EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

*European Journal of Neuroscience, pp. 1–7, 2009* doi:10.1111/j.1460-9568.2009.06860.x

# Behaviorally evoked transient reorganization of hippocampal spines

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*Keywords*: Arc/Arg3.1, dendritic spine, immediate-early gene, plasticity, sparse coding

## Abstract

Dendritic spines, microstructures that receive the majority of excitatory synaptic inputs, are fundamental units to integrate and store neuronal information. The morphological reorganization of spines accompanies the functional alterations in synaptic strength underlying memory-relevant modifications of network connectivity. Here we report the rapid dynamics of cell population-selective spine reorganizations related to behavioral experiences. In Thy1-GFP transgenic mice, hippocampal CA1 pyramidal neurons that were putatively activated during environmental explorations were detected with their *post hoc* immunoreactivity for Arc, an activity-dependent immediately-early gene. Immediately after a 60-min exposure to a familiar environment, the spine densities of Arc-positive and Arc-negative neurons were differently distributed. This density imbalance was due exclusively to changes in the number of small, rather than large, spines. The change disappeared within 60 min after mice were returned to the home cages. Thus, spines possess the ability to rapidly and reversibly alter their morphology in response to a brief environmental change. We propose that these transient spine dynamics represent a latent preliminary stage for longer-term plasticity on demand.

## Introduction

Dendritic spines are tiny ($\sim$0.1 $\mu m^3$) protrusions arising from neuronal dendritic shafts. They express glutamate receptors on their surface (Nusser *et al.*, 1998; Takumi *et al.*, 1999; Matsuzaki *et al.*, 2001) and are the major postsynaptic component of excitatory synapses in the CNS (Beaulieu & Colonnier, 1985; Harris & Stevens, 1989). The spines are structurally diverse and undergo activity-dependent morphological changes. The induction of long-term potentiation is associated with long-lasting enlargement and *de novo* emergence of spines (Engert & Bonhoeffer, 1999; Matsuzaki *et al.*, 2004), while long-term depression leads to spine shrinkage and retraction (Nagerl *et al.*, 2004; Zhou *et al.*, 2004). The morphological alterations are linked to functional changes, such as postsynaptic current size and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor content (Matsuzaki *et al.*, 2004). Because of their flexible dynamics, the spine reorganization is hypothesized to be pivotal for the tuning of network connectivity that underlies learning and memory. This notion, however, has so far been supported only partially by *in vitro* studies; rapid spine changes that occur within several hours have not been described *in vivo*, although chronic or long-lasting experiences of animals are well known to affect the spine morphology (Turner & Greenough, 1985; Moser *et al.*, 1994, 1997; Geinisman *et al.*, 2001; Leuner *et al.*, 2003; Stranahan *et al.*, 2007).

We recently reported, for the first time, a rapid form of spine reorganization that takes place *in vivo* within 60 min during exposure to a novel enriched environment (Kitanishi *et al.*, 2009). This finding was made by histological experiments designed to combine two imaging techniques, i.e. (i) by visualizing the structure of spines in brain sections from mice expressing membrane-targeted green fluorescent protein (mGFP) (Thy1-mGFP transgenic mice), and (ii) by dividing CA1 neurons into two subsets based on expression of activity-regulated cytoskeletal-associated protein (Arc, also called Arg3.1). Arc is an immediate-early gene induced by intense neuronal activity (Link *et al.*, 1995; Lyford *et al.*, 1995) and, hence, its expression works as a putative cellular marker of neuronal activity prior to death (Guzowski *et al.*, 1999; Ramirez-Amaya *et al.*, 2005). Using this experimental design, we found that environmental exploration induces a rapid and spine size-selective change in spine density in Arc(+) pyramidal neurons in the hippocampal CA1 region. However, several important questions have to yet be clarified: (i) whether the reorganization is linked to learning of novel environments or simply reflects environmental changes; (ii) whether the sensitive fraction of spines is flexible in association with animal experience; and (iii) how long this change persists. To address these questions, in the present study, Thy1-mGFP mice were repeatedly challenged to the same environment, and the spine morphology between Arc(+) and Arc(−) neurons was compared.

