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Various firing patterns of many cortical neurons represent information processing in the brain. The microarchitecture of synaptic connections control information processing in cortical circuits. The structure and location of synapses determine and modify the strength of this information processing. The aim of our laboratory is to reveal how information is formed, maintained, selected, and decoded in the brain at the levels of single cells and of single synapses. To do so, we mainly use two-photon microscopy that allows us to see fluorescence signals from deep within living tissue, developing novel photostimulation methods and animal behavioral tasks. The goals of our recent studies are to reveal how voluntary movement is memorized and represented in cortical circuits. One of the most important problems in neuroscience is how a variety of spatio-temporally heterogeneous neural activity in the cortex emerges moment-by-moment at multiple stages of a movement.

I. Two distinct layer-specific dynamics of cortical ensembles during learning of a motor task.

The primary motor cortex (M1) possesses two intermediate layers upstream of the motor-output layer: Layer 2/3 (L2/3) and layer 5a (L5a). Although repetitive training often improves motor performance and movement coding by M1 neuronal ensembles, it is unclear how neuronal activities in L2/3 and L5a are reorganized during motor task learning. We conducted two-photon calcium imaging in the mouse M1 during 14 training sessions of a self-initiated lever-pull task (Figure 1).

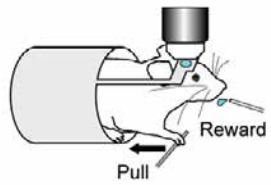

Figure 1. Schematic showing the self-initiated lever-pull task for a head-restrained mouse.

Mice were trained over 14 sessions (1 h/d) to use their right forelimb to pull a lever over a distance of 5 mm for 700 ms to acquire a water reward. Training was initiated 2–3 weeks after injection of an adeno-associated virus (AAV) 2/1 encoding GCaMP3 into the left forelimb M1. Two-photon calcium imaging was performed on the left forelimb M1 of 9 mice without apparent deterioration of the neuronal responsiveness over repeated sessions in L2/3 or L5a.

We assessed the effect of repetitive training on the neuronal coding of lever movement because the lever movement provided a good representation of forelimb movement. We determined whether the accuracy of the lever trajectory predicted from neuronal ensemble activity improved with learning. To evaluate the predictive information carried by the neurons ($I_{ensemble}$), we calculated the mutual information between predicted and recorded lever trajectories. In L2/3, there was no significant change in $I_{ensemble}$ from early sessions (sessions 1-4) to late sessions (sessions 11-14; Figure 2). By contrast, in L5a, $I_{ensemble}$ was significantly higher in the late sessions than in the early sessions (Figure 2). No consistent changes in L2/3 $I_{ensemble}$ were observed as learning progressed, whereas increases in L5a $I_{ensemble}$ were associated with improved task performance.

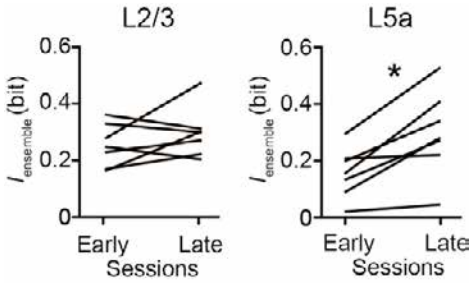

Figure 2. $I_{ensemble}$ averaged over early and late sessions for each field in L2/3 (left; n=7) and L5a (right; n=7). *$P < 0.05$.

Next, to determine how each neuron changed its predictive information (I_{single}) during learning, we analyzed I_{single} of neurons. Neurons that increased or decreased I_{single} across sessions were defined as increase- or decrease-neurons, respectively. In L2/3, there was a similar proportion of increase- and decrease-neurons (Figure 3), suggesting that the overall change in L2/3 I_{single} was balanced across all imaged fields. By contrast, in L5a, there was a higher proportion of increase-neurons than decrease-neurons (Figure 3). Many L5a increase-neurons displayed an increase in activity associated with lever-pull movements during learning. Increase-neurons were critical for the correlation between L5a $I_{ensemble}$ and task performance.

Each neuron within a field in a given session was ranked according to I_{single} and the rank was then normalized between 1 (top) and 100 (bottom). The $I_{ensemble}$ of the top 20% of ranked neurons was larger than the $I_{ensemble}$ of the bottom 50%

Note: Those members appearing in the above list twice under different titles are members whose title changed during 2014. The former title is indicated by an asterisk (*).

