Learning-Induced Enduring Changes in Functional Connectivity among Prefrontal Cortical Neurons

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Current thinking about how memories are stored in the brain has been profoundly influenced by Donald O. Hebb's cell assembly hypothesis, which posits that (1) learning produces a stable alteration in patterns of connectivity among repeatedly coactivated neurons, and (2) memory retrieval involves reactivation of those altered patterns of connectivity. However, learning-induced changes in connectivity that persist over long periods of time have not been clearly demonstrated. In the present study, two spatial navigation tasks and a long-term ensemble recording technique are used to describe long-lasting modifications in functional connectivity (FC) (defined as changes in synchronous firing) of prefrontal cortical neurons in behaving rats. Animals were initially trained to alternate visiting two spatial locations on a figure-8-shaped maze to obtain a reward (alternating task 1). Afterward, while continuing on task 1, animals were additionally trained to visit only one spatial location on the same maze to obtain a reward (unilateral task 2). Multiple single units were recorded while rats were undergoing acquisition, retention, and performance of both tasks. Our data indicate that correlated firing of prefrontal cortical neurons changed significantly in early phases of training when learning rate was maximal but became progressively smaller in later phases when learning reached asymptote. After animals became proficient, FC remained constant, although neuronal activities varied across two different tasks. The present finding of negatively accelerated changes in FC confirms associative learning theories and provides crucial neurophysiological evidence for Hebb's hypothesis.

Key words: learning; memory; functional connectivity; cell assembly; prefrontal cortex; ensemble recording

Introduction

A central tenet in learning and memory is that the formation of memory trace (or engram) in the brain involves changing connection strength among neurons, thereby forming new functional cell assemblies (Hebb, 1949). In support, long-lasting activity-dependent synaptic plasticity has been discovered (Bliss and Lomo, 1973), and ensuing studies have generated a wealth of evidence supporting the crucial role of synaptic plasticity in memory (Martin et al., 2000). A crucial missing link between synaptic plasticity and memory, however, is the evidence that connectivity among neurons undergoes stable changes across time (paralleling memory).

Hebb originally proposed "some growth process or metabolic change" to account for the formation of new connectivity. Neu-

rophysiologically, the connectivity between two neurons can be assessed in behaving animals by measuring the degree of synchronous firing (i.e., cross-correlation) between two spike trains (Perkel et al., 1967), which is commonly referred to as functional connectivity (FC). Using this method, several studies demonstrated changes in FC with learning, albeit with limited success. For instance, McNaughton and colleagues showed that neuron pairs in the hippocampus, neocortex, and ventral striatum that were coactive during a reward-obtaining task enhanced their synchronous firing during subsequent rest period (Wilson and McNaughton, 1994; Qin et al., 1997; Hoffman and McNaughton, 2002; Pennartz et al., 2004). However, because recordings were made in well trained animals in a familiar environment and because enhanced FC returned to the baseline within ~30 min (except in the ventral striatum in which the change persisted ~40 min) postbehavior rest period, it is unclear that the changes in FC were the product of encoding new information and stable over time. Similarly, Ahissar et al. (1992) found short-lived (12–13 min) alterations in FC in the auditory cortex of monkeys well trained in a cellular classical conditioning task. Changes in FC have also been reported in the amygdala during fear conditioning in rats (Quirk et al., 1995), in which the recording duration lasted ~3 h and the number of neuron pairs was too small to draw a significant conclusion. More recently, Schoenbaum et al. (2000) showed that FC changes in the amygdala and orbitofrontal cortex during odor discrimination (and reversal) learning in rats. How-

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Figure 1. Behavioral tasks and recording sites. **A, B,** Training consisted of two phases. In phase I (an alternation task 1) (left), animals placed in the middle arm were required to run unidirectionally to the junction, make either left or right arm entry to obtain a reward, continue forward to reenter the middle arm (for the next trial), and then make an alternate arm entry for a reward (e.g., LRLR...). If animals make the same arm entry as the preceding trial (e.g., LL or RR), the reward is not presented. Reward locations are denoted by ®. In phase II, each daily session initiated with task 1 for 25 trials, which was then immediately followed by animals requiring to make repetitive unilateral turns for reward (i.e., RRRR...; a unilateral task 2) (right). If animals make alternate arm entry, the reward is not presented. **C,** Recordings were made in prelimbic (PL) and infralimbic (IL) cortices. **D–G,** Mean $\pm$ SEM percentage accuracy over the course of phase I (task 1), phase II (tasks 1 and 2), and overtraining phase ($n = 4$ animals). Data are plotted for alternating days in **G**.

ever, the observed alterations did not persist across sessions, making it difficult to link FC changes with encoding and maintenance of memory. To our knowledge then, there exists no definitive evidence for Hebb's hypothesis that relates FC changes with long-term memory.