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*Received 24 February 2009, revised 20 May 2009, accepted 23 June 2009*

## Materials and methods

### *Environmental change procedures*

Experiments were performed according to the guide for the care and use of laboratory animals of the University of Tokyo and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Behavioral procedures were performed as previously described (Kitanishi *et al.*, 2009). Male Thy1-mGFP mice (line 21, gift from Dr V. de Paola and Dr P. Caroni; De Paola *et al.*, 2003), which express mGFP in a small number of neurons (Richards *et al.*, 2005), were handled and were not exposed to a novel environment for at least 7 days before they were subjected to the experiments at 8–11 weeks old. Half of the Thy1-mGFP mice were placed in a new environment, i.e. a particular plastic cage (37D × 21W × 15H cm), that was larger than their home cages for 60 min. Five objects and four small food pellets were placed in the cage, and four distinct markings were displayed on the walls, all of which were absent in the home cages. This environmental exposure was repeated at a 23-h interval over a 6-day period. Immediately after the exposure on the final day, the animals were killed for histological inspections (ENV group). Age-matched littermates always remained in their home cages as a control (HC group). Like the ENV group, some mice were exposed to the environment for 6 days, but after the exposure on the final day they were returned to the home cages for 60 min and killed.

After the behavioral sessions, the mice were anesthetized by inhalation of diethyl ether and perfused transcardially with chilled phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. It took less than 10 min from the end of the behavioral sessions to the start of the transcardiac perfusion. The brains were removed, post-fixed overnight in the same fixative at 4°C, and coronally sectioned (ca. bregma −1.3 to −2.0 mm) at a thickness of 100 $\mu$m with a microslicer (ZERO 1; Dosaka, Kyoto, Japan).

### *Histological procedures*

Coronal sections were incubated in 0.2% Triton X-100 for 30 min and treated in 1% $H_2O_2$ diluted in PBS for 15 min. After blocking with 2% normal goat serum for 60 min, the sections were incubated in anti-Arc antibody (rabbit-polyclonal, 1 : 8000; Lyford *et al.*, 1995) for 48 h at 4°C, followed by anti-rabbit biotinylated secondary antibody (1 : 500; Vector Laboratories, Burlingame, CA, USA) containing NeuroTrace 435/455 blue-fluorescent Nissl stain (1 : 50; Molecular Probes, Eugene, OR, USA) 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, USA). All binding procedures were followed by three PBS washes.

### *Confocal microscopy*

Images of the dorsal hippocampal CA1 region were captured with a confocal microscope (LSM510; Zeiss, Oberkochen, Germany) equipped with 405-nm diode, 488-nm argon and 543-nm helium/neon lasers. To classify mGFP-positive pyramidal cells as Arc(+) or Arc(−), image stacks (1.0 $\mu$m thickness × 21 planes) of Arc, mGFP and Nissl from the pyramidal cell layer were collected using a 63× oil immersion objective (NA = 1.4). To reduce sampling bias or false-positive noise detection, mGFP-positive pyramidal neurons whose somata were located within 16 $\mu$m (7.8 ± 3.7 $\mu$m) from the slice surface were selected so as to yield sufficient mGFP fluorescence intensity and Arc immunoreactivity. All cells that satisfied the criteria were adopted from the entire CA1 middle-lateral subfields, because there were no apparent differences in Arc expression and spine morphology along CA1a, CA1b or CA1c. The magnified images of basal dendrites of mGFP-positive pyramidal cells were collected at a *Z*-stack interval of 0.25 $\mu$m (33 planes) with 3 × digital zoom (0.05 $\mu$m/pixel). To avoid interference from dendrites of other mGFP-positive neurons, dendritic segments that were not spatially isolated from the nearest dendrites were excluded from the subsequent analyses. In this line of Thy1-mGFP mice, the expression of mGFP was nearly an all-or-none fashion among neurons, and the fluorescence intensity was also invariant among mGFP-positive cells (Richards *et al.*, 2005). In addition, to minimize the possible invisibility of the fluorescence in deeper sections, only dendritic segments close to the surface (typically ≤ 15 $\mu$m) were analysed. Neither unexpected fluorescence variations nor sampling bias was likely to affect our spine morphometry. The point spread function was estimated with a 175-$\mu$m-diameter bead (PS-Speck Microscope Point Source Kit component B; Molecular Probes). Its full widths at the half maximum on the horizontal and vertical axes were 0.26 ± 0.01 and 1.01 ± 0.08 $\mu$m, respectively. To calculate the percentage of Arc(+) neurons, a 20× objective (NA = 0.5) was used to broadly image the CA1 region.

### *Image analyses*

The confocal mGFP images were processed by medial filtration and deconvolution with the nearest-neighbor method (Koh *et al.*, 2002) using METAMORPH software (Molecular Devices, Downingtown, PA, USA). Spine detection and measurements were performed semi-automatically by the NEURONSTUDIO software (Rodriguez *et al.*, 2006, 2008). The NEURONSTUDIO operates on serially sectioned confocal images in a three-dimensional algorithm, and thus it resolves the stereoscopic metrics of spines, which are unmeasurable with conventional two-dimensional tools. Furthermore, the system works more accurately and faster than the existing tools. Once the starting points of dendritic tracing are manually determined, dendritic shafts are automatically detected. Because thick shafts near the soma were occasionally detected in error, these errors were manually corrected by eye. The dendritic segments on the top and the bottom confocal planes were excluded from the analysis because spines could be truncated. Then, the individual spines were automatically detected. Erroneous detection, such as short dendritic branches and optical noise, was manually corrected. Finally, very closely located spines were not separable with NEURONSTUDIO and thus manually corrected. These manual corrections were carefully carried out by referring to their *xz* and *yz* views in addition to the *xy* view. Then, several morphological parameters of individual spines (diameter of a head, diameter of a neck, length), dendritic shafts (length and position) and the soma (position) were morphometrically measured with the same software. The spine density was defined as the number of spines per micrometer along the dendrite longitudinal axis. The spine shape was classified into three groups, i.e. mushroom spine, thin spine and stubby spines, according to the criteria proposed in Harris *et al.* (1992). In brief, a spine with the head diameter being >0.4 $\mu$m and the ratio of the head diameter to the neck diameter being ≥ 1.1 was defined as a mushroom spine. In the remaining spines, a spine with the total length being ≥ 1.0 $\mu$m was defined as a thin spine. The other spines were defined as stubby. To obtain a sufficient data number ($n \geq 10$ for all 10-$\mu$m bins) in the same dendritic location, data were collected from spines on basal dendrites within 20–50 $\mu$m of the soma, and each 10-$\mu$m length along the longitudinal axis of the focused dendrite was counted as a dendritic segment (referred here to as *n*). Tissue shrinkage was not corrected.

We validated the outputs automatically generated by NEURONSTUDIO with three independent assessments. First, the head sizes of the identical spines were compared between those measured by NEURONSTUDIO and manual measurement (Kitanishi *et al.*, 2009), and were found to show a significant linear relationship (Supporting information, Fig. S1). Second, spines three-dimensionally reconstructed by NEURONSTUDIO had no apparent spatial bias in their density, head sizes, lengths or stem angles (supporting Fig. S2). Third, the number of the detected spines and head-size and length distributions were not significantly affected by multiplying the pixel intensity of the original image or by adding dot noise to the image, which mimicked a possible variability in mGFP brightness and noise of a photon multiplier tube, respectively (supporting Fig. S3). Thus, the NEURONSTUDIO data were not only accurate and reproducible, but also robust and noise-resistant, thus being enough to analyse in the following study.

To separate Arc(+) and Arc(−) neurons, the threshold intensity of the Arc signal was automatically determined with the METAMORPH software. Then, when more than one-third of the soma area determined by Nissl stain was covered by pixels with the signal intensity greater than the threshold value, the cell was defined as an Arc(+) neuron. All of the 13 mGFP(+) HC neurons examined for spine morphometry in this study were Arc(−). The Nissl-positive cells in a pyramidal cell layer, ranging from 72 to 275 (153 ± 50) cells/animal, were defined as the total neuron population used to calculate the percentage of Arc(+) neurons. The classification of Arc expression and the spine analyses were performed independently and blind to the experimental conditions.

### *Statistics*

To test the significance of the bidirectional disparity in spine density distributions between the Arc(+) and Arc(−) populations across the HC baseline, we measured the maximum differences between the normalized cumulative distribution functions of spine density, i.e. $D_1$ [the maximal difference between the Arc(+) and HC distributions] and $D_2$ [the maximal difference between the HC and Arc(−) distributions], and calculated the Euclidean distance $D \equiv \sqrt{D_1^2 + D_2^2}$ as an index of bidirectional disparity. The significance of the observed $D$ value was determined based on the statistical population distribution estimated from 1000 surrogate data. The surrogates were made by randomly shuffling the observed spine density across the Arc(+), Arc(−) and HC groups with maintaining the total number of dendritic segments in each group. The chance $D$ values from surrogates were 0.20 ± 0.07. Immediately after the 6-day exploratory experiences (the ENV group), the $D$ value was 0.35 and significantly larger than the surrogates ($P$ = 0.02; Fig. 1). After 1-h rest in the home cages, the $D$ value was 0.15 and not significantly different from that of the surrogate ($P$ = 0.73; Fig. 3).

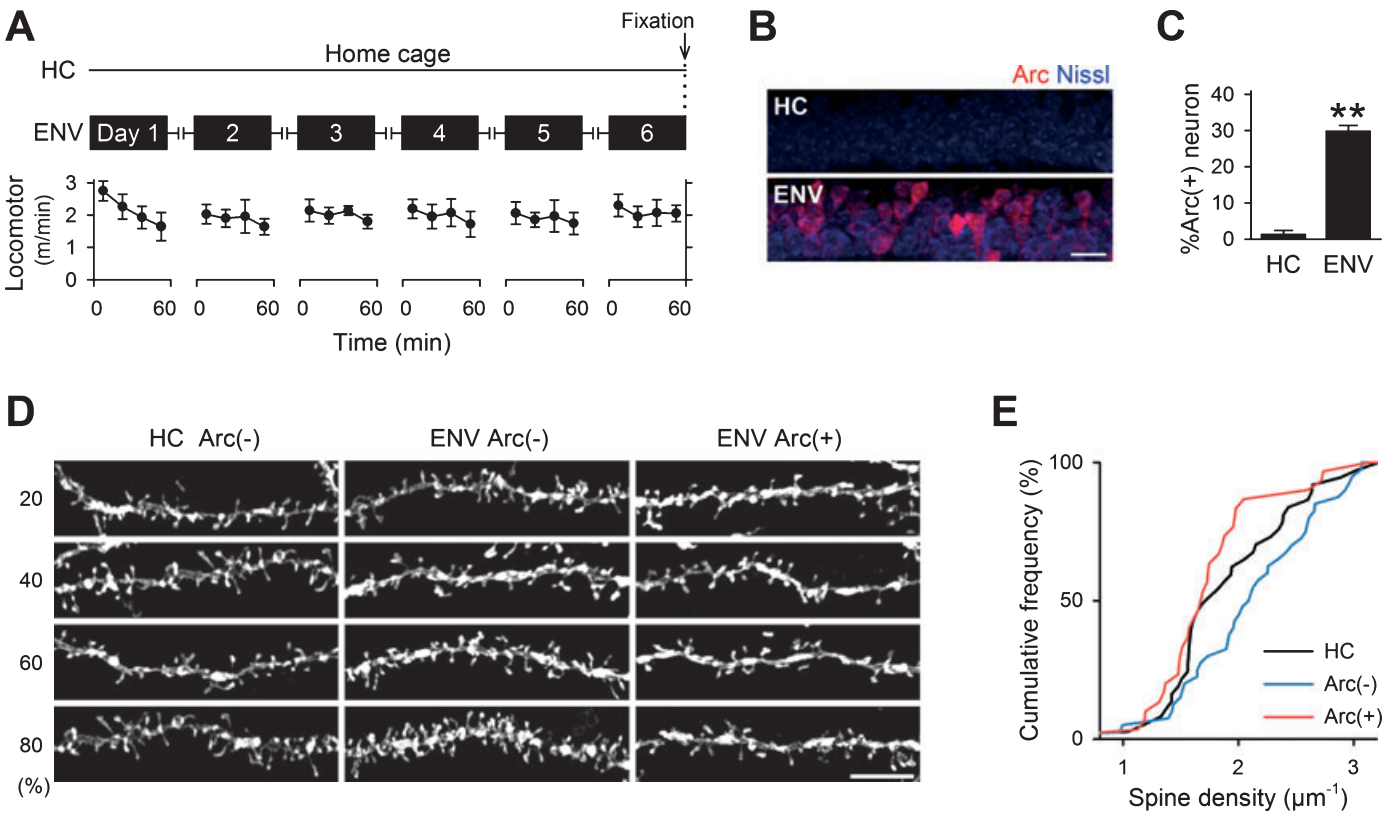


FIG. 1. Cell population-selective alterations in spine density after environment change. (A) Diagram of the experimental schedule. Thy1-mGFP mice were exposed to the same environment for 60 min per day for 6 days (the ENV group), whereas control mice were always kept in their home cages (the HC group). Mice were killed immediately after the behavioral session on Day 6. The bottom plot indicates the locomotor activity of the ENV-group mice during the environmental exposures. Data are shown as means ± SD of 8 mice. (B) Confocal immunohistochemical images for Arc (red) in the hippocampal CA1 pyramidal cell layer counterstained with blue-fluorescent Nissl stain. Scale bar: 20 μm. (C) Percentage of Arc(+) neurons to the total neurons in the pyramidal cell layer. **$P$ < 0.01, Welch's test. Mean ± SEM of 9 mice (HC) and 14 mice (ENV). (D) Representative images of basal dendritic spines from the 20–80th percentile in spine density visualized with mGFP fluorescence. Scale bar: 5 μm. (E) Cumulative spine density in cell populations. $n$ = 37 (HC), 40 [ENV-Arc(−)] and 30 dendritic segments [ENV-Arc(+)]. $P$ < 0.01 between ENV-Arc(−) and ENV-Arc(+), Kolmogorov–Smirnov test.

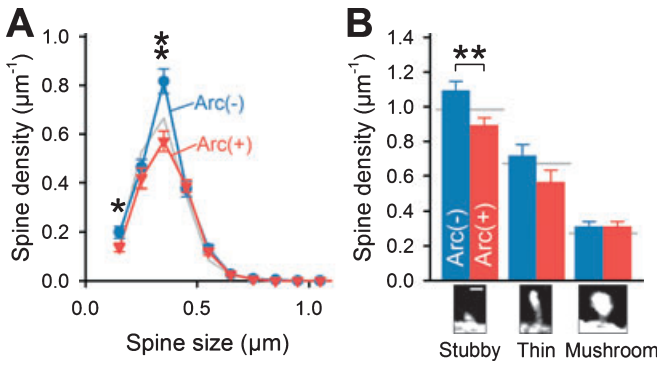


FIG. 2. Selective alterations in small stubby spines in response to environment change. (A) Distributions of spine-head size in Arc(+) and Arc(−) neurons of the ENV group. $*P < 0.05$, $**P < 0.01$ between Arc(−) and Arc(+), Student's *t*-test after repeated-measures two-way ANOVA. The gray line indicates the mean of the HC-group cells. (B) Distribution of stubby, thin and mushroom spines, which were classified based on their head size, neck diameter and length. The representative spine image in each category is shown at the bottom. $**P < 0.01$, Student's *t*-test after two-way ANOVA, mean ± SEM of 40 Arc(−) and 30 Arc(+) dendritic segments. Scale bar: 0.5 μm.

We report the averaged values as the means ± standard deviations in the text.

## Results

To investigate how natural experience affects the spine morphology of Arc(+) and Arc(−) neurons, we exposed Thy1-mGFP mice six times to the same environment for 1 h every 24 h (ENV group). Control mice remained in their home cages throughout the 6 days (HC groups; Fig. 1A). This procedure was aimed to make the mice accustomed to the environment. We already found that one-shot exposure to this environment is enough to induce the reduction in spine density in Arc(+) neurons (Kitanishi *et al.*, 2009), but the previous data cannot determine whether the effect results from a sudden exposure to a novel environment or from an environmental change *per se*, in other words, whether the effect reflects some type of learning or merely senses changes in the surrounding conditions.

To evaluate the environmental adaptation of the ENV-group mice, locomotor activity was monitored during the 60-min exposures. The locomotor was highest at the beginning of Day 1, decreased within 60 min and, thereafter, it was almost constant throughout the subsequent 5 days (Fig. 1A). This suggests that the mice were rapidly familiarized to the environment, and that on Day 6 the environment was no longer novel to the mice.

The ENV-group mice were killed immediately after the environmental exposure on Day 6, together with the HC-group mice. In the ENV group, Arc immunoreactivity was detected in 30.0 ± 5.5% of hippocampal CA1 neurons (2309 neurons were examined in total from 14 mice), whereas only 1.5 ± 2.5% (1500 neurons were examined in nine mice) were Arc(+) in the HC group (Fig. 1B and C). This Arc(+) expression ratio was comparable to the level seen after the single-shot exposure, as reported in our and other previous studies (Guzowski *et al.*, 1999; Ramirez-Amaya *et al.*, 2005; Kitanishi *et al.*, 2009). This suggests that, although the locomotor activity was reduced on Day 6, hippocampal neurons were still activated to a similar degree during the exploration.

In the hippocampal CA1 region of Thy1-mGFP mice, mGFP was sparsely expressed in a limited fraction of neurons, but their mGFP(+) dendrites were spatially overlapped with each other. To

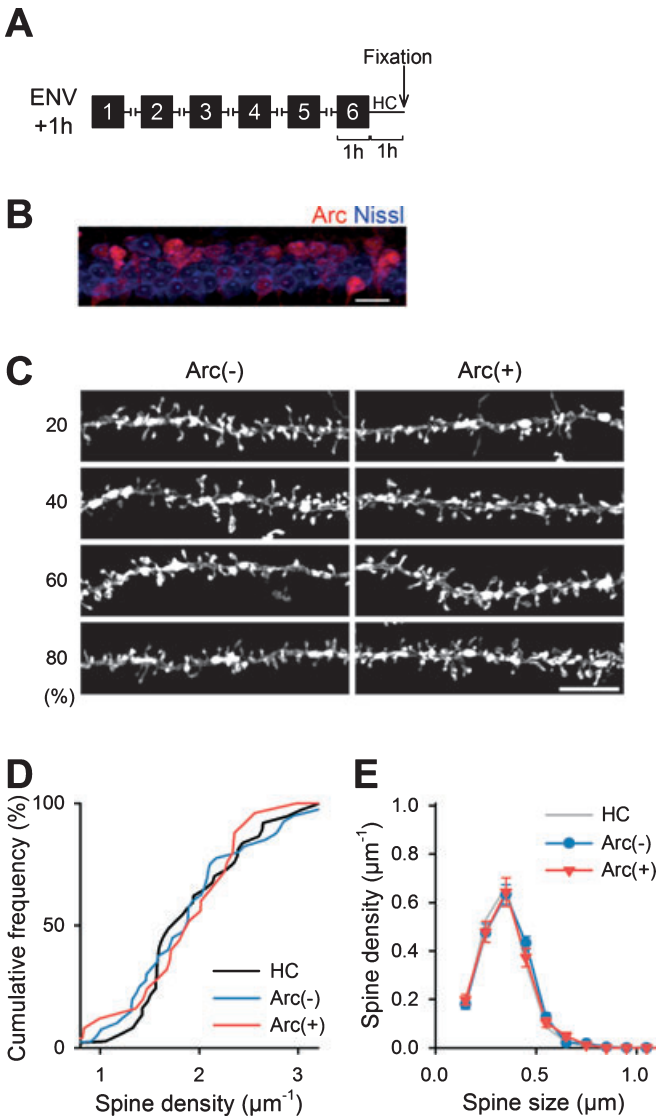


FIG. 3. Rapid reverse of morphological changes following return to home cage. (A) After the sixth exposure, mice were returned to their home cages. After 60 min, they were killed. (B) Immunohistochemistry for Arc. Scale bar: 20 μm. (C) Representative images of basal dendritic segments from the 20–80th percentile in spine density visualized with mGFP. Scale bar: 5 μm. (D) Cumulative spine density in cell populations. $n = 40$ Arc(−) and 25 Arc(+) dendritic segments. $P > 0.05$ between Arc(−) and Arc(+), Kolmogorov–Smirnov test. (E) Distributions of spine-head size. $P > 0.1$ by repeated-measures two-way ANOVA. Mean ± SEM.

reliably select mGFP(+) dendrites that arose from Arc(+) neurons and avoid interference from neighboring mGFP(+)/Arc(−) dendrites, we examined the spine morphology only in basal-dendrite segments close to the soma of mGFP(+) pyramidal cells. In three neuron groups, i.e. HC-Arc(−) cells, ENV-Arc(−) cells and ENV-Arc(+) cells, the spine density and morphology were three-dimensionally reconstructed and analysed with the automated software (Fig. 1D).

First, we found that Arc(+) neurons in the ENV-group had a lower spine density than ENV-Arc(−) neurons [1.7 ± 0.5 $\mu m^{-1}$ for Arc(+) and 2.1 ± 0.6 $\mu m^{-1}$ for Arc(−); Fig. 1E]. This is consistent with the change found after the single-shot exposure to a novel environment (Kitanishi *et al.*, 2009). We next compared these ENV-group data to

those of the control HC group (Fig. 1E). Interestingly, the distributions of the spine density of ENV-Arc(−) and ENV-Arc(+) neurons showed a bidirectional disparity from that of the HC neurons ($1.9 \pm 0.5\ \mu m^{-1}$ for HC, $P = 0.02$; see Materials and methods for statistics).

Because spines are diverse in their morphology, we measured the size and type of spines in the ENV group to determine the fraction that was responsive to the environmental exposure. A significant difference between Arc(−) and Arc(+) neurons was found only in relatively small spines with head sizes of less than 0.4 $\mu$m (Fig. 2A). This is also consistent with the result after the single-shot exposure (Kitanishi *et al.*, 2009). As for the spine types (Harris *et al.*, 1992), the difference was observed in stubby-type spines, but not in thin-type or mushroom-type spines (Fig. 2B). In contrast to the changes observed in small spines, we found no change in the density of large spines with head sizes more than 0.4 $\mu$m. This contrasted with the case of a single-shot exposure, found in our previous study, which showed that fractions of large and small spines increased and decreased, respectively, after a single environmental exposure (Kitanishi *et al.*, 2009). These results suggest that small and large spines are reorganized in a different manner along repeated behavioral experiences.

How long do the morphological differences last? Our findings can be interpreted in two different ways: (i) the spine reorganization induced by single-shot experience persists for many days; and (ii) the spine reorganization occurs whenever the environment changes, but the change reverses spontaneously. We found that the latter is the case. Thy1-mGFP mice were returned to their home cages after environmental exposure on Day 6 and killed 60 min later (Fig. 3A and B). In this group, Arc expression was observed in 26.5 ± 8.2% (1554 neurons were examined in total from 12 mice) of CA1 neurons, the ratio being similar to that of the ENV group (see Fig. 1B and C). However, no significant difference between Arc(−) and Arc(+) neurons was detected in either spine density (Fig. 3C and D) or spine size (Fig. 3E), nor was the spine density in either Arc(−) or Arc(+) neurons different from that in the HC group [$1.9 \pm 0.5\ \mu m^{-1}$ for Arc(+) and $1.9 \pm 0.6\ \mu m^{-1}$ for Arc(−), $P = 0.73$; for statistics see Materials and methods].

## Discussion

The present work has demonstrated that natural environmental changes rapidly induce spine reorganization in CA1 pyramidal cells. The reorganization is accompanied by the bidirectional Arc(−) vs. Arc(+) disparity.

Previous studies examining experience-dependent spine morphogenesis have focused mainly on very slow dynamics that ranges from days to months (Turner & Greenough, 1985; Moser *et al.*, 1994, 1997; Geinisman *et al.*, 2001; Leuner *et al.*, 2003; Stranahan *et al.*, 2007) and, hence, they might have overlooked rapid, transient forms of spine reorganization over tens of minutes. We captured them by taking advantage of the rapid kinetics of Arc expression and by separating cell populations according to Arc expression.

### *Size-selective spine reorganization and its association with spatial novelty*

The spine reorganization took place in small spines with exposure to a familiar environment. This is consistent with our previous result in a single-shot exposure to a novel environment (Kitanishi *et al.*, 2009). This suggests that small spines are consistently sensitive even to a familiar environment. In general, smaller spines are more dynamic, exhibiting greater head enlargement after the induction of long-term potentiation (Matsuzaki *et al.*, 2004), whereas large spines appear structurally stable, perhaps reflecting long-lasting steady memory (Trachtenberg *et al.*, 2002; Kasai *et al.*, 2003; Yasumatsu *et al.*, 2008).

We failed to find a difference in large spines. It is intriguing to find that an increase in large spines occurred in Arc(+) cells in our previous single-shot-exposure experiments (Kitanishi *et al.*, 2009). Because the present study employed the same behavioral procedures and the same mice line as before, the difference in reorganization is attributable to single or repeated environmental exposures. In other words, large spines exhibit reorganization selectively in response to novel environments, but no longer to familiar ones. It should be noted, however, that the present study does not completely rule out the possibility that large spines are still dynamic. Because our observation time points were limited by *post hoc* histochemical imaging, the detection sensitivity is lower than that of time-lapse imaging that can trace morphological changes in individual spines. In addition, spine turnover with constant spine density, if any (Holtmaat *et al.*, 2006), may make it difficult to detect the possible change. Time-lapse monitoring with *in vivo* deep-brain imaging techniques (Mizrahi *et al.*, 2004; Kuga *et al.*, 2008) would be required to address this issue.

### *On input layers into CA1 pyramidal cells*

Is the present finding a general or specific phenomenon across dendritic compartments? We focused on basal dendrites in the stratum oriens (s.o.), but the apical dendrites of CA1 pyramidal cells run through anatomically distinct layers, i.e. the stratum radiatum (s.r.) and stratum lacunosum-moleculare (s.l.m.). Although some behavioral tasks are known to affect spines both in s.o. and s.r. in a similar manner (Kozorovitskiy *et al.*, 2005; Restivo *et al.*, 2006; Stranahan *et al.*, 2007), growing in an enriched environment (Moser *et al.*, 1997) and the eye-blink conditioning task (Leuner *et al.*, 2003) affect the number of spines selectively in s.o., that is, spines in s.o. and s.r. that both receive inputs from CA3 pyramidal cells can be differentially regulated. This may result from different rules governing synaptic plasticity between s.o. and s.r. (Kaibara & Leung, 1993; Haley *et al.*, 1996). Thus, further studies on s.o. and s.r. spines, together with the present study, are necessary to reveal the possible spine function specific for dendritic compartments. In s.l.m., spine sizes are larger than those in s.r. and s.o. (Megías *et al.*, 2001). It would also be important to clarify whether those large sizes are capable of showing dynamic reorganization in relation to behavioral exploration.

### *Mechanisms of spine reorganization*

It has not been determined whether Arc itself mediates spine reorganization. Arc induction depends on *N*-methyl-D-aspartate (NMDA) receptor activation (Steward & Worley, 2001). In addition, Arc interacts with actin cytoskeleton and the endocytic machinery in spines, and mediates AMPA-receptor trafficking (Chowdhury *et al.*, 2006; Shepherd *et al.*, 2006). These pieces of evidence imply the involvement of Arc in the regulation of spine shapes, because the spine shapes are primarily regulated by actin cytoskeleton (Fischer *et al.*, 1998; Honkura *et al.*, 2008), and AMPA-receptor trafficking is also tightly associated with spine morphology (Kopec *et al.*, 2007). There is, however, a contradictory report showing that Arc-knockout

mice did not show any changes in the density and length of spines (Plath *et al.*, 2006). Therefore, spine reorganization and Arc expression could be parallel phenomena that partially share the same signal transduction. Another possibility to explain this contradiction is that Arc mediates synapse competition across neurons, an effect that is undetected in Arc-knockout mice and may also explain the distribution disparity between Arc(+) and Arc(−) cells observed in this study.

### *Biological functions of transient spine reorganization*

It is striking that the spine reorganization rapidly occurred during a 60-min period of exploration, but disappeared within 60 min after the return to the home cages. Two interpretations can account for this observation: (i) spines showed transient reorganizations; and (ii) the once evoked spine reorganization indeed persisted for the 60-min return to the home cages, but it was technically undetectable merely because of a time-dependent drift of Arc-expressing cell populations. We consider that the former is the case because Arc-protein expression lasts 2 h after environmental changes (Ramirez-Amaya *et al.*, 2005) and, after return to the home cage, the Arc induction occurs almost exclusively in the same cell population as that already expressed Arc during the environment exploration (Marrone *et al.*, 2008). In the present study, therefore, the Arc-expressing cell population was thought to be the same between the mice killed immediately and 60 min after the environmental challenge. This favors that the disappearance of the spine responses reflects a spontaneous recovery process, rather than observations of different Arc(+) populations.

What is the functional significance of this transient spine change? In our experimental paradigm, mice were not forced to acquire some type of memory, e.g. food location, social members and electric shock but, in general, the environmental changes are a sign for situations where memory is possibly needed. We speculate that the spine reorganization during a brief environmental change represents an extra readiness for associative-memory formation, which can be consolidated into a long-lasting form, e.g. through protein synthesis-dependent mechanisms on demand (Tanaka *et al.*, 2008).

## Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Comparison of measurement methods of spines.

Fig. S2. Validation of the automated three-dimensional spine detection.

Fig. S3. Robustness of the automated spine detection against image noise.

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## Acknowledgements

We thank Dr Vincenzo de Paola (Imperial College London) and Dr Pico Caroni (Friedrich Miescher Institute) for supplying Thy1-mGFP TG mice, and Dr Paul F. Worley (Johns Hopkins University School of Medicine) for anti-Arc antibody.

## Abbreviations

AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; Arc/Arg3.1, activity-regulated cytoskeletal-associated protein; ENV, mice killed immediately after the sixth environmental exposure; HC, home-caged control mice; mGFP, membrane-targeted green florescent protein; PBS, phosphate-buffered saline; s.l.m., stratum lacunosum-moleculare; s.o., stratum oriens; s.r., stratum radiatum.

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