obtain a sufficient number of data (n = 5, bin 10 μm) in the same location, data were collected from the spines on basal dendrites within 10–50 μm from the soma. The effect of distance from the soma is shown in Figure S4B–D. To classify Arc expression, the threshold intensities of Arc signals were determined automatically using MetaMorph software. Then, the cells that had the signal intensity greater than the threshold value that covered at least one third of the soma area defined by mGFP (or by Nissl stain) were defined as the total neuron population used to calculate the percentage of Arc(+) neurons (Fig. 1 and Fig. S3). All mGFP(+) HC cells (n = 38) analyzed in this study were Arc(+). The classification of Arc expression and the spine analyses were performed independently and blind to the experimental conditions.

**Distributions of Spines with Large Heads**

Spine data from the 3 groups of cells [Arc(-) cells of N15/N60, Arc(+) cells of N15/N60, and HC Arc(-) cells] were mixed and ranked in descending order according to spine head size. Then, the proportion of spines in group m in the top x% of head size, P(x,m), were calculated, where m represents one of the 3 cell groups: Arc(-) cells of HC, Arc(-) cells of N15 (or N60), or Arc(+) cells of N15 (or N60). Because P(x,m) was biased by the length of dendrites analyzed in each group, the data were divided by dendrite length as follows, % of fraction of group m = (P(x,m)/L(m))/Σ(L(m)/I(L)), where L(m) is analyzed length of the dendrites in m (one of the above-mentioned 3 cell groups) and the denominator represents the sum of normalized proportions of the 3 groups in a dataset. Dendrite length and spine number were as follows: in the N15 experiment; HC, 840 μm, 1,119 spines in 22 cells from 4 mice; Arc(-), 1,100 μm, 1,600 spines in 27 cells; Arc(+), 398 μm, 561 spines in 10 cells from 4 mice (Fig. 3A–C); in the N60 experiment; HC, 683 μm, 851 spines in 16 cells; Arc(-), 988 μm, 1,283 spines in 19 cells; Arc(+), 330 μm, 350 spines in 8 cells; from 8 mice (Fig. 5D–F). The Pvalues of the % of fraction of group m were calculated with the 200 surrogate data points made by random shuffling of the ranking of head size for cellular groups (Microsoft Excel).
Results

Arc Expression after Exposure to the Novel, Enriched Environment

Thy1-mGFP mice were exposed to a novel, enriched environment for 15 min (N15), 60 min (N60) or kept in their HCs. Immediately after the 60-min session, the brains were collected. Approximately 25% of the hippocampal CA1 neurons in the N15 and N60 brains were Arc(+), whereas only 3% of the CA1 neurons in HC samples were Arc(+). The proportion of cells that was Arc(+) as well as the intensity of Arc immunoreactivity were similar between the N15 and N60 groups (Fig. 1B,C). In the N60 group, administration of the muscarinic receptor antagonist scopolamine, which impairs the formation of hippocampal-dependent spatial memory (Buresova et al. 1986), before placing the mice in the environment decreased the proportion of Arc(+) cells to 1% (Fig. S3). This finding supports a possible link between Arc expression and memory formation, and suggests that the Arc expression was not merely due to mental and physical stress or other physical differences between HC and N15/N60.

Time-Dependent Reduction in the Number of Small Spines in Arc(+) Cells

Morphological analysis of mGFP-labeled spines on the basal dendrites of CA1 pyramidal cells (Fig. 1D, see also Fig. S1B for additional images of dendritic segments) revealed that overall spine density in N15 (mean ± SEM: 1.47 ± 0.05/μm dendrite) and N60 (1.26 ± 0.06/μm dendrite) was similar to that of the respective littermate HC control group cells (1.38 ± 0.06 and 1.26 ± 0.07/μm dendrite, respectively; both P’s > 0.05 by Student’s t-test), suggesting that the total number of spines was not altered by the environmental exposure. Segregation of Arc(+) and Arc(−) cells in the same samples, however, revealed that the spine density of Arc(+) cells was lower than that of Arc(−) cells in the N60 group (Fig. 2C), but not in the N15 group (Fig. 2A,B). The difference in the N60 group was also confirmed by comparing the averaged data from individual animals (Arc(−), 1.29 ± 0.08; Arc(+), 1.06 ± 0.07/μm dendrite; n = 5 animals, P < 0.01 by paired t-test). The difference in the spine density became more evident if only spines with a head size of less than 0.5 μm in diameter were considered (Fig. 2D–F). Namely, Arc(+) cells in the N60 group had lower small spine density compared with Arc(−) cells in both HC and N60 group (Fig. 2D). This difference was more evident in dendrites 30–50 μm from soma (Fig. S4B,D). Furthermore, we prepared Figure S4A,C to assuage a concern on the location-related bias, because the somata of Arc(+) cells were preferentially localized nearer to stratum oriens in the cell layer in both N60 and N15 groups, although the underlying mechanism is not known. The figure shows that the Arc(+) cells had fewer small spines, regardless of the location of the soma.

Increase in Large-Head Spines in Arc(+) Neurons

Arc(+) and Arc(−) neurons also exhibited differences in large-head spines. We analyzed 2 pooled datasets of spines from the 3 groups. 1) Arc(−) cells in HC (there were no mGFP-labeled, Arc(+) cells in HC), 2) Arc(−) cells in either N15 or N60, and 3) Arc(+) cells in either N15 or N60 as 2 datasets for each condition (N15/N60). We first defined large-head spines as spines whose size was among the largest 5% of all measured spines in the each of the pooled datasets and evaluated the size distribution among the 3 groups. In the dataset including N15 mice, allocation of large-head spines in each group was close to 33%, which is chance level (Fig. 3A). In the dataset including N60 mice, however, large-head spines were more frequently found on Arc(+) cells (Fig. 3D). This divergence was robust, even if the definition of a large-head spine was expanded to the largest 25% (Fig. 3E). To examine whether this distribution was within possible stochastic fluctuations, we created surrogate data by randomly shuffling the rank order of spine size in the each pooled dataset. A significant deviation from the random data was rarely found in the N15 dataset (Fig. 3C), HC versus N15 Arc(−), HC versus N15 Arc(+), and HC versus N60 Arc(−) (Fig. S5A–C), but many more large spines were present in Arc(+) cells in the N60 mice (Fig. 3F and Fig. S5D) than expected by stochastic fluctuation.

The reason that significant difference was not detected in average density of large spines in Figure 2D may be due to the large deviations among density from cells within the Arc(+) group (Chen et al. 2007). To show the information, we plotted parameters of each cell, and actually found that very-large spine density of Arc(+) cells varied greatly in the N60 group (Fig. 3G–I).

In addition, we found that the spine size for the N15 and N60 group resulted in a significant inverse correlation between the densities of small (<0.5 μm) and very-large (>0.8 μm, approximately correspond to the largest 5% of all spines) spines in the N60 group (Fig. 3HL; the HC control group showed no statistically significant correlation (Fig. 3G).

The time course of these shifts in spine size frequency might be related to changes in neuronal networks demonstrated by the mouse’s behavior. In separate groups of mice re-exposed to the same environment the next day, the locomotor activity of mice in the N60 group was significantly lower than that of the N15 group, suggesting recognition memory of the previous day’s experience in the N60 mice (Fig. S6).

It was confirmed that mGFP expression did not interfere with Arc detection or the intensity and pattern of Arc expression (Fig. 1 and Fig. S7D,E). Furthermore, Thy1-mGFP transgenic mice did not differ in the locomotor activity compared with wild-type mice, suggesting that the mGFP expression also did not affect the behavior (Fig. S7A–C).

Discussion

Dendritic Spine Changes in Living Animals

The novel finding of the present study was that rapid structural changes in hippocampal spines were induced by exposure to a novel, enriched environment. There were no clear differences in the spines between the Arc(−) and Arc(+) cells in the N15 group, thereby excluding the possibility that only neurons that already had spines with a different morphology preferentially expressed Arc during exploratory behavior. Although it is possible that spine morphology was also changed within the 15 min of exposure to the novel environment in the N15 group and that this effect was reversed during the subsequent 45-min period in the HC, the fact remains that the changes persisted between the Arc(−) and Arc(+) cells in the N60 group. These data together suggest that the spine changes occurred as a result of the duration of the exposure to a novel, enriched environment. That is, the reduction in the number of small spines is likely due to spine elimination or shrinkage during...
exploration of the environment, whereas the increase in the number of large spines likely reflects enlargement of existing spines and/or de novo emergence of large spines.

**Relationship between Behavior and Spine Changes**

The structural differences between N15 and N60 seemed to be related with mouse behavior in the re-exposure session (Fig. S6). Significantly suppressed exploratory behavior in N60 on day 2 suggests that the extent and/or quality (Bevins and Besheer 2006) of familiarization during the exposure to the environment on day 1 was greater in the N60 than N15, and that spine reorganization may underlie memory formation in the behaving animals.

**Rapid Spine Changes in a Subset of Neurons**

The present findings indicated that relatively rapid (but not immediate, <60 min) structural changes occurred in hippocampal pyramidal cell spines. Various behavioral paradigms such as eye-blink conditioning, exposure to an enriched environment, and chronic stress induce structural reorganization of spines that has been observed from 1 day to several months later (Rampon et al. 2000; Leuner et al. 2003; Silva-Gomez et al. 2003; Mitra et al. 2005), whereas in in vitro experiments, bidirectional spine plasticity has been described within 1 h of stimulation (Engert and Bonhoeffer 1999; Matsuzaki et al. 2004; Zhou et al. 2004). This is the first report, however, of the detection of rapid spine reorganization after stimulation of living animals with short exposure to a stimulus.

**Figure 2.** Effects of exposure to a novel, enriched environment on spine density. Spine density per micron of dendrite length and distributions of spine head sizes in N15 (A, B) and N60 (C, D) cells are shown. Arc(-) cells in the N60 group possessed fewer small spines compared with Arc(-) cells in both the N60 and HC groups, whereas there was not a statistically significant difference in the N15 group. (A, B) HC, n = 19 (1081 spines) from 4 mice; Arc(-), n = 23 cells (1513 spines); Arc(+), n = 10 cells (564 spines) from 4 mice. (C, D) HC, n = 16 cells (851 spines) from 6 mice; Arc(-), n = 19 cells (1283 spines); Arc(+), n = 8 cells (350 spines) from 8 mice. Error bars indicate standard error of the mean. *P < 0.05 by Tukey’s post hoc test in (A, C). **P < 0.01/3, *P < 0.05/2 by Bonferroni–Holm test after repeated-measures 2-way ANOVA in (B, D). These significant differences were reproduced in another independent experiment. (E) There was a pronounced decrease in spines smaller than 0.5 μm on Arc(+) cells in the N60 group (red), but not in the N15 (gray) group. (F) Spine density ratio between the Arc(+) and Arc(-) cells for the small (<0.5 μm) and large (>0.5 μm) spines. Note the distinctly opposite patterns for small and large spines in the N60 group.
We were able to detect rapid spine changes by dividing the cells into Arc(+) and Arc(-) groups to isolate those cells that had recently been active. Thus, even when it is difficult to detect the structural changes in spines averaged across the entire cell population, this method allowed us to detect clear rapid changes of spines in a specific subset of neurons that were activated by the stimulus to the animals.

Possible Effects by Reduction of Small Spines after Exploring Activity

The depressed densities of small (<0.5 μm) spines imply changes in functional neuronal circuits. Although silent synapses would be included in these decreased ones, some spines should form functional synapses (Harris and Stevens 1989; Noguchi et al. 2005). Thus, the decrease of spines would mean some form of depression in synaptic transmission at the time. Further, it might lead to long-term depression accompanied by spine shrinkage and retraction (Nagerl et al. 2004; Zhou et al. 2004).

Another important point is that smaller spines have greater potentiality to undergo long-term potentiation (Matsuzaki et al. 2004). Assuming that some of Arc(+) neurons commit to convey specific information thereafter, the reduction of small spines might contribute to the functional differentiation.

Enlargement of Spines in Limited Number

It is notable that exposure to the novel, enriched environment in our experiment enlarged only a limited number of spines.
These findings are similar to those of a recent study in which the authors estimated the number of spines with detectable transport of newly synthesized glutamate receptors (GluR1), implying enhanced transmission, during fear conditioning; only ~3% of all spines in Fos-positive neurons had preferential transport of newly synthesized GluR1 (Matsuo et al. 2008). Our results, however, are not consistent with the ~30% increases in the density of distinct spines observed 24 h after eye-blink conditioning (Leuner et al. 2003). The difference might reflect the difference in the strength and quality of stimulations to the brain region. Future studies to evaluate the strength of the relationship between learning or neuronal network stimulation and spine reorganization are necessary. We speculate that although only a minor proportion of spines would be enlarged, the more substantial inputs they would create would be critically important for competitive neural circuit reactivation. Previous findings that a larger spine evokes a larger EPSP (Matsuzaki et al. 2001) and that spatiotemporally clustered large inputs can be supra-linearly summed due to the initiation of a dendritic spike (Losonczy and Magee 2006) support this notion.

Conclusions

The present study provides the first evidence of rapid, coordinated spine enlargement and spine elimination in neurons activated by an animal's exposure to a novel environment, and provides an estimate of the extent of structural synaptic changes that occur during a natural animal experience. The ability to monitor structural changes in activated and nonactivated populations of neurons provides an important new and simple paradigm for studying the molecular and synaptic mechanisms of natural structural reorganization.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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Notes

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