In the present study, we used a long-term ensemble recording technique and investigated FC changes during both learning and memory retention across multiple days. Specific questions addressed are as follows: (1) whether FC changes in parallel with behavioral learning, (2) whether FC changes repeatedly during different learning tasks, and (3) whether FC changes are maintained stably for a long time (analogous to memory). To do so, rats chronically implanted with a microdrive array of 12 tetrodes into the prefrontal cortex (PFC) were trained to learn two distinct task rules on a figure-8-shaped maze (see Fig. 1A–C). The PFC is an ideal structure for analyzing FC because its neural activities change in parallel with learning (Maxwell et al., 1994; Rainer and Miller, 2000; Baeg et al., 2001, 2003; Mulder et al., 2003), because long-term synaptic plasticity has been demonstrated (Hirsch and Crepel, 1990; Larocque et al., 1990; Herry et al., 1999; Gemmell and O'Mara, 2000; Kim et al., 2003), and because PFC neurons discharge liberally [compared with other structures implicated in

learning such as the hippocampus (O'Keefe and Dostrovsky, 1971; Jung and McNaughton, 1993; Jung et al., 1998)] for quantifying functional relationship in a large number of neuron pairs. The results of the present study indicate large changes in FC during early (but not latter) stages of training and that these changes are maintained stably for a long time.

Materials and Methods

Behavioral task. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Ajou University School of Medicine. After postoperative recovery, four male Sprague Dawley rats (9–13 weeks old) underwent behavioral training on a figure-8-shaped maze (Fig. 1A) as described previously (Baeg et al., 2003). Training consisted of two phases. In phase I, animals were trained to alternate two spatial locations on the maze to obtain water reward [task 1 (Fig. 1A, left); 29.79 ± 2.17 trials per day]. In phase II, each daily session started with the animals performing task 1 (25 trials). Immediately afterward, a wooden block was briefly placed on the upper branching point to block access to the left goal and to signal the beginning of the task 2 (Fig. 1A, right), in which animals now had to make repetitive right turns to obtain water reward for the next 25 trials. Those animals that were trained for >14 d in the second phase of training were considered overtrained.

Unit recording. Multiple single units were recorded in the prelimbic and infralimbic cortices as described previously (Baeg et al., 2003). Briefly, a microdrive array was loaded with 12 tetrodes and implanted in the left or right medial PFC (2.7 mm anterior, 0.7 mm lateral from bregma) under sodium pentobarbital (50 mg/kg) anesthesia. Before reaching the overtraining phase, tetrodes were not advanced to record the same unit signals repeatedly, unless unit signals from a given tetrode disappeared. Unit signals were amplified 10,000–20,000 $\times$, filtered between 0.6 and 6 kHz, digitized at 32 kHz, and stored on a personal computer using the Cheetah data acquisition system (Neuralynx, Tucson, AZ). The head position of the animal was recorded at 60 Hz. When recordings were completed, small marking lesions were made and recording locations were verified histologically.

Unit analysis. Single units were isolated by projecting the four-channel relative amplitude data two dimensionally and manually applying boundaries to each subjectively apparent unit cluster (cf. Baeg et al., 2003). Spike width was also used as an additional spike waveform characteristic for unit isolation. Consistency of unit signals across multiple days was determined based on spike waveforms, baseline firing rates, autocorrelograms, interspike interval histograms, and clustering patterns (supplemental data, available at www.jneurosci.org as supplemental material).

Unit activity across two tasks was compared by computing pixel-by-pixel correlation between two spatial firing rate maps (cf. Song et al., 2005). The 117 $\times$ 117 cm camera field containing the maze (90 $\times$ 50 cm) was divided into 64 $\times$ 64 pixels, and the firing rate (total number of spikes divided by total occupancy time) for each pixel was calculated. “Adaptive binning” was used as described previously (Jung et al., 1994) to optimize the tradeoff between sampling error and resolution. Only the central section and the right side of the maze were subjected to the construction of a firing rate map. Pixel-by-pixel correlations between two firing rates maps were then computed and transformed to Fisher’s z for normalization (Rosner, 1995). In all ensuing analysis, FC was defined as follows: $FC = (A - B)/(A + B)$, where A denotes the observed number of synchronous firing between two units within a 50 ms time period, and B denotes the expected number of synchronous firing assuming that two units are independent, homogeneous Poisson processes with the equivalent average firing rates. Two alternative methods were used to validate our FC analysis. In the first method, we calculated B by counting the number of synchronous firing between two surrogate spike trains that were generated based on spike density functions of original spike trains (Gaussian kernel with $\delta_t = 100$ ms was applied to each spike) assuming that each spike train is a rate-modulated, independent gamma process with shape parameter equals two (Oram et al., 1999; Baker and Lemon, 2000). For this, we treated the entire recording session (both baseline period and behavioral session) as a set of single continuous spike trains. In the second method, FC was defined as the degree of synchronous firing within shorter (10–40 ms) time intervals. These two alternative methods essentially yielded the same results (data not shown).

Linear regression analysis, Student’s t test (two-tailed), and one-way ANOVA were used for statistical comparisons. A p value < 0.05 was used as the criterion for a significant difference. Data are expressed as mean $\pm$ SEM.

Results

Behavioral learning

The animals’ performance on task 1 improved appreciably during phase I training (Fig. 1D) ($36.87 \pm 6.80\%$ on day 1 to $85.77 \pm 1.40\%$ on day 9). In phase II, task 2 learning improved from $63.08 \pm 3.74\%$ on day 1 to $96.21 \pm 0.21\%$ on day 14 (Fig. 1F). While animals were learning a new task 2, performance on previously acquired task 1 fluctuated considerably (Fig. 1E), presumably because the animals were executing two different tasks (alternation vs unilateral turning responses). In the overtraining phase, animals performed both tasks at asymptotic levels (Fig. 1G). Linear regression analysis indicated that the slope of task 1 performance was significantly greater than 0 ($r = 0.863$; $p = 0.003$) in phase I when animals were learning the task but not in

phase II ($r = 0.083$; $p = 0.773$) when animals already learned the task. The slope of task 2 performance in phase II was also significant ($r = 0.926$; $p < 0.000$). These data clearly indicate that animals effectively learned task 1 (in phase I) and then were able to learn an additional task 2 (in phase II) while concurrently retaining/expressing previously acquired task 1.

Changes in functional connectivity during learning

A total of 355 neurons and 4952 neuron pairs were recorded during training phases I and II (Fig. 1B,C). Because the majority (94.8%) of neuron pairs were recorded from separate tetrodes, essentially the same results were obtained when analyses were performed excluding those neuron pairs recorded from the same tetrode to ensure unequivocal separation of unit signals (data not shown). Changes in FC (ΔFC) were calculated as the difference in FC across 2 successive days of neuron pairs recorded over multiple days with ≥ 300 emitted spikes during each baseline period, wherein animals sat quietly on a pedestal in the recording room for ~ 10 min before the task (2366 and 1534 neuron pairs in phases I and II, respectively). Throughout the study, only neurons with ≥ 300 spikes were used in the analysis to avoid the influence of spuriously correlated discharges, and neuron pairs with ΔFC values beyond 3 SDs from the mean were excluded to avoid the influence of outliers (this analysis criterion did not affect the overall results). As can be seen from an example neuron pair (Fig. 2A–C), FC changed significantly during learning. However, because both enhancement and reduction in FCs were observed in comparable numbers of neuron pairs, averaged net changes were near 0 at all time points in both training phases (Fig. 3A,B).

However, when we examined the absolute value of ΔFC regardless of enhancement or reduction, strikingly large FC changes were observed during early training sessions, and these changes became smaller across later sessions. This observation was confirmed by comparing the variance in ΔFC across the population of neuron pairs in the course of behavioral training. Specifically, the slopes of ΔFC variances were significantly less than zero for both phase I ($r = -0.841$; $p = 0.009$) and phase II ($r = -0.556$; $p = 0.049$), indicating that the amount of ΔFC became progressively smaller as a function of learning tasks 1 and 2 (Fig. 3C,D). Note that the ΔFC variance was smaller in phase II than in phase I, which is likely attributable to tasks 1 and 2 sharing common features (e.g., same reward locations on the maze). Similar results were obtained when ΔFC was calculated based on neuronal activities during the animal’s behavior (Fig. 3E–G). The slopes of ΔFC variances were significantly less than 0 for both phase I (task 1, 2610 pairs, $r = -0.806$, $p = 0.016$) and phase II (task 1, 1098 pairs, $r = -0.612$, $p = 0.026$; task 2, 1456 pairs, $r = -0.614$, $p = 0.026$).

To test the possibility that ΔFC might be affected by factors other than learning, we examined whether variations of mean firing rate or burst firing could account for the observed learning-associated changes in ΔFC during the baseline period. Burst firing was assessed by calculating the proportion of interspike intervals < 50 ms. Because changes in these parameters over successive days can contribute to changes in ΔFC (with mean firing rate and burst firing remaining constant across days), we also examined the variances of firing rate change (Δrate) and burst firing change (Δburst) across consecutive days. As shown in Figure 4, A and B, no significant variation was observed in mean firing rates (linear regression analysis; phase I, $r = 0.302$, $p = 0.430$; phase II, $r = -0.482$, $p = 0.081$) or in the degree of burst firing (phase I, $r = 0.524$, $p = 0.147$; phase II, $r = 0.235$, $p = 0.418$) across training sessions. Significant variation was detected in neither variance of

Δ rate (phase I, $r = 0.388$, $p = 0.343$; phase II, $r = -0.254$, $p = 0.402$) nor Δ burst (phase I, $r = -0.192$, $p = 0.649$; phase II, $r = -0.069$, $p = 0.823$) (Fig. 3C,D). These results indicate that negatively accelerated changes in FC cannot be accounted for by variations in firing rate or spiking pattern.

To further confirm the above conclusion, we selected neuron pairs whose mean and burst firing rates changed in relatively small degrees ($<50\%$) across 2 d and performed the same analysis for phase I, which (unlike phase II) has a relatively large number of unit pairs. In both conditions, the Δ FC variance progressively decreased over training days (mean firing rate group, 1179 pairs, $r = -0.869$, $p = 0.005$; burst firing rate group, 1364 pairs, $r = -0.820$, $p = 0.013$), consistent with previous analysis. These results indicate that the observed learning-associated changes in Δ FC cannot be accounted for by variations of mean firing rate or burst firing. In addendum, when we normalized FC to the value that takes into account local variations in spike discharge (by generating surrogate spike trains based on spike density functions of original spike trains; see Materials and Methods), we obtained progressive decreases in Δ FC in both training phases I and II, further corroborating our conclusion.

Stable maintenance of altered FC after learning

If learning-induced changes in FC encode long-term memory of the task, then the altered FC should remain stable over time. In the overtraining phase (i.e., >14 d of training in phase II), the Δ FC variance of neuron pairs recorded for at least 2 d ($n = 102$) was 0.0046, a value similar to the Δ FC variance in latter parts of phase I and II training when animals were performing the task at asymptotic levels. This level of variance in FC likely reflects measurement noise from innate fluctuation of FC and/or limited sampling. Thus, the magnitude of FC changes decreased as training progresses and remained at a low level in the overtraining phase. These results indicate that learning-induced changes in FC are stably maintained for a long time.

Neuronal activity and functional connectivity across two behavioral tasks during overtraining

Theoretical studies propose that multiple patterns of memory can be encoded and retrieved correctly out of a common set of functional connectivity, provided that stored patterns are sufficiently different (i.e., overlapping and distributed representation of memories) (Marr, 1971; Kohonen, 1977; Hinton and Anderson, 1981; Hopfield, 1984; McClelland and Rumelhart, 1986; Mc-

Figure 2. An example of FC change during learning. **A**, A neuron pair (red and green clusters) was recorded for the initial 5 d of training in phase I. Clustering patterns of spike waveform parameters and averaged spike waveforms are shown. For the determination of same unit signals across multiple days, see supplemental data (available at www.jneurosci.org as supplemental material). **B**, Corresponding daily cross-correlograms. Cross-correlograms were constructed in 5 ms time bins. Dotted horizontal lines indicate the expected level of cross-correlation between two independent Poisson processes with equivalent firing rates in each cross-correlogram. **C**, FC of the example neuron pair during 5 d of recordings.

Naughton and Morris, 1987; Rolls and Treves, 1998). We tested this postulation by recording PFC neural activities across two different behavioral tasks on the same maze, which made it possible to compare memory-related neural activities under similar sensory input and motor response conditions, thereby effectively minimizing potentially confounding variables (Fig. 1A).

Initially, we examined whether neuronal activities are differ-

Figure 3. Time course of FC change across training sessions. **A, B**, Box plots showing distribution of ΔFC of neuron pairs recorded over multiple days in phases I and II. Positive–negative numbers on the ordinate denote enhancement–reduction in FC across 2 d. The numbers representing integer i on the abscissas of the graphs shown in **A–G** denote the time period between sessions i and $i + 1$. The diagram shows median values (horizontal lines inside boxes), interquartile distances, and upper and lower 10% values. **C, D**, The time course of ΔFC variance in each training phase. ΔFC was computed based on neural activity during the baseline period before each session. **E–G**, ΔFC was computed based on neural activity during task 1 or 2 performance. Solid lines were determined by linear regression.

ent across two tasks and then whether the difference is attributable to factors other than memory. Comparisons between (alternation) task 1 and (unilateral) task 2 were made based on recording data obtained in the central section and the right side of the maze (excluding the left side unless noted), thereby matching the sensory inputs and motor responses between two tasks. A total of 766 neurons were recorded from four rats overtrained in both tasks 1 and 2 (Fig. 1G) (>14 d of training in the phase II). Although animals ran on the same maze, the patterns of neuronal activity were quite different across two tasks (Fig. 5A). As can be seen, the neuronal ensemble showed partially overlapping patterns of activity across two tasks, in which some neurons showed similar firing patterns whereas others showed radically different firing patterns across two tasks. For a quantitative comparison, we divided the tasks into two halves and compared neuronal activities between two halves of task 1 (T1–T1), between those of task 2 (T2–T2) and between the second half of task 1 and the first half of task 2 (T1–T2). As a negative control, we compared T1–T1

Figure 4. Changes in mean firing rate and discharge pattern during learning. **A, B**, No significant variation was found in the mean firing rate or the degree of burst firing in the course of training phase I or II. The abscissa denotes training sessions. Data are mean $\pm$ SEM. **C, D**, Significant variation was detected in neither the variance of Δrate nor Δburst . The numbers representing integer i on the abscissas denote the time period between sessions i and $i + 1$.

neuronal activities of randomly shuffled neuron pairs (so that neurons are randomly paired). Specifically, neuronal activities were compared by computing pixel-by-pixel correlation between two spatial firing rate maps (cf. Song et al., 2005). The firing rate map correlation was significantly lower for T1–T2 compared with T1–T1 or T2–T2 (Fig. 5B) (repeated-measures ANOVA, $F_{(2,890)} = 72.341$, $p < 0.000$; T1–T1 vs T1–T2, $p < 0.000$; T2–T2 vs T1–T2, $p < 0.000$). However, T1–T2 correlation was significantly higher than randomly shuffled T1–T1 correlation (unpaired t test, $t_{(1437)} = 26.300$, $p < 0.000$). Similar results were observed when we compared (1) only the central section of the maze, in which working memory demand and animal trajectory (i.e., where the animal came from and where to go next) were different between the two tasks (Fig. 5C) (repeated-measures ANOVA, $F_{(2,878)} = 63.725$, $p < 0.000$; T1–T1 vs T1–T2, $p < 0.000$; T2–T2 vs T1–T2, $p < 0.000$; shuffle comparison, T1–T2 vs shuffled data, unpaired t test, $t_{(1390)} = 6.815$, $p < 0.000$) or (2) the rest of the maze excluding the central section (Fig. 5D) (repeated-measures ANOVA, $F_{(2,890)} = 18.710$, $p < 0.000$; T1–T1 vs T1–T2,

$p < 0.000$; T2–T2 vs T1–T2, $p < 0.000$; shuffle comparison, T1–T2 vs shuffled data, unpaired t test, $t_{(1437)} = 23.142$, $p < 0.000$). These findings strongly indicate that neuronal activities during performances on tasks 1 and 2 are considerably different but also share partially overlapping properties.

The average firing rates during tasks 1 and 2 were 5.42 ± 0.23 and 5.41 ± 0.23 Hz ($n = 766$ units), respectively, which did not vary significantly (paired t test, $t_{(765)} = 0.256$, $p = 0.798$). Neither spatial distribution of instantaneous speed nor occupancy was significantly different across the two tasks (data not shown). Moreover, similar patterns of neuronal activity were observed when a task was repeated within the same day or across days, indicating that activity patterns for a given task were maintained stably across time and intervening behaviors (Fig. 6). These results show that changes in neuronal activity across tasks 1 and 2 cannot be attributed to variations in animal behavior or passage of time. It appears then that different PFC neural activities observed during two tasks likely represent two different memories.

Next, a total of 1796 neuron pairs recorded from four animals were examined to compare the stability of FC across two tasks in the overtraining phase. FC was maintained similarly regardless of changes in individual neuronal activity (Fig. 7A). A group comparison indicated that there was no significant difference in FC between the two tasks (Fig. 7B) (paired t test, $t_{(1795)} = -1.341$, $p = 0.180$). In the similar manner described for comparing neuronal activity across two tasks (above), we also divided the trials within T1 and T2 tasks into two halves and compared FC of T1–T1, T1–T2, and T2–T2 pairs of halved sessions (Fig. 7C, scatter plots comparing FC within and across two tasks). Significant positive correlations were observed in all three cases ($r = 0.500$, 0.423 , and 0.534 for T1–T1, T1–T2, and T2–T2, respectively; $p < 0.000$ in all cases, linear regression analysis). We also converted each point into the difference in FC and compared their distributions (Fig. 7D). The difference in FC in T1–T1, T1–T2, and T2–T2 pairs of halved sessions did not vary significantly (repeated-measures ANOVA, $F_{(2,4404)} = 0.606$, $p = 0.546$), further indicating that FC remains similar regardless of the animal's performance on the maze.

We further examined the relationship between the change in neuronal activity and the difference in FC. If FC remains similar regardless of changes in individual neuronal activity across tasks 1 and 2, then variation in FC across tasks 1 and 2 should not correlate with the difference in neuronal activities across tasks 1 and 2. As predicted, there was no significant correlation between the sum of task 1 versus task 2 firing rate map correlations and the difference in FC of a neuron pair across tasks 1 and 2 (Fig. 7E) ($r = -0.021$, $p = 0.375$, linear regression analysis). Similar results were obtained when the analysis was confined to the data obtained from the central section only (Fig. 7F) ($r = -0.021$, $p =$

Figure 5. Different patterns of neural activity across tasks 1 and 2 in the overtraining phase. **A**, An example of neuronal ensemble activity in tasks 1 and 2 in the overtraining phase. Neurons were arranged according to the similarity of firing rate maps between the two tasks. Each spatial firing rate map shows spatial distribution of firing rate over the maze. Red indicates maximum firing (at least 1 Hz), which is different for each neuron, and dark blue indicates no firing. Pixel-by-pixel correlation is indicated for each pair of firing rate maps on the right. Correlations in this and subsequent figures indicate z transforms of correlation coefficients. **B**, Correlations of firing rate map between two halves of task 1 (T1–T1), between the second half of task 1 and the first half of task 2 (T1–T2), between two halves of task 2 (T2–T2), and between randomly paired neurons (task 1) (Shuffle). **C**, Correlations of firing rate maps in the central section only (Central). **D**, Correlations of firing rate maps excluding the central section (Central excluded). Data are plotted as mean $\pm$ SEM. Asterisks denote significant differences.

0.332) or the rest of the maze excluding the central section (Fig. 7G) ($r = -0.013$, $p = 0.685$). These results corroborate the conclusion that FC remains similar across two tasks in the overtraining phase regardless of the degree of neuronal activity change.

Effects of afferent neural activity

Considering that a large proportion of afferent projections to the PFC arise from other cortical areas including sensory association and motor cortical areas (Conde et al., 1995; Fuster, 1997), afferent activities are expected to be different on two sides of the maze. In the present study, the maze was placed near one corner of the recording room with rich visual cues, making the sensory cues on the left versus right side of the maze quite distinct. Regarding the motor aspect, the animals made left (or right) turns only on the left (or right) side of the maze. Previous work has shown that $\sim 30\%$ of neurons exhibited turn-specific activities in cortical areas that project to the PFC (McNaughton et al., 1994). Furthermore, neurons in the ventral hippocampus exhibit place-specific firing (Jung et al., 1994; Poucet et al., 1994) and, via direct projections to the PFC (Swanson, 1981; Ferino et al., 1987; Jay and Witter, 1991), exert powerful influence on the discharges of PFC neurons (Siapas et al., 2005). If afferent neuronal activity is an important factor determining synchronous firing of PFC neu-

Figure 6. Stably maintained neural activity across intervening behavior and time. **A**, An example of neurons that were recorded in the sequence of task 1 (t_1')–task 2–task 1 (t_1''). **B**, Group data ($n = 81$ neurons). Correlations of firing rate maps across three sessions are shown. There was a significant effect of the task on firing rate map correlation (repeated-measures ANOVA, $F_{(2,160)} = 10.203$, $p < 0.000$), and t_1' – t_1'' firing rate map correlation (1.022 ± 0.062) was significantly higher than t_1' –task 2 (0.8 ± 0.062 , $p < 0.000$) or t_1' –task 2 correlation (0.847 ± 0.069 , $p = 0.002$). **C**, Two examples of neurons that were recorded for 2 consecutive days. **D**, **E**, Group data. Each task was divided into two halves, and correlations of firing rate maps within (day 1, D1–D1; day 2, D2–D2) and across 2 successive days (D1–D2) were calculated ($n = 91$ neurons). The correlations across successive days (task 1, 1.163 ± 0.057 ; task 2, 1.283 ± 0.053) were similar to the correlations of the same day (task 1: day 1, 1.26 ± 0.062 ; day 2, 1.242 ± 0.058 ; repeated-measures ANOVA, $F_{(2,180)} = 1.738$; $p = 0.179$; task 2: day 1, 1.345 ± 0.06 ; day 2, 1.337 ± 0.059 ; repeated-measures ANOVA, $F_{(2,180)} = 1.273$; $p = 0.282$), indicating that neural activity in each task is stably maintained across time and intervening behaviors. Data represent mean $\pm$ SEM. Asterisks denote significant differences.

ron pairs, then FC should be different on the left versus right side of the maze.

Three different analyses reveal that this may not be the case. First, the comparison of FC between left and right sides of the maze during task 1 performance did not produce a significant difference ($n = 2243$ neuron pairs recorded in the overtraining phase, paired t test, $t_{(2242)} = -0.006$, $p = 0.995$). Second, the variation in FC across the two regions of the maze was compared with the variation in FC of the same region of the maze. Specifically, we compared FC on the left versus right side of the maze, and, as a positive control, we divided neuronal activity data on the right side of the maze into two halves and compared FC of each neuron pair across two epochs. In both cases, as expected, significant correlation was detected (Fig. 8A,B) (left vs right, $r = 0.435$, $p < 0.000$; right vs right, $r = 0.502$, $p < 0.000$; $n = 2243$ neuron pairs, linear regression analysis). We then calculated the difference in FC under two conditions for all neuron pairs (right vs left and right vs right) and compared their distributions. The two distributions did not vary significantly (Fig. 8C,D) (paired t test,

$t_{(2242)} = 0.881$, $p = 0.378$). Third, in addition to FC, we compared Δ FC from left versus right side of the maze during phase I learning. If afferent neuronal activity is an important factor determining synchronous firing of PFC neuron pairs, then Δ FC measured in one part of the maze should not correlate with Δ FC measured in another part of the maze. However, there was a significant correlation between the two measures (Fig. 8E) ($n = 1899$ pairs, $r = 0.407$, $p < 0.000$, linear regression analysis). Although we cannot completely exclude the possibility of unknown afferents influencing activities of recorded PFC neuron pairs, the present results suggest that the observed FC changes (Fig. 3) cannot be fully explained by altered afferent neural activity.

Discussion

It is widely believed that memories are stored in the brain by altering synaptic strengths and thereby forming new cell assemblies that become capable of functioning together (Hebb, 1949). A crucial evidence to support Hebb's hypothesis is the demonstration that FC among neurons changes during learning and that the changes are stably maintained for a long time. Our study provides supporting evidence for Hebb's hypothesis by showing that (1) FC changes in parallel with behavioral learning, (2) FC changes repeatedly during multiple episodes of learning, and (3) changed FC is stable for a long time. To demonstrate these findings, we used a long-term ensemble recording technique to measure FC among PFC neurons as rats learned two different spatial navigation tasks over multiple days. Behavioral data clearly show that rats learned a specific behavioral task rule on a figure-8-shaped maze, and, while simultaneously retaining and expressing that information across days, animals accurately acquired a different task rule on the same maze. Because alternation turning (task 1) and unilateral turning (task 2) shared common features (e.g., maze, reward locations), there were indications of both generalized learning from task 1 to task 2 and retroactive interference during the initial transitions from task 1 to task 2. In parallel to behavior, ensemble recording data show that pairs of neurons in PFC demonstrated robust correlated firing changes (both increases and decreases) during the early phase of training (when the learning rate was maximal) but not during the latter phase of training (when learning reached asymptote). This pattern was repeatedly observed in the course of phase I and II sessions. Once animals were overtrained, FC remained similar regardless of neural activity. Together, these findings suggest that specific experience-dependent, long-lasting changes in FC among PFC neurons are involved in the formation of memory trace.

In principle, synchronous firing can be induced by local interactions and/or common afferent inputs. However, it is unlikely that the present finding of learning-induced changes in FC can be accounted solely by altered afferent neural activity. First, the majority of synaptic connections in the cortex arise from nearby neurons (Peters, 1987). Second, if afferent neuronal activity strongly influences synchronous firing of PFC neuron pairs, then FC values from the left versus right side of the maze should be different because afferent neural activities are expected to be different in the two regions, which was not the case. Third, not only FC but also Δ FC during learning was similar between left and right sides of the maze. Fourth, changes in FC were observed in the course of learning regardless of behavioral task (alternating vs unilateral) and behavioral state (baseline vs performance). For these reasons, learning-associated changes in synaptic plasticity in the PFC likely account for the observed changes in FC. This view is consistent with evidence of synaptic plasticity in the PFC

(Hirsch and Crepel, 1990; Laroche et al., 1990; Herry et al., 1999; Gemmell and O'Mara, 2000; Kim et al., 2003) and findings indicating that PFC encodes specific task rules (Winocur and Moscovitch, 1990; Jung et al., 1998; Wallis et al., 2001; Genovesio et al., 2005; Johnston and Everling, 2006). A major caveat of this interpretation is the fact that direct learning-induced changes in synaptic plasticity (i.e., behavioral stimulus or motor-evoked synaptic changes that parallel memory formation) have yet to be demonstrated in the PFC (but for other mnemonic structures, see Rogan et al., 1997; Rumpel et al., 2005; Whitlock et al., 2006).

Interestingly, during the course of learning, both enhancement and reduction of FC were observed such that the net change in FC was near 0. The present result is somewhat different from those reported by previous studies in which the overall pattern of FC was an enhancement (Ahissar et al., 1992; Wilson and McNaughton, 1994; Schoenbaum et al., 2000). However, considering that the enhancement of FC was short lasting and observed in overtrained animals, this pattern of change may reflect a process different from the encoding of long-term memory, such as short-term synaptic plasticity. In contrast, long-lasting changes in FC associated with encoding of long-term memory may involve both enhancement and reduction so that overall FC is maintained constant. In line with this possibility, other studies have shown that synaptic weight increases in some synapses are compensated by co-occurrence of synaptic weight decreases in other synapses (Lynch et al., 1977; Royer and Pare, 2003). We also found that long-term potentiation induction in the hippocampal CA3 region altered FC among CA3 neurons, but the changes were bidirectional and thus yielded no net change in overall FC (Yun et al., 2007). A number of neural network models use similar normalization algorithms to maintain overall synaptic weights constant and provide a robust synaptic model for information storage (Arbib, 1995). Such long-lasting changes may overlap with short-lasting FC changes but may have been undetected in previous studies. Conversely, because the FC change was assessed over days in our study, short-lasting FC changes may have been undetected. Additional studies are necessary to determine whether the observed “zero-sum” change in FC is a general characteristic underlying long-term memory encoding.

The present study also provides supporting evidence for the theoretical concept that memories are represented in a distributed and overlapping manner in a network of neurons by changing synaptic strengths among neurons (Marr, 1971; Kohonen, 1977; Hinton and Anderson, 1981; Hopfield, 1984; McClelland and Rumelhart, 1986; McNaughton and Morris, 1987; Rolls and

Figure 7. FC remained similar across tasks 1 and 2 in the overtraining phase. **A**, Example cross-correlograms of task 1 (blue) and task 2 (red) during the overtraining phase. Dotted horizontal lines indicate the expected level of cross-correlation between two independent Poisson processes with equivalent firing rates. Spatial firing rate maps of the neuron pair are also shown. Although neuronal activity of one neuron changed, cross-correlograms were similar across two tasks. **B**, Group data. The distributions of FC were similar in tasks 1 and 2. **C**, Each point in the scatter plot denotes FC of a neuron pair in session 1 (abscissa) and session 2 (ordinate). The point above and below the 45° line denotes enhanced and reduced FC, respectively, in session 2 compared with that in session 1. FC was compared for T1–T1, T1–T2, and T2–T2 pairs of halved sessions. **D**, Each point in **C** was converted into the difference in FC. The distributions of FC differences were similar across T1–T1, T1–T2, and T2–T2 session pairs. **E–G**, No significant correlation between the similarity of neuronal activity and FC difference. Each point in the scatter plot denotes the sum of task 1 versus task 2 firing rate map correlations (ordinate) and the difference in FC of a neuron pair between tasks 1 and 2 (abscissa). Analyses were limited to the central section in **F** and to the rest of the maze excluding the central section in **G**.

Treves, 1998). This theory predicts (1) that changes in synaptic weight (FC) should be detected during each episode of learning, and (2) that, once multiple patterns of memory are stored, partially overlapping patterns of neuronal activity will be retrieved out of a common set of synaptic weights. Consistent with the first prediction, we observed that FC changed repeatedly as new learning transpired. However, because of the technical difficulty in recording the same pair of neurons all through learning tasks 1 and 2 (>2 weeks), we were not able to ascertain whether FC between the same neuron pairs changes continuously in the course of learning different tasks. As an alternative, different populations of neurons were recorded in an overlapping manner in the present study. Nevertheless, our results suggest rather strongly that synaptic weight changes take place associated with

Figure 8. Similar values of FC and Δ FC on different parts of the maze. **A**, FCs measured on the left and right sides of the maze in task 1 were compared. **B**, Neural data on the right side of the maze (task 2) were divided into two halves, and their FCs were compared. **C**, **D**, The data in **A** and **B** were converted into differences in FC, and their distributions are plotted. No significant difference was found between the two distributions. **E**, This plot shows Δ FC measured from left versus right side of the maze during phase I training, which were significantly correlated. The 45° line is shown in each scatter plot.

each episode of new learning. Also, in agreement with the second prediction, different activity patterns emerged out of the same FC in the overtraining phase. Specifically, some neurons exhibited similar activity patterns across the two tasks, whereas others showed radically different activity patterns (that is, partially overlapping patterns of activity). As detailed in our analysis, different neural activity across the two tasks cannot be attributed to differences in sensory input, motor output, or unknown time-dependent variables. Furthermore, neural activities representing task 1 and task 2 rules were stably maintained and retrieved reliably across multiple days with intervening behaviors. Conversely, despite the difference in neuronal activities for two tasks, FC remained similar. These results are consistent with the hypothesis that partially overlapping patterns of neural activity are retrieved out of a common set of functional connectivity. It should be noted that the present results do not disprove the possibility of local representation (Barlow, 1972). Nonetheless, in combination with other available evidence (Rolls and Treves, 1998), our results provide strong supporting evidence that multiple patterns of memory are represented in a distributed and overlapping manner in a network of neurons.

In conclusion, this study showing learning-induced changes in FC in PFC neurons provides crucial evidence for the notion that memories are stored in the brain by altering patterns of synaptic strengths among coactivated neurons (i.e., Hebb's cell

assembly hypothesis). The finding that the magnitude of FC changes was large during the early stages of training when learning rate was maximal but became progressively smaller as learning reached asymptote is also consistent with learning theories and connectionists models espousing that fundamental changes in associative mechanisms exhibit a negatively accelerated rate of change (Rescorla and Wagner, 1972; Mackintosh, 1975; Pearce and Hall, 1980; Sutton and Barto, 1998; Christian and Thompson, 2003). Hence, FC may be an important component or electrophysiological marker of associative mechanisms underlying negatively accelerated learning.

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