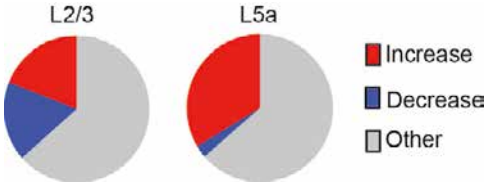

Figure 3. Proportion of increase-, decrease-, and other-neurons across all fields in L2/3 (left) and L5a (right).

of ranked neurons in > 85% of sessions in L2/3 and L5a. Thus, the top 20% of ranked neurons were considered to be highly ranked neurons that strongly contributed to the ensemble representation. L2/3 increase-neurons rose through the ranks from the early sessions to occupy 31% of the top 20% of ranked neurons in the late sessions. Forty-six percent of the top 20% of ranked neurons in the early sessions were also in the top 20% of ranked neurons in the late sessions. We call these neurons stable, highly ranked neurons. The mean activity of the stable, highly ranked neurons in L2/3 during successful lever-pull movements did not significantly change from the early to late sessions. These results suggest that L2/3 $I_{ensemble}$ was maintained by balanced activity of increase- and decrease-neurons, and by activity of stable, highly ranked neurons. In contrast to L2/3, the rank of L5a increase-neurons gradually increased during the middle to the late sessions, and increase-neurons accounted for 73% of the top 20% of ranked neurons in the late sessions. In L5a, only 23% of the top 20% of ranked neurons in the early sessions were in the top 20% of ranked neurons in the late sessions. These results indicate that the increase in L5a $I_{ensemble}$ was supported by the conversion of increase-neurons into highly ranked neurons during the middle to late sessions.

We also found that 31% of the examined L5a crossed corticostriatal (CCS) neurons were increase-neurons and 3% were decrease-neurons. Of the top 20% of the ranked CCS neurons observed in the early sessions, only 17% were in the top 20% of the CCS neurons observed in the late sessions. Twenty-six percent of examined L5a corticospinal (CSp) neurons were increase-neurons and 5% were decrease-neurons. Of the top 20% of the ranked CSp neurons observed in the early sessions, 100% were in the top 20% of the CSp neurons observed in the late sessions. These results suggest that increase-neurons were more prevalent than decrease-neurons for both L5a CCS and CSp neurons and that a subset of L5a CCS neurons was likely recruited into the newly formed ensemble that represented the lever-pull movement.

The balanced changes in L2/3 neurons could reflect the fact that L2/3 neurons with highly plastic activity process a variety of information from other areas of the brain to maintain a constant overall level of network activity for homeostasis. L2/3 neurons may play an important role in rapid acquisition of novel movements or rapid adaptation to some disturbance by combining sensory feedback and motor primitives. As such, L2/3 may work as a driver for motor output throughout learning. L5a $I_{ensemble}$ increased during learning, and this was maintained by a gradual increment in I_{single} ranks of a subset of L5a neurons. These neurons were directly associated with lever-pull movement and presumably worked as a potent driver for motor output in the late sessions. Changes in L5a neurons in M1 may represent the formation of motor memory that accompanies memory transfer from the prefrontal area to M1 and the basal ganglia. Stable, highly ranked CSp neurons may be directly associated with muscle movements or motor primitives. The distinct dynamic networks in L2/3 and L5a, the two intermediate layers of M1, are clearly core elements that drive the L5b motor output for well-learned movements.

II. Reward-timing-dependent bidirectional modulation of cortical microcircuits during optical single neuron operant conditioning

Animals rapidly adapt to environmental change. To reveal how cortical microcircuits are rapidly reorganized when an animal recognizes novel reward contingency, we conducted two-photon calcium imaging of layer 2/3 motor cortex neurons in mice and simultaneously reinforced the activity of a single cortical neuron with water delivery.

Head-restrained mice were trained to perform a self-initiated lever-pull task using the right forelimb before single-neuron operant conditioning by two-photon calcium imaging (2pSNOC). After 5–14 lever-pull task training sessions, two-photon calcium imaging of L2/3 motor cortical neurons was performed during SNOC. Each 2pSNOC session consisted of three periods: a pre-conditioning period (10 min), a conditioning period (15 min), and a post-conditioning period (10 min). 2pSNOC was performed during the conditioning period. Active neurons during the pre-conditioning period were reconstructed and classified into two groups: neurons with high activity during lever-related periods, and other neurons ("lever-unrelated neurons"). One neuron was targeted in the conditioning period. During the conditioning period, the mouse performed a 2pSNOC task. During the 2pSNOC task, water drops were successfully delivered to the mouse immediately after the $\Delta F/F$ of a single target neuron increased above a threshold (Figure 4).

During the conditioning period, for lever-unrelated target neurons, activity increased by approximately 50%, whereas, for lever-related target neurons, activity did not change (Figure 5). The reward frequency increased by approximately 50% when lever-unrelated neurons were targeted, and did not change when lever-related neurons were targeted. The rapid operant conditioning of single lever-unrelated neurons was successful even though the activity of non-target neurons slightly changed. When lever-related neurons were targeted, the mouse performed goal-directed lever-pull movements from the onset of the conditioning period without recognition of SNOC. This

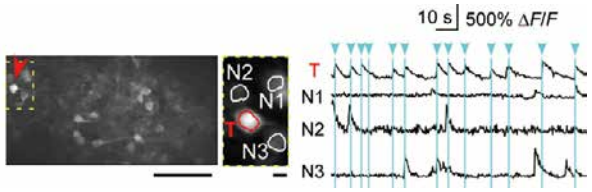

Figure 4. Left: A time-averaged image of a L2/3 field during 2pSNOC. Red arrowhead, a target neuron. Scale bar, 100 μm. Middle: Expanded image of the rectangle in left. Scale bar, 10 μm. Right: $\Delta F/F$ of the target (T) and the three neighboring (N1, N2, and N3) neurons. Cyan bars, reward delivery.

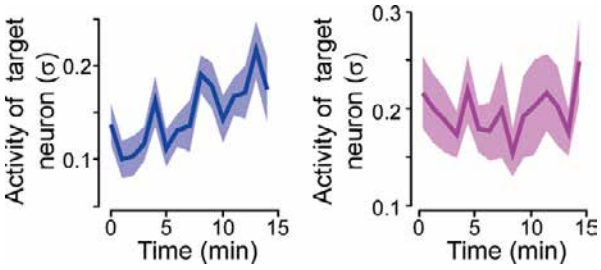

Figure 5. Time course of the activity of lever-unrelated (left; n=11) and lever-related (right; n=13) target neurons during the conditioning period. The activity is Z scored for each neuron. Thick lines and shading indicate the mean ± s.e.m.

may explain why the activity gain (the ratio of the mean activity in the last 5 min of the conditioning period to the mean activity in the first 5 min of the conditioning period minus 1) for lever-related target neurons was not significantly positive. In the following analyses, only data from sessions with a lever-unrelated target neuron were used.

Non-target neurons whose activity was temporally, but not spatially, associated with the target neuron increased their activity. Non-target neurons should have been affected by the water delivery because it was the reinforcer. For each non-target neuron, the ratio of the sum of the activity in a 0.33-s time bin t s after reward delivery to the sum of the remaining activity in the same bin was defined as the reward synchronization index (t) (RSI(t)). For each time bin, activity gain was averaged across the non-target neurons with RSI values in the top 5%. The activity gain of non-target neurons with RSI in the top 5% was significantly larger than zero when t ranged from –0.33 s to 0.33 s, and significantly smaller than zero when t was 2.3, 3, and 4 s. We refer to this phenomenon as reward-timing-dependent bidirectional modulation (RTBM), and refer to neurons that had RSI (–0.33 s to 0.33 s) in the top 5% as SR neurons and neurons that had RSI (2 s to 4 s) in the top 5% as AR neurons. The activity gain of SR and AR neurons was substantially positive and negative, respectively. The percentage of lever-related and lever-unrelated neurons was similar between SR and AR neurons. The distance of SR and AR neurons from the target neuron was similar to the distance of other neurons from the target neuron. The pairwise correlation with the target neuron was higher for SR neurons than for AR neurons and other neurons during both the conditioning period and pre-conditioning period without water delivery. This indicates that SR neurons were frequently active together with the target neuron. By contrast, AR neurons did not appear different from other non-target neurons in terms of their associations with the target neuron. Neither SR nor AR neurons were specifically related to licking. Likewise, neither SR nor AR neurons were specifically related to lever-pull in response to the reward delivery.

To validate whether the activity timing relative to the reward delivery is sufficient to induce the neuronal activity changes, we performed repetitive pairing of the activity of a random set of neurons and reward delivery with different time intervals. In each photostimulation session, 60 photostimuli were delivered during a 15 min period to induce firing in channelrhodopsin-2-expressing neurons in the field of view and a reward was given 0.25 s after or 2.5 s before each photostimulation (potentiation protocol and depression protocol, respectively). The photostimuli-induced activity of photostimuli-responsive neurons significantly increased and decreased in the potentiation and depression protocols, respectively (Figure 6). The activity of the other neurons did not change in either protocol. Thus, repetitive pairing of neuronal activity with reward delivery at different time intervals was sufficient to recreate RTBM.

Our results suggest that the microcircuit has the capability to strengthen the activity of neurons that were previously included in the same ensemble without strong spatial constrains. RTBM may be one of possibly many processes that underlie rapid reorganization of the L2/3 cortical microcircuit during fast adaptation to environmental changes, which occurs during BMI/BCI learning, motor adaptation, and skill learning.

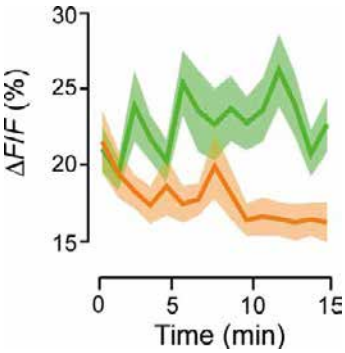

Figure 6. Time course of the mean $\Delta F/F$ of photostimuli-responsive neurons in the potentiation (green, n=78 neurons) and depression (orange, n=40 neurons) protocols. Thick lines and shading indicate the mean ± s.e.m.

Publication List

〔Original papers〕

- Hira, R., Ohkubo, F., Masamizu, Y., Ohkura, M., Nakai, J., Okada, T., and Matsuzaki, M. (2014). Reward-timing-dependent bidirectional modulation of cortical microcircuits during optical single neuron operant conditioning. Nature Commun. *5*, 5551.
- Masamizu, Y., Tanaka, Y.R., Tanaka, Y.H., Hira, R., Ohkubo, F., Kitamura, K., Isomura, Y., Okada, T., and Matsuzaki, M. (2014). Two distinct layer-specific dynamics of cortical ensembles during learning of a motor task. Nature Neurosci. *17*, 987-994.