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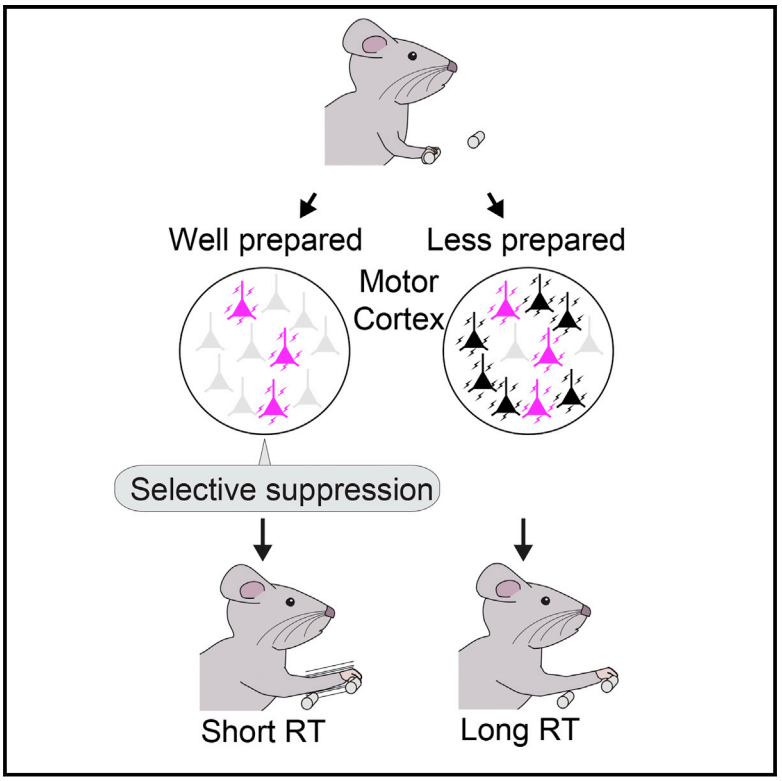
# Selective Suppression of Local Circuits during Movement Preparation in the Mouse Motor Cortex

## Graphical Abstract

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## In Brief

After establishing a delayed reaching task in mice, Hasegawa et al. identify a specific pattern of motor circuit activity during motor preparation. These circuit activity patterns are mediated by the suppression of a majority of neurons in the motor cortex and may be important contributors to motor preparation.

## Highlights

- We developed a reaching task to model motor preparation in mice
- A distinct group of motor cortex neurons increased activity during a waiting period
- Other motor cortex neurons suppressed activity during a preparation period
- Specific patterns of circuit activity and suppression accompany motor preparation

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# Selective Suppression of Local Circuits during Movement Preparation in the Mouse Motor Cortex

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## SUMMARY

**Prepared movements are more efficient than those that are not prepared for. Although changes in cortical activity have been observed prior to a forthcoming action, the circuits involved in motor preparation remain unclear. Here, we use in vivo two-photon calcium imaging to uncover changes in the motor cortex during variable waiting periods prior to a forepaw reaching task in mice. Consistent with previous reports, we observed a subset of neurons with increased activity during the waiting period; however, these neurons did not account for the degree of preparation as defined by reaction time (RT). Instead, the suppression of activity of distinct neurons in the same cortical area better accounts for RT. This suppression of neural activity resulted in a distinct and reproducible pattern when mice were well prepared. Thus, the selective suppression of network activity in the motor cortex may be a key feature of prepared movements.**

## INTRODUCTION

Voluntary movements can be facilitated by prior preparation (Coles, 1989; Rosenbaum, 1980). Such motor preparation is often manifested as a shorter reaction time (RT; quicker response) to execute a particular movement. For example, subjects can respond more quickly when instructions are given in advance and when a signal to initiate an action is presented after a certain waiting period (e.g., "ready, set, go" in a 100-m dash in a track and field competition).

Preparation for a forthcoming movement is thought to be achieved through changes in cortical activity during the waiting period (Wise, 1985). Previous studies have examined cortical neuronal activity during motor preparation using extracellular unit recording mainly in primates (Crammond and Kalaska, 2000; Riehle and Requin, 1989; Tanji and Evarts, 1976; Wise, 1985). These studies used behavioral paradigms in which the instruction cues were separated from subsequent execution cues by a waiting period. During the waiting period, neurons in the primary motor cortex (M1) and premotor areas showed increased firing rates (Tanji and Evarts, 1976; Wise et al., 1983). The increased neural activity during the waiting period is thought to reflect motor preparation based on the assumption that cortical neurons have a trigger threshold to initiate motor actions (Hanes and Schall, 1996; Riehle and Requin, 1989). In other words, motor preparation coincides with the increased firing rate to reach the trigger threshold. This interpretation, however, refers to a narrow subset of cortical neuronal activity. More recent studies have examined a new aspect of motor preparation in the framework of population activity in the motor areas. For example, it has been proposed that motor control behavior becomes well prepared for an intended action when cortical neurons exhibit specific patterns of activities (Afshar et al., 2011; Churchland et al., 2006; Michaels et al., 2015). However, the nature of the activity patterns and the information processing mechanisms required for efficient motor preparation remain unclear at the level of local circuits.

To understand the circuit mechanisms underlying motor preparation, we visualized and quantified the activity patterns of cortical neurons on a large scale using in vivo calcium imaging of a population of motor cortical neurons in mice (Komiyama et al., 2010; Masamizu et al., 2014; Peters et al., 2014). A previous study showed robust preparatory activity of neurons just before mice initiated a licking behavior (Guo et al., 2014;


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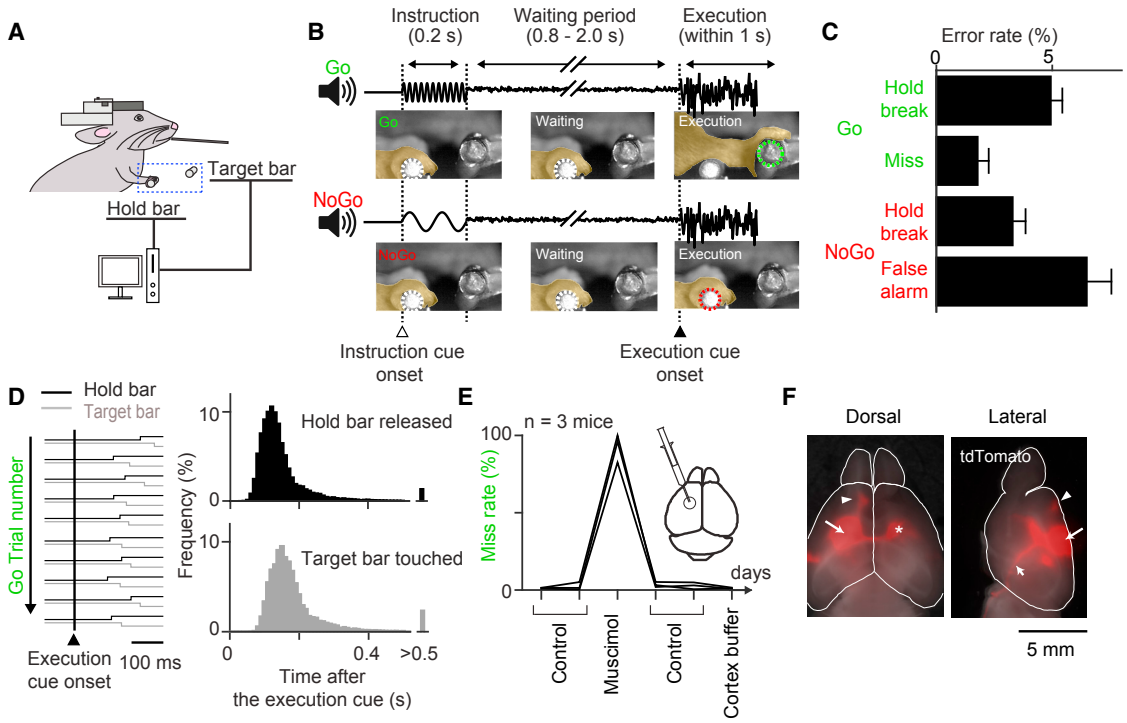



**Figure 1. Delayed Go/NoGo Reaching Task Controlled by the Mouse Motor Cortex**

(A) Schematic of the experimental design. A head-fixed mouse touches either a hold bar or target bar to obtain a water reward from a spout. The dotted square shows a schematic view of the paw images shown in (B).

(B) Task structure. Sound trace and images at the top show Go trials, and those at the bottom show NoGo trials. The dotted circles in the images indicate the correct forepaw position for each time point. In the Go trials, the right forepaw moved from the hold bar to the target bar after the execution cue was presented (indicated in green). In the NoGo trials, the forepaw stayed at the hold bar during the trial (indicated in red).

(C) Percentages of different types of errors. The errors consisted of 4.9% hold-break and 1.8% miss trials in Go trials (top two rows) and 3.3% hold-break and 6.5% false alarm in NoGo trials (bottom two rows) (n = 16 mice). Error bars indicate mean ± SEM.

(D) Distribution of the time between execution cue onset and forepaw movements. Left: examples of square-wave signals from the hold bar and target bar in ten representative trials from a single session. The mouse responded quickly in some trials but slowly in others. Square-wave signals indicate the electrical signal from the hold bar (black lines, lower position, touched; upper position, released) or from the target bar (gray lines, lower position, touched; upper position, not touched). Because the target bar was placed far from the hold bar, the mice always released the hold bar before they touched the target bar. Right top: distribution of the time between execution cue onset and forepaw movements defined as the release of the hold bar (top). Right bottom: time between execution cue onset and touch of the target bar (bottom).

(E) Injection of muscimol into the M1 impairs the reaching behavior. This sequential experiment consisted of 2 days of control experiments, 1 day of muscimol injection, 2 days of control experiments, and 1 day of cortex buffer injection. Injection of muscimol significantly increased the miss rate ($p < 0.02$ for all three mice), whereas injection of cortex buffer did not affect performance ($p > 0.9$ for all three mice).

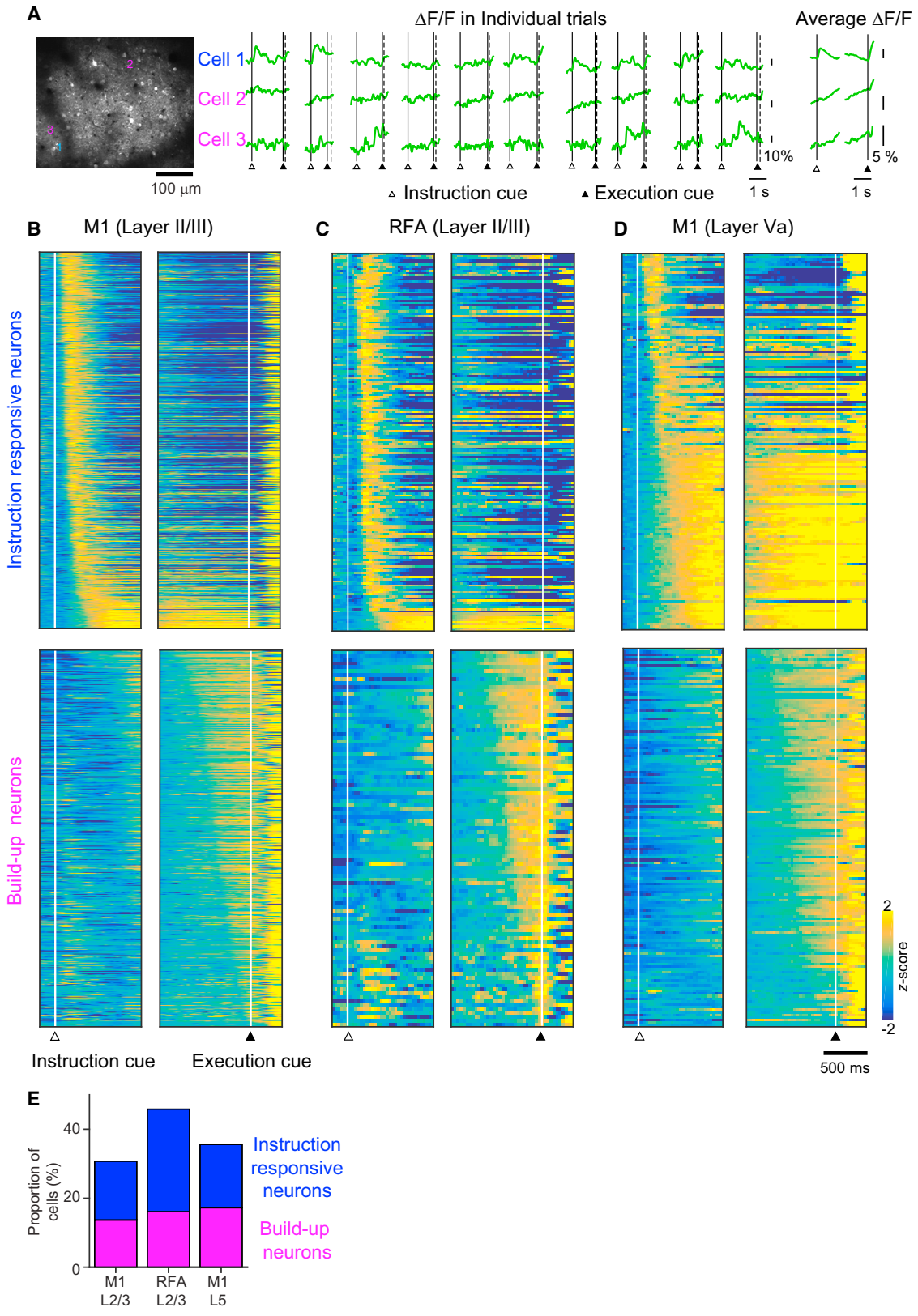
(F) Whole-brain images of M1 projections. Virus (AAV2/1-EF1a-tdTomato) was injected into the M1 as an anterograde tracer, and transparent brains were prepared by Clear, Unobstructed Brain Imaging Cocktails and Computational Analysis (CUBIC). Left: dorsal view. Right: lateral view. Long white arrows, M1; arrowheads, RFA; asterisk, contralateral M1; short arrow, cortico-spinal pathway.

Li et al., 2015). In this study, we investigated how preparatory activity is related to motor performance at the circuit level. For this goal, we developed a reaching task with variable waiting periods, which allowed us to determine whether the mouse was prepared by measuring RT (Churchland et al., 2006). When the RT was short, we assumed that the mouse was well prepared during the waiting period, and when the RT was long, the mice were unprepared. We found that preparation during the waiting period was accompanied by selective suppression of neural networks rather than activation of specific neurons in the motor cortex. Our findings suggest that the suppressive state of the local circuits might improve the signal-to-noise ratio for specific neural activity that increases during preparation.

## RESULTS

### Delayed Go/NoGo Reaching Tasks in Mice

To investigate the neural circuit activity underlying the preparation of voluntary movements, we developed a behavioral paradigm for mice: a delayed Go/NoGo reaching task. This task consisted of a series of auditory cues (instruction, waiting period, and execution; Figures 1A and 1B; Movie S1). Each trial began with a brief auditory instruction cue (200 ms; Figure 1B) that signaled the mice to prepare for a specific behavior depending on the sound: a high-frequency tone (14 kHz) signaled the mice to reach a target bar with their forepaw (Go), and a low-frequency tone (6 kHz) signaled the mice to withhold the forepaw

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movement (NoGo). Immediately after the instruction cue, we presented a weak white noise to indicate a waiting period (0.8–2.0 s) for the forthcoming movement. We then presented a louder white noise as an execution cue to signal the mice to perform the prepared movements. After several weeks of training, the mice demonstrated a high degree of success (>90% accuracy) with few errors (Figure 1C). The high success rate, albeit with the randomized waiting period, suggests that the mice did not rely on internal timing to initiate the movement. Indeed, even when the mice encountered rare trials with a prolonged waiting period (3.0 s, only 5%–10% of total trials, n = 3 mice), they successfully performed the task without prior training (94.1%, 94.4%, and 100% in trials with a prolonged waiting period; 97.9%, 96.7%, and 98.4% in normal trials; $p > 0.85$ for all three mice, Pearson's chi-square test). Hold break error rates were similarly low (2.9%, 5.6%, and 0.0% versus 0.6%, 3.0%, and 0.8% and $p > 0.15$, $p > 0.4$, and $p > 0.7$ for the three mice), indicating that the mice relied on the execution cue to initiate forepaw movements.

Although mouse performance was accurate in the majority of trials, the RTs varied from trial to trial (Figure 1D). The RT, defined as the delay between execution cue onset and hold bar release, was an average of 150.5 ms ± 90.8 ms (mean ± SD). Based on previous studies (Churchland et al., 2006; Riehle and Requin, 1989), we assumed that the variability in RT reflected how well the mice were prepared; when the RT was short, the mice were well prepared, and when the RT was long, the mice were less prepared. Because RT can be defined only in Go trials for which mice execute prepared movements, our analysis on network activity and RT focused on Go trials. Performance on this task, especially in Go trials, was significantly impaired both by reversible inactivation (Figures 1E; Figure S1A; Movie S2) and by chronic lesion (Figure S1B) of the M1.

## Classification of Neural Activity during the Waiting Period

In pursuing the networks responsible for preparation during the waