Functional Connectivity of Multiple Brain Regions Required for the Consolidation of Social Recognition Memory

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Social recognition memory is an essential and basic component of social behavior that is used to discriminate familiar and novel animals/humans. Previous studies have shown the importance of several brain regions for social recognition memories; however, the mechanisms underlying the consolidation of social recognition memory at the molecular and anatomic levels remain unknown. Here, we show a brain network necessary for the generation of social recognition memory in mice. A mouse genetic study showed that cAMP-responsive element-binding protein (CREB)-mediated transcription is required for the formation of social recognition memory. Importantly, significant inductions of the CREB target immediate-early genes *c-fos* and *Arc* were observed in the hippocampus (CA1 and CA3 regions), medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), and amygdala (basolateral region) when social recognition memory was generated. Pharmacological experiments using a microinfusion of the protein synthesis inhibitor anisomycin showed that protein synthesis in these brain regions is required for the consolidation of social recognition memory. These findings suggested that social recognition memory is consolidated through the activation of CREB-mediated gene expression in the hippocampus/mPFC/ACC/amygdala. Network analyses suggested that these four brain regions show functional connectivity with other brain regions and, more importantly, that the hippocampus functions as a hub to integrate brain networks and generate social recognition memory, whereas the ACC and amygdala are important for coordinating brain activity when social interaction is initiated by connecting with other brain regions. We have found that a brain network composed of the hippocampus/mPFC/ACC/amygdala is required for the consolidation of social recognition memory.

Key words: brain network; consolidation; CREB; IEGs; social recognition memory

Significance Statement

Here, we identify brain networks composed of multiple brain regions for the consolidation of social recognition memory. We found that social recognition memory is consolidated through CREB-mediated gene expression in the hippocampus, medial prefrontal cortex, anterior cingulate cortex (ACC), and amygdala. Importantly, network analyses based on *c-fos* expression suggest that functional connectivity of these four brain regions with other brain regions is increased with time spent in social investigation toward the generation of brain networks to consolidate social recognition memory. Furthermore, our findings suggest that hippocampus functions as a hub to integrate brain networks and generate social recognition memory, whereas ACC and amygdala are important for coordinating brain activity when social interaction is initiated by connecting with other brain regions.

Introduction

Social behaviors contain multiple components including social approach, interaction, and recognition/discrimination (Thor

and Holloway, 1982; Colgan, 1983; Lai et al., 2005; Gabor et al., 2012). In particular, social recognition memory is an essential and basic component of social behavior used to discriminate fa-

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Figure 1. Exposure to a juvenile mouse for 3 min, but not 1 min, generates LTM of social recognition. **A, B,** Mice exposed to a juvenile male mouse twice for a long (3 min) or short (1 min) period with an interval of 24 h. **A,** Recognition index. * $p < 0.05$ compared with the 1 min group. **B,** Comparisons of social investigation time. 3 min group, $n = 14$; 1 min group, $n = 14$. * $p < 0.05$ compared with the first exposure (paired t test). **C,** Mice exposed to a juvenile male mouse for 3 or 1 min and 24 h later, exposed to a familiar or novel juvenile for 3 min. * $p < 0.05$ compared with the first exposure (paired t test). 3 min–3 min: familiar group, $n = 12$; novel group, $n = 12$; 1 min–3 min: familiar group, $n = 12$; novel group, $n = 12$. Error bars indicate SEM.

miliar and novel animals/humans (Berry and Bronson, 1992; Jiming et al., 1994; Kogan et al., 2000).

Memory consolidation is the process underlying the formation of a long-term memory (LTM) by stabilizing a labile short-term memory (STM) (Squire et al., 1995; Dudai, 1996; McGaugh, 2000). A critical biochemical feature of memory consolidation is the requirement for new gene expression (Flexner et al., 1965; Davis and Squire, 1984; Abel et al., 1997; Silva et al., 1998; Martin et al., 2000; McGaugh, 2000). Importantly, the gene expression necessary for consolidation is activated by transcription factor cAMP-responsive element-binding protein (CREB)-mediated transcription, which is known as a master regulator of neural activity-dependent transcription (Bourtchuladze et al., 1994; Kida et al., 2002; Pittenger et al., 2002; Josselyn et al., 2004; Kitamura et al., 2012).

CREB activates the expression of the immediate-early genes (IEGs) *c-fos* and *Arc* in an activity- and learning-dependent manner (Sheng et al., 1990; Abraham et al., 1993; Worley et al., 1993; Guzowski et al., 1999; Montag-Sallaz et al., 1999; Guthrie et al., 2000; Kaczmarek and Robertson, 2002; Kawashima et al., 2009). Importantly, abundant studies have shown that brain regions showing learning-induced IEG expression play essential roles in gene-expression-dependent memory processes including consolidation, reconsolidation, and extinction (Morrow et al., 1999; Santini et al., 2004; Mamiya et al., 2009; Zhang et al., 2011; Fukushima et al., 2014). Therefore, the expression of IEGs has been widely accepted as a marker to identify brain regions that are activated in response to learning or memory retrieval (Guzowski et al., 2001; Frankland et al., 2004; Frankland et al., 2006; Mamiya et al., 2009; Zhang et al., 2011; Fukushima et al., 2014).

Previous studies have shown that multiple brain regions regulate social behaviors and the formation of social recognition memory. The hippocampus, amygdala, and anterior cingulate cortex (ACC) are critical regions for the formation/consolidation of social recognition memory in mice (Kogan et al., 2000; Suzuki et al., 2011; Hitti and Siegelbaum, 2014; Garrido Zinn et al., 2016). Conversely, the medial prefrontal cortex (mPFC) and amygdala are involved in the regulation of social behaviors such as social interaction and approach. However, distinct roles for multiple brain regions in the consolidation of social recognition memory and connectivity of these regions to generate this memory remain unclear.

In the social recognition memory task in mice, an adult mouse is allowed to recognize a juvenile mouse through investigations of the juvenile mouse (Fukushima et al., 2008; Suzuki et al., 2011; Nomoto et al., 2012; Ishikawa et al., 2014; Inaba et al., 2016b).

The difference in social investigation times between the first and second exposures to a juvenile mouse reflects the familiarity of the two mice. In this study, to understand the mechanisms for the consolidation of social recognition memory at the anatomical level, we first clarified the brain regions required for the consolidation of social recognition memory by analyzing the expression of IEGs and then examining the effects of inhibiting gene expression on this process. Finally, we examined interregional functional connectivity using network analysis to gain further insight into how these brain regions work as a network to generate social recognition memory.

Materials and Methods

Animals. All experiments were conducted according to the *Guide for the Care and Use of Laboratory Animals* (Japan Neuroscience Society and Tokyo University of Agriculture). All animal experiments performed in this study were approved by the Animal Care and Use Committee of Tokyo University of Agriculture (authorization #250008). All surgical procedures were performed under Nembutal anesthesia and every effort was made to minimize suffering. Male C57BL/6N mice were obtained from Charles River Laboratories. Transgenic mice expressing an inducible CREB repressor (CREB^{IR} mice) were backcrossed to C57BL/6 (Kida et al., 2002; Suzuki et al., 2008; Mamiya et al., 2009; Fukushima et al., 2014). The mice were housed in groups of five or six, maintained on a 12 h light/dark cycle, and allowed *ad libitum* access to food and water. The mice were at least 8 weeks of age at the start of the experiments and all behavioral procedures were conducted during the light phase of the cycle. All experiments were conducted blinded to the treatment condition of the mice.

Social recognition task. The social recognition test was performed as described previously (Kogan et al., 2000; Fukushima et al., 2008; Suzuki et al., 2011; Nomoto et al., 2012; Ishikawa et al., 2014; Inaba et al., 2016b). Adult mice were placed into individual plastic cages in an experimental room under dim light. The cages were identical to those in which the mice were normally housed (plastic, 30 × 17 × 12 cm). After a period of 60 min, a juvenile mouse was placed into the cage with a subject for a first-exposure trial lasting 0, 1, or 3 min. The duration of social investigation behavior exhibited by the adult mouse was determined with a hand-held stopwatch. Social investigation was measured as described previously (Thor and Holloway, 1982). Memory was reassessed 2 h (STM) or 24 h (LTM) later by recording the length of investigation time exhibited by the subject to a familiar (same) or novel (new) juvenile (second exposure). To evaluate the differences of ability to form social memory between the groups of mice, we calculated a recognition index: the ratio of the duration of the second and first investigation times.

For the first experiment (Fig. 1A,B), we investigated the training condition that allows mice to form social recognition memory. Mice were exposed to a juvenile mouse twice for 1 or 3 min at an interval of 24 h.

Figure 2. CREB-mediated transcription is required for the formation of social recognition LTM. **A, B**, Social recognition STM. WT/p.o., $n = 6$; WT/TAM, $n = 6$; CREB^{IR}/p.o., $n = 6$; CREB^{IR}/TAM, $n = 6$. **C, D**, Social recognition LTM. WT/p.o., $n = 12$; WT/TAM, $n = 14$; CREB^{IR}/p.o., $n = 12$; CREB^{IR}/TAM, $n = 12$. **A, C**, Recognition index. * $p < 0.05$ compared with the other groups. **B, D**, Investigation time. * $p < 0.05$ compared with the first exposure (paired t test). Error bars indicate SEM.

For the second experiment, we investigated whether 1 min exposure to a juvenile mouse enables the generation of social recognition memory (Fig. 1C). The mice were exposed to a juvenile mouse for 3 min or 1 min at the first exposure and, 24 h later, were exposed to a familiar or novel juvenile mouse for 3 min (second exposure).

For the third experiment (Fig. 2), transgenic mice were used that express an inducible CREB repressor (CREB^{IR}) in the forebrain, where a dominant-negative CREB protein is fused with the ligand-binding domain of a mutant estrogen receptor. As described previously, systemic injection of tamoxifen into these transgenic mice inhibits CREB activity in the forebrain (Kida et al., 2002). CREB^{IR} and wild-type (WT) mice were administered an intraperitoneal injection of 16 mg/kg 4-hydroxytamoxifen (TAM; Sigma-Aldrich), which was dissolved in 10 ml of peanut oil (p.o; Sigma-Aldrich), or vehicle (VEH; a similar volume of peanut oil) at 6 h before the first exposure (Kida et al., 2002; Suzuki et al., 2008; Mamiya et al., 2009; Fukushima et al., 2014). At 2 or 24 h after the first exposure session, the mice received the second exposure.

For the fourth experiment (immunohistochemistry of c-fos and Arc; Figs. 3, 4), the mice were divided into 4 groups: 3, 1, and 0 min groups were exposed to a juvenile mouse for 3, 1, or 0 min, respectively (the 0 min group was placed into individual plastic cages, but not exposed to a juvenile mouse). These groups were perfused at 90 min after exposure. The home cage group was left undisturbed in their home cage throughout the experiment and anesthetized, as above, after they were taken from their home cages.

For the fifth experiment (Figs. 5, 6), the mice were trained as described above and received a microinfusion of the protein synthesis inhibitor anisomycin (ANI, 62.5 μ g; Sigma-Aldrich) or artificial CSF (ACSF) into the hippocampus, mPFC, ACC, or amygdala immediately after the first exposure. At 2 or 24 h after the first exposure session, the mice received the second exposure. ANI was dissolved in vehicle solution (ACSF) and adjusted to pH 7.0–7.4 with NaOH.

Immunohistochemistry. Immunohistochemistry was performed as described previously (Mamiya et al., 2009; Suzuki et al., 2011; Zhang et al., 2011; Fukushima et al., 2014; Inaba et al., 2015 and 2016a). After anes-

thetization, all mice were perfused with 4% paraformaldehyde. Brains were then removed, fixed overnight, transferred to 30% sucrose, and stored at 4°C. Coronal sections (30 μ m) were cut in a cryostat. The sections were pretreated with 4% paraformaldehyde for 20 min and 3% H₂O₂ in methanol for 1 h, followed by incubation in blocking solution (PBS plus 1% goat serum albumin, 1 mg/ml bovine serum albumin, and 0.05% Triton X-100) for 3 h at 4°C. Consecutive sections were incubated with a polyclonal rabbit primary antibody for anti-c-fos (1:5000; Millipore catalog #PC38, RRID: AB_2106755) or anti-Arc (1:1000; Santa Cruz Biotechnology catalog #sc-15325, RRID: AB_634092) in the blocking solution for 2 nights at 4°C. Subsequently, the sections were washed with PBS and incubated for 4 h at room temperature with biotinylated goat anti-rabbit IgG (SAB-PO kit; Nichirei Biosciences), followed by 1 h at room temperature in the streptavidin-biotin-peroxidase complex (SAB-PO kit). Immunoreactivity was detected with a DAB substrate kit (Nichirei Biosciences). Structures were anatomically defined according to the atlas of Franklin and Paxinos (1997). Quantification of c-fos- or Arc-positive cells in sections (100 $\times$ 100 μ m) of the olfactory bulb (OB; bregma between +4.28 and +3.92 mm), mPFC (bregma between +2.10 and +1.98 mm), ACC (bregma between +0.8 and +1.0), medial preoptic area (MPOA; bregma between +0.14 and +0.02 mm), amygdala (bregma between -1.22 and -1.34 mm), dorsal hippocampus (bregma between -1.46 and -1.82 mm), visual cortex (VC; bregma between -3.88 and -4.00), temporal cortex (TC; bregma between -3.88 and -4.00), perirhinal cortex (PRh; bregma between -3.88 and -4.00), and entorhinal cortex (EC; bregma between -3.88 and -4.00) was performed using a computerized image analysis system (WinROOF version 5.6 software; Mitani). Immunoreactive cells were counted bilaterally with a fixed sample window across at least three sections by an experimenter blinded to the treatment condition. The number of c-fos- or Arc-positive cells in each group was expressed as the ratio of the averaged values in the home cage control group.

Surgery for drug microinfusion. Surgeries were performed as described previously (Suzuki et al., 2008; Mamiya et al., 2009; Suzuki et al., 2011; Zhang et al., 2011; Fukushima et al., 2014; Guimaraes et al., 2015; Inaba et al., 2015 and 2016a). Under Nembutal anesthesia and using standard

Figure 3. *c-fos* expression in distinct brain regions after exposure to a juvenile mouse. **A**, Representative immunohistochemical staining of CA1, PL, ACC, and BLA *c-fos*-positive cells from the indicated mice. Scale bar, 50 μ m. **B–H**, Number of *c-fos*-positive cells was measured after exposure to a juvenile mouse for 0, 1, or 3 min or remaining in the home cage. **B**, CA1, CA3, and DG regions of the hippocampus. **C**, PL and IL regions of the mPFC. **D**, ACC. **E**, LA, BLA, and CeA regions of the amygdala. **F**, OB. **G**, MPOA. **H**, VC, TC, PRh, and EC regions of the cortex. *c-fos* expression for each group is expressed as the ratio of the home cage group to the other groups. Home cage, $n = 12$; 0 min, $n = 11$; 1 min, $n = 12$; 3 min, $n = 12$. * $p < 0.05$ compared within the group. Error bars indicate SEM.

stereotaxic procedures, stainless steel guide cannulae (22 gauge) were implanted into the dorsal hippocampus (-1.8 mm, ± 1.8 mm, -1.9 mm), mPFC (2.7 mm, ± 0 mm, -1.6 mm), ACC (1.8 mm, ± 0 mm, -1.6 mm), or amygdala (-1.3 mm, ± 3.3 mm, -4.4 mm). The mice were allowed to recover for at least 1 week after surgery. After this, they were handled for 1 week before the commencement of the social recognition task. Infusions into the dorsal hippocampus, mPFC, ACC, or amygdala (0.5 μ l) were made at a rate of 0.25 μ l/min. This dose of locally infused ANI inhibits $>90\%$ of protein synthesis for at least 4 h (Rosenblum et al.,

1993). Cannula tip placements are shown in Figures 5I and 6I. Only mice with a cannula tip within the boundaries of the hippocampus, mPFC, ACC, or amygdala were included in the data analysis.

Network construction and graph theoretical analysis. Correlation matrices were generated using Pearson r values from interregional *c-fos* expression data at 0, 1, and 3 min (Fig. 7A). For comparisons of average correlations between groups, Pearson r values were Fisher z -transformed, statistics were calculated, and the data were retransformed back to r values. All interregional correlations between hippocampal, mPFC, ACC, or amygdala regions

Figure 4. Arc expression in distinct brain regions after exposure to a juvenile mouse. **A**, Representative immunohistochemical staining of CA1, PL, ACC, and BLA Arc-positive cells from the indicated mice. Scale bar, 50 μ m. **B–H**, Number of Arc-positive cells measured after exposure to a juvenile mouse for 0, 1, or 3 min or remaining in the home cage. **B**, CA1, CA3, and DG regions of the hippocampus. **C**, PL and IL regions of the mPFC. **D**, ACC. **E**, LA, BLA, and CeA regions of the amygdala. **F**, OB. **G**, MPOA. **H**, VC, TC, PRh, and EC regions of the cortex. Arc expression for each group is expressed as the ratio of the home cage group to the other groups. Home cage, $n = 12$; 0 min, $n = 12$; 1 min, $n = 12$; 3 min, $n = 12$. * $p < 0.05$ compared within the group. Error bars indicate SEM.

were used to assess time-dependent changes in functional connectivity (Fig. 7D–K).

Graph theoretical analysis was used to characterize the social recognition memory networks. Networks were constructed by thresholding correlations of interregional c-fos counts at an uncorrected significance level of $p < 0.05$ to generate unweighted adjacency matrices for the 0, 1, and 3 min groups. The centrality measures of degree and betweenness were calculated and normalized to the maximum and minimum values for each individual network. Clusters were identified through enumeration of all potential community structures and finding the configuration that optimized the

modularity (Newman and Girvan, 2004). Measures of within-community (within-module z-scores) and between-community (participation coefficient) connectivity were calculated as defined in Guimerà and Amaral (2005). All graph theoretical analysis was performed in R (version 3.2.2) using the igraph (version 1.0.1; Csardi and Nepusz, 2006) and brainGraph (version 0.62.0) packages. Graph visualization was performed using Cytoscape (version 3.2.1; Shannon et al., 2003).

Data analysis. One-way or two-way factorial ANOVA followed by a *post hoc* Newman–Keuls comparison were used to analyze the effects of time, genotype, group, and drug. A paired *t* test was used to analyze the

Figure 5. Protein synthesis in the hippocampus, mPFC, ACC, and amygdala is not required for social recognition STM. **A, B**, Protein synthesis inhibition in the hippocampus. VEH, $n = 12$; ANI, $n = 12$. **C, D**, mPFC. VEH, $n = 12$; ANI, $n = 11$. **(E, F)** ACC. VEH, $n = 12$; ANI, $n = 12$. **G, H**, amygdala. VEH, $n = 11$; ANI, $n = 11$. **A, C, E, G**, Recognition index. * $p < 0.05$ compared with the VEH group. **B, D, F, H**, Comparisons of social investigation time. * $p < 0.05$ compared with the first exposure (paired t test). Error bars indicate SEM. **I**, Cannula tip placements.

differences in social investigation times within each group between the first and second exposure in the social recognition task. All values in the text and figure legends represent the mean $\pm$ SEM.

Results

Formation of social recognition memory after exposure to a juvenile mouse for 3 min, but not 1 min

We first investigated whether the training conditions used in this study enabled mice to form social recognition memory. Similar to our previous studies (Fukushima et al., 2008; Suzuki et al., 2011; Nomoto et al., 2012; Ishikawa et al., 2014; Inaba et al., 2016b), adult male mice were exposed to a juvenile male mouse twice for a long period (3 min) or a short period (1 min) at an interval of 24 h and the time taken to investigate the juvenile during the first (training) and second (test) exposures was assessed. To evaluate the strength of social recognition memory, we assessed the recognition index (Fukushima et al., 2008; Suzuki et al., 2011), the ratio of the second and first investigation time. One-way ANOVA followed by a *post hoc* Newman–Keuls test

revealed significant effects of exposure time (Fig. 1A, $F_{(1,26)} = 12.147$, $p < 0.05$). The 3 min group showed a significantly better recognition index than the 1 min group (Fig. 1A, $p < 0.05$), suggesting that the 3 min group formed a stronger social recognition memory than the 1 min group. Consistently, the 3 min group, but not the 1 min group, showed a significant decrease in social investigation time at the second exposure compared with the first exposure (Fig. 1B; paired t test, $p < 0.05$), suggesting that only the 3 min group formed a social memory. These results suggest that mice form a social recognition memory when they are exposed to a juvenile mouse for 3 min, but not for 1 min.

However, the possibility remained that a short exposure (1 min) to a juvenile mouse is sufficient to generate a social memory, but the social memory is masked because the 1 min exposure is too short to allow us to identify a decrease in social investigation time at the second exposure. To address this possibility, similar to the previous experiment (Fig. 1A, B), adult mice were exposed to a juvenile male mouse for 3 min or 1 min at the first

Figure 6. Protein synthesis in the hippocampus, mPFC, ACC, or amygdala is required for social recognition LTM. **A, B**, Protein synthesis inhibition in the hippocampus. VEH, $n = 12$; ANI, $n = 12$. **C, D**, mPFC. VEH, $n = 12$; ANI, $n = 10$. **E, F**, ACC. VEH, $n = 14$; ANI, $n = 13$. **G, H**, Amygdala. VEH, $n = 11$; ANI, $n = 12$. **A, C, E, G**, Recognition index. * $p < 0.05$ compared with the VEH group. **B, D, F, H**, Comparisons of social investigation time. * $p < 0.05$ compared with the first exposure (paired t test). Error bars indicate SEM. **I**, Cannula tip placements.

exposure, but were exposed to the familiar or novel juvenile male mouse for 3 min, but not 1 min, at 24 h after the first exposure (second exposure). Consistent with the previous observation, mice exposed to a juvenile mouse for 3 min at the first exposure showed a significant decrease in investigation time at the second exposure when they were exposed to a familiar, but not novel, mouse (Fig. 1C, $p < 0.05$) and, importantly, showed significantly less investigation time for a familiar mouse compared with a novel mouse, indicating that 3 min groups formed a social recognition memory and discriminated familiar and novel mice. In contrast, the 1 min group showed comparable investigation time to familiar and novel mice even when they were exposed to the mice for 3 min at the second exposure, indicating that the 1 min group failed to discriminate familiar and novel mice. These results suggest that a 1 min exposure to a juvenile mouse is insufficient to allow mice to form a social recognition memory. According to these findings, we used the 1 min group as a negative control that was unable to form a social recognition memory when exposed to a juvenile mouse.

Role of CREB-mediated transcription in the consolidation of social recognition memory

CREB-mediated transcription is required for the consolidation of fear, conditioned taste avoidance, and spatial memories (Kida et al., 2002; Josselyn et al., 2004; Kitamura et al., 2012). To understand the molecular mechanisms underlying the consolidation of social recognition memory, we examined the effects of loss-of-CREB function using CREB^{IR} transgenic mice (Kida et al., 2002). CREB^{IR} and WT littermate control mice were exposed to a juvenile male mouse twice for 3 min at an interval of 2 h (STM) or 24 h (LTM). Six hours before the first exposure, the mice received a systemic injection of TAM or VEH to inhibit CREB activity (Kida et al., 2002).

Two-way ANOVA comparing recognition index of STM revealed no significant effect of genotype (WT vs CREB^{IR}) or drug (VEH vs TAM) and no genotype versus drug interaction (Fig. 2A, genotype, $F_{(1,20)} = 0.775$, $p > 0.05$; drug, $F_{(1,20)} = 0.023$, $p > 0.05$; genotype vs drug, $F_{(1,20)} = 0.123$, $p > 0.05$), suggesting that TAM-injected CREB^{IR} mice showed normal STM. Consistently, TAM-injected CREB^{IR} mice and other control mice showed a

Figure 7. Generation of social recognition memory networks. **A**, Matrices showing interregional correlations for *c-fos* expression at 0, 1, or 3 min exposure. Colors reflect correlation strength based on Pearson's r (scale, right). **B**, Network graphs were generated by significant positive correlations ($p < 0.05$). **C**, Mean r was calculated from all interregional correlation coefficients. $*p < 0.05$ compared within the group. **D, F, H, J**, Color-coded matrices showing interregional correlations for *c-fos* expression between the hippocampus (**D**), mPFC (**F**), ACC (**H**), or amygdala (**J**) and other regions in the 0, 1, and 3 min groups. **E, G, I, K**, Mean r was calculated from interregional correlation coefficients. $*p \leq 0.05$ compared within the group. Error bars indicate SEM.

significant decrease in social investigation time at the second exposure compared with the first exposure (Fig. 2*B*, paired t test, $p < 0.05$).

In contrast to the results for STM, two-way ANOVA comparing recognition index of LTM revealed significant effects of genotype and drug and a genotype versus drug interaction (genotype, $F_{(1,46)} = 5.193$, $p < 0.05$; drug, $F_{(1,46)} = 14.938$, $p < 0.05$; genotype vs drug, $F_{(1,46)} = 4.41$, $p < 0.05$). Importantly, TAM-

injected CREB^{IR} mice showed a significantly worse recognition index than the other groups (Fig. 2*C*; $p < 0.05$). Consistently, only TAM-injected CREB^{IR} mice failed to show a significant decrease in social investigation time at the second exposure (Fig. 2*D*; paired t test, $p > 0.05$). These results indicated that the inhibition of CREB-mediated transcription in the forebrain blocked the formation of long-term social recognition memory without affecting short-term social recognition memory, suggesting that

activation of CREB-mediated transcription is required for the consolidation of social recognition memory.

Induction of c-fos expression in multiple brain regions after training for social recognition

To identify the brain regions expressing IEGs targeted by CREB when social recognition memory is generated, we performed immunohistochemistry to measure the expression of c-fos. We prepared 4 experimental groups (3, 1, and 0 min exposure and home cage groups). Similar to Figure 1, the mice were exposed to a juvenile male mouse for 3 min or 1 min and only the 3 min group formed a social memory. The other groups of mice were exposed to the investigation cage (novel environment) without exposure to a juvenile male mouse (0 min group) or stayed in their home cages when the other groups of mice (0, 1, and 3 min groups) underwent exposure. The number of c-fos-positive cells was measured in the hippocampus [CA1, CA3, and dentate gyrus (DG) regions], mPFC [prelimbic (PL) and infralimbic (IL) regions], ACC, amygdala [lateral (LA), basolateral (BLA), and central (CeA) regions], OB, MPOA, and other cortical regions (VC, TC, PRh, and EC) at 90 min after the exposure session.

One-way ANOVA revealed significant effects of group on the CA1, CA3, PL, IL, ACC, and BLA regions, but not on the DG, LA, and CeA regions (Fig. 3B–E; CA1, $F_{(3,43)} = 10.427, p < 0.05$; CA3, $F_{(3,43)} = 9.166, p < 0.05$; PL, $F_{(3,43)} = 5.564, p < 0.05$; IL, $F_{(3,43)} = 7.214, p < 0.05$; ACC, $F_{(3,43)} = 6.244, p < 0.05$; BLA, $F_{(3,43)} = 8.072, p < 0.05$; DG, $F_{(3,43)} = 2.238, p > 0.05$; LA, $F_{(3,43)} = 0.235, p > 0.05$; CeA, $F_{(3,43)} = 0.695, p > 0.05$). The 3 min group showed significantly more c-fos-positive cells in the CA1, CA3, PL, IL, ACC, and BLA regions compared with the other groups (Fig. 3B–E; $p < 0.05$), whereas the other groups showed comparable numbers of c-fos-positive cells in these brain areas ($p > 0.05$). These results indicated significant c-fos induction in the hippocampal CA1 and CA3, PL, and IL regions of the mPFC, ACC, and BLA region of the amygdala only in the 3 min group (Fig. 3B–E), suggesting that c-fos expression is induced in these brain regions when social recognition memory is generated.

Importantly, in addition to the 3 min group, the 1 min group showed more c-fos-positive cells in the CA1 region than the 0 min and home cage groups, although a significant difference was only observed when compared with the home cage group ($p < 0.05$), not the 0 min group ($p > 0.05$). These results suggest that the hippocampal CA1 region is activated in response to exposure to a juvenile mouse, but shows an exposure-time-dependent increase in c-fos expression.

Similarly, one-way ANOVA revealed significant effects of group on the OB and MPOA (Fig. 3F,G; OB, $F_{(3,43)} = 7.355, p < 0.05$; MPOA, $F_{(3,43)} = 5.031, p < 0.05$). In contrast to the results shown above, the 3, 1, and 0 min groups showed significantly more c-fos-positive cells in the OB than the home cage group ($p < 0.05$), although the 3, 1, and 0 min groups showed comparable numbers of c-fos-positive cells ($p > 0.05$), suggesting that exposure to a novel environment is sufficient to induce c-fos expression in the OB. In contrast, the 3 and 1 min groups showed significantly more c-fos-positive cells in the MPOA region than the home cage group ($p < 0.05$), although the 3 and 1 min groups showed comparable numbers of c-fos-positive cells ($p > 0.05$), suggesting that exposure to a juvenile mouse induces c-fos expression in the MPOA even though exposure duration was only 1 min.

In contrast, one-way ANOVA revealed no significant effect of group on the VC, TC, PRh, and EC (Fig. 3H; VC, $F_{(3,43)} = 1.704, p > 0.05$; TC, $F_{(3,43)} = 0.343, p > 0.05$; PRh, $F_{(3,43)} = 0.732, p > 0.05$; EC, $F_{(3,43)} = 0.865, p > 0.05$). These results indicated that

c-fos expression is not induced in these cortical regions after exposure to a mouse and/or novel environment.

Together, our anatomical analyses indicated that c-fos expression is induced in the hippocampus, mPFC, ACC, and amygdala when social memory is generated, raising the possibility that gene expression in these brain regions contributes to the formation of social recognition memory.

Induction of Arc expression in multiple brain regions after training for social recognition

To clarify further the brain regions showing IEG expression after training for social recognition, the expression of another activity-dependent gene targeted by CREB, Arc (Kaczmarek and Robertson, 2002; Guzowski et al., 1999; Montag-Sallaz et al., 1999; Guthrie et al., 2000; Kawashima et al., 2009), was measured in the same brain regions of the four groups as above. Similar to the results for c-fos, one-way ANOVA revealed significant effects of group on the CA1, CA3, PL, IL, ACC, BLA, and OB, but not the DG, LA, CeA, MPOA, and cortical regions (Fig. 4B–H; CA1, $F_{(3,44)} = 6.208, p < 0.05$; CA3, $F_{(3,44)} = 9.064, p < 0.05$; PL, $F_{(3,44)} = 21.266, p < 0.05$; IL, $F_{(3,44)} = 24.581, p < 0.05$; ACC, $F_{(3,44)} = 19.487, p < 0.05$; BLA, $F_{(3,44)} = 11.809, p < 0.05$; DG, $F_{(3,44)} = 2.562, p > 0.05$; LA, $F_{(3,44)} = 2.710, p > 0.05$; CeA, $F_{(3,44)} = 1.652, p > 0.05$; OB, $F_{(3,44)} = 3.555, p < 0.05$; MPOA, $F_{(3,44)} = 1.453, p > 0.05$; VC, $F_{(3,44)} = 1.384, p > 0.05$; TC, $F_{(3,44)} = 0.310, p > 0.05$; PRh, $F_{(3,44)} = 2.551, p > 0.05$; EC, $F_{(3,44)} = 2.123, p > 0.05$). Importantly, the 3 min group showed significantly more Arc-positive cells in the CA1, CA3, PL, IL, ACC, and BLA regions than the other groups (Fig. 4B–E; $p < 0.05$), suggesting that Arc expression is activated in hippocampal CA1 and CA3, mPFC (PL and IL), ACC, and BLA of the amygdala when social recognition memory is generated. These results confirmed the results of c-fos expression and suggested that CREB-mediated gene expression (Arc and c-fos) is induced in the hippocampus, mPFC, ACC, and amygdala when social recognition memory is formed. In contrast to the results of c-fos expression, the OB showed significantly more Arc-positive cells in the 3 min group than in the home cage group, but not in the other groups ($p < 0.05$), suggesting that c-fos and Arc expression is regulated differently in the OB.

Requirement for gene expression in the hippocampus, mPFC, ACC, and amygdala for the consolidation of social recognition memory

Gene expression in the hippocampus is required for the consolidation of social recognition memory (Kogan et al., 2000; Suzuki et al., 2011). Our gene expression analyses indicated that gene expression is activated in the hippocampus, mPFC, ACC, and amygdala after social recognition learning, suggesting that new gene expression in these brain regions contributes to the formation of social recognition memory. To test this, we examined the effects of inhibiting protein synthesis in the hippocampus, mPFC, ACC, or amygdala on the formation of social recognition memory. Male mice were exposed to a juvenile male mouse twice for 3 min at an interval of 2 h (STM) or 24 h (LTM). Immediately after the first exposure, the mice received a microinfusion of ANI (62.5 μg) or VEH into the brain region under examination. Canula tip placements are shown in Figures 5I and 6I.

One-way ANOVA comparing recognition indexes of STM revealed no significant effect of drug (Fig. 5A,C,E,G; hippocampus, $F_{(1,22)} = 0.089, p > 0.05$; mPFC, $F_{(1,21)} = 0.385, p > 0.05$; ACC, $F_{(1,22)} = 0.195, p > 0.05$; amygdala, $F_{(1,20)} = 0.193, p > 0.05$). Consistently, the VEH and ANI groups displayed signifi-

cant decreases in social investigation time at the second exposure compared with the first exposure ($p < 0.05$). These results indicated that all of the ANI groups showed normal STM.

In contrast to the results for STM, one-way ANOVA comparing recognition indexes of LTM revealed significant effects of drug (Fig. 6*A,C,E,G*; hippocampus, $F_{(1,22)} = 10.292$, $p < 0.05$; mPFC, $F_{(1,20)} = 28.414$, $p < 0.05$; ACC, $F_{(1,24)} = 8.940$, $p < 0.05$; amygdala, $F_{(1,21)} = 16.887$, $p < 0.05$). The ANI groups displayed a significantly worse recognition index than the VEH group when ANI was microinfused into the hippocampus, mPFC, ACC, or amygdala (Fig. 6*A,C,E,G*; $p < 0.05$). Consistently, the ANI groups showed comparable social investigation times during the first and second exposures ($p > 0.05$), although the VEH groups showed significant decreases in social investigation time at the second exposure ($p < 0.05$; Fig. 6*B,D,F,H*). These results indicate that inhibition of protein synthesis in the hippocampus, mPFC, ACC, or amygdala blocks the formation of social recognition LTM.

Our observations that inhibition of protein synthesis in the hippocampus, mPFC, ACC, or amygdala impairs LTM without affecting STM suggest that activation of gene expression in these brain regions is required for the consolidation of social recognition memory. These results confirmed previous findings showing essential roles for the hippocampus in social memory consolidation and extended the observations that the mPFC, ACC, and amygdala are also required for memory consolidation.

Brain network composed of multiple brain regions for the consolidation of social recognition memory

Memory is not stored in only a single brain area, but in a network composed of multiple regions (Phillips and LeDoux, 1992; Kim et al., 1993; Fanselow and LeDoux, 1999; Zhang et al., 2008; Mamiya et al., 2009; Zhang et al., 2011; Wheeler et al., 2013; Fukushima et al., 2014). Our findings that the formation of social recognition memory requires the activation of gene expression in multiple brain regions led us to analyze functional connectivity by computing covariance across subjects to infer interactions between neural elements (Horwitz et al., 1995; McIntosh, 1999). To do this, we first computed a complete set of interregional correlations in the groups of mice at each time exposure (0, 1, or 3 min) using the results of c-fos expression shown in Figure 3 (Fig. 7; Wheeler et al., 2013). Figure 7*A* shows matrices that display interregional correlations for the number of c-fos-positive cells at each exposure. Increases in higher positive correlations of c-fos expression between two brain regions are observed with exposure time to a juvenile mouse (e.g., the number of red pixels, indicating Pearson's $r \geq 0.6$: 0 min, 42; 1 min, 68; 3 min, 102). On the basis of these matrices, we generated network graphs for each exposure where the nodes represent brain regions and the connections between nodes (edges) represent significant correlations ($p < 0.05$; Fig. 7*B*). Changes in network density were observed at a global level with exposure time to a juvenile mouse (number of edges: 0 min, 21; 1 min, 41; 3 min, 54). Furthermore, comparisons of Pearson's r value among all brain regions analyzed confirmed these observations and revealed that the 3 min group had significantly increased interregional correlation coefficients compared with the 0 and 1 min groups (Fig. 7*C*, one-way ANOVA, $F_{(2,312)} = 22.77$, $p < 0.05$; Newman–Keuls test, $p < 0.05$).

We next examined changes in the connectivity of the brain regions (hippocampus, mPFC, ACC, and amygdala) required for social recognition LTM with other brain regions (Fig. 7*C–J*). Interestingly, c-fos expression between the hippocampus or

mPFC and other regions was more strongly correlated in the 3 min group than in the 1 and 0 min group (Fig. 7*D–G*, one-way ANOVA, hippocampus, $F_{(2,114)} = 21.56$, $p < 0.05$; mPFC, $F_{(2,78)} = 6.8$, $p < 0.05$; Newman–Keuls test, $p < 0.05$), suggesting that functional connectivity is increased in the hippocampus and mPFC when social recognition memory is generated. In contrast, c-fos expression between the ACC or amygdala and other regions showed comparable correlations between the 1 and 3 min groups, but was more correlated in the 1 and 3 min groups compared with the 0 min group, although significantly higher c-fos expression in these regions was observed in the ACC and amygdala of the 3 min group than in the 1 min group (Fig. 7*H–K*, one-way ANOVA, ACC, $F_{(2,39)} = 5.08$, $p < 0.05$; amygdala, $F_{(2,114)} = 25.04$, $p < 0.05$; Newman–Keuls test, $p \leq 0.05$). In contrast to the results for the hippocampus and mPFC, these observations suggest that exposure to a juvenile mouse is sufficient to increase functional connectivity of the ACC and amygdala with other brain regions.

Identification of social recognition memory network hubs

We applied graph theoretical analysis to our networks to discover if the relative importance of any of the brain regions changes during the formation of a social recognition memory. We determined brain region importance in two ways. First, we examined how the relative ranking of the centrality measures of degree and betweenness differed in our networks (Fig. 8*A,B*). Degree is the number of edges that are associated with a given node and betweenness is the number of shortest path lengths in the network on which a node lies (Sporns et al., 2007). Second, we identified the community structure for each network (Fig. 8*C*) and used this to compute the within-community z-scores and participation coefficients for each region. Within-community z-scores measure how well connected a region is to its own community, whereas the participation coefficient measures how connected a region is to other communities (Guimerà and Amaral, 2005). Regions that are moderately high in both within-community z-scores and participation coefficients are considered important hubs that may help to mediate the interactions of disparate parts of a network (Fornito et al., 2015). In the 3 min group, the hippocampus (CA1, CA3, and DG regions) showed a higher rank of both degree and betweenness than in the 0 and 1 min groups (Fig. 8*A,B*). Furthermore, the DG of the hippocampus had both a high within-community z-score and participation coefficient in the 3 min group compared with the 0 and 1 min groups (Fig. 8*D*). In contrast, the mPFC regions were lower in both degree and betweenness in the 3 min group compared with the hippocampus, ACC, and amygdala, all of which are required for social recognition LTM. These results suggest that the hippocampus, but not the mPFC, functions as a connector hub to coordinate the interaction of modules within the brain to support social recognition memory. Interestingly, and consistent with the c-fos expression analyses (Fig. 7*I,H*), the ACC and amygdala had higher ranks of both degree and betweenness in the 1 min group than in the 0 and 3 min groups (Fig. 8*A,B*). These regions were also high in both within-community z-scores and participation coefficients (Fig. 8*D*). These results suggest that the ACC and amygdala play distinct roles from the hippocampus and function as hubs that are important primarily in mediating activation in response to the exposure to a novel mouse.

Discussion

A critical biochemical feature of memory consolidation is a requirement for gene expression, especially CREB-mediated gene expression (Flexner et al., 1965; Davis and Squire, 1984;

Figure 8. Identification of hub regions in the social recognition memory network. **A, B**, Brain regions ranked in descending order for degree (**A**) and betweenness (**B**). Degree and betweenness are normalized. **C**, Network summarizing functional connections at 0, 1, or 3 min exposure. Colors represent the communities identified via modularity maximization. The widths of the edges are proportional to the strength of the correlations and the size of the nodes is proportional to the degree of the node. **D**, Within-community z-scores and participation coefficients for each brain region in the networks.

Bourtchuladze et al., 1994; Abel et al., 1997; Silva et al., 1998; Martin et al., 2000; McGaugh, 2000; Kida et al., 2002; Pittenger et al., 2002; Kozus et al., 2004). In this study, we identified brain regions that play essential roles in the formation of social recognition memory through the activation of gene expression. We

first found that CREB-mediated transcription in the forebrain is required for the consolidation of social recognition memory. We next showed that the expression of *c-fos* and *Arc*, which are CREB target genes (Abraham et al., 1993; Worley et al., 1993; Guzowski et al., 1999; Montag-Sallaz et al., 1999; Guthrie et al., 2000; Kac-

zmarek et al., 2002; Kawashima et al., 2009), is significantly induced in the hippocampus (CA1 and CA3), mPFC (PL and IL), ACC, and amygdala (BLA) (Figs. 3A–D, 4A–D) when social recognition memory is generated. Finally, we showed that protein synthesis in these brain areas is required for the consolidation of social recognition memory (Figs. 5, 6). Our findings indicated that the consolidation of social recognition memory depends on new gene expression in the hippocampus, mPFC, ACC, and amygdala. Furthermore, our network analyses using correlations of *c-fos* expression among brain regions suggest distinct roles for the hippocampus, mPFC, ACC, and amygdala in social recognition memory consolidation. Importantly, the hippocampus may be a connector hub functioning to coordinate the generation of social recognition memory through its interactions with other brain regions.

The hippocampus is a critical region for the formation/consolidation of social recognition memory in mice (Kogan et al., 2000; Suzuki et al., 2011; Hitti and Siegelbaum, 2014). Our results confirmed and supported these previous findings. Interestingly, our network analyses suggested that the hippocampus showed more increases in connectivity with other brain regions compared with the other regions when social recognition memory is generated. Importantly, the hippocampus of the 3 min group showed the highest rank according to both degree and betweenness of functional connectivity and the DG in particular may play an important role in coordinating network activity. These observations suggest that the hippocampus plays a role as a hub to generate social recognition memory by interacting with other brain regions. Interestingly, a recent study by Hitti and Siegelbaum (2014) showed that the hippocampal CA2 region is essential for social recognition memory. It is important to investigate further and compare the roles of hippocampal subregions (CA1, CA2, and CA3) in social recognition memory because we failed to detect an obvious induction of *c-fos* and *Arc* in the CA2 region of the 3 min group (data not shown).

Similar to the hippocampus, our biochemical and behavioral results showed that the mPFC plays an essential role in the consolidation of social recognition memory. Furthermore, the mPFC is also suggested to increase connectivity with other brain regions when social recognition memory is formed. However, in contrast to the hippocampus, the mPFC was not ranked highly according to the degree and betweenness of connectivity during the consolidation of social recognition memory compared with the hippocampus, ACC, and amygdala. These observations suggest that the mPFC is a critical brain region for the formation of social recognition memory, but plays a different role to the hippocampus. Importantly, previous studies have shown that the mPFC is involved in the regulation of social behaviors (Jodo et al., 2010; Yizhar et al., 2011; Felix-Ortiz et al., 2016). Changes in the neuronal activity of the mPFC are correlated with social approach and interaction (Jodo et al., 2010). Consistently, social approach/interaction are blocked by an imbalance of excitation and inhibition in the mPFC (Yizhar et al., 2011). Together, these findings indicate that the mPFC is a critical brain region regulating both social approach/interaction and social recognition memory, suggesting that the mPFC plays central roles in social behaviors. The mPFC may contribute to social recognition/discrimination by referring to previously consolidated social recognition memory.

We showed that, in addition to the hippocampus and mPFC, gene expression in the amygdala and ACC is required for the consolidation of social recognition memory (Figs. 5E,F, 6E,F). However, in contrast to the results for the hippocampus and mPFC, the amygdala and ACC did not show further increases in

connectivity even when exposure time to a juvenile mouse was increased (Fig. 7F–I, 3 vs 1 min group). Consistently, the ACC and amygdala of the 1 min group showed the highest rank in both the degree and betweenness of functional connectivity and were identified as important hubs based on the community structure of the network. This suggests that the ACC and amygdala are important for coordinating brain activity when social interaction is initiated. Similar to our findings, previous studies using rats showed that blocking protein synthesis or the β -adrenoreceptor or activating the D1/D5 dopamine receptor or H2 histaminergic receptor (Garrido Zinn et al., 2016) in the BLA impairs the formation of social recognition memory. Lesioning of the ACC impairs social recognition STM in mice (Rudebeck et al., 2007). More importantly, the BLA–ventral hippocampus and BLA–mPFC pathways regulate social interaction (Felix-Ortiz and Tye, 2014; Felix-Ortiz et al., 2016). Consistently, electrophysiological recordings revealed changes in neuronal activity in the BLA during social interaction (Katayama et al., 2009). In addition to these rodent studies, neuroimaging studies in humans suggest that the amygdala plays a functional role in social processing and cognition (Killgore and Yurgelun-Todd, 2005; Schultz, 2005; Bickart et al., 2011) and that the ACC is active when participants engage in social interaction (Frith et al., 1999; Rilling et al., 2002; Rilling et al., 2004). Lesioning the ACC in macaques impairs social interaction (Hadland et al., 2003; Rudebeck et al., 2006). Together, these findings suggested that the ACC and amygdala are not only required for the consolidation of social recognition memory, but may also play regulatory roles in social interaction.

Previous studies have shown that activation of gene expression in the hippocampus and amygdala is required for consolidation of contextual fear memory. More importantly, previous work using a combination of *c-fos* expression and network analyses found that long-term contextual fear memory is stored in a brain network that has a thalamic–hippocampal–cortical signature (Wheeler et al., 2013). In addition, mPFC and thalamus may function as a hub-like region that plays privileged roles in memory expression (Wheeler et al., 2013). Together with our findings in this study, social recognition and contextual fear memories display distinct brain networks to memory formation/recall, suggesting that distinct types of memory show their own functional memory networks. Further studies focusing on other types of memory such as spatial and object recognition memories are required to understand brain networks for memory storages.

Mice discriminate familiar and novel mice based on social recognition memory (Kogan et al., 2000; Fig. 1). Although we identified brain networks among the hippocampus, mPFC, ACC, and amygdala to generate social recognition memory, the brain systems that allow for the discrimination of the familiar and novel still remain unknown. In the future, it will be important to examine and compare neuronal activity and activation of gene expression in those brain regions during and after exposure to familiar and novel mice.

In this study, we found that the consolidation of social recognition memory requires CREB-mediated transcription. Importantly, we showed that the expression of CREB-target IEGs (*c-fos* and *Arc*) was induced in the hippocampus, mPFC, ACC, and amygdala when social recognition memory was generated. Consistently, we showed that new gene expression in these brain regions was required for the consolidation of social recognition memory. These findings suggest that social recognition memory is encoded in a network of these brain regions. Furthermore, our network analyses suggest that the hippocampus, mPFC, ACC, and amygdala show distinct roles in learning and memory of

social recognition and, importantly, that the hippocampus functions as a connector hub to generate social recognition memory by coordinating regional interactions.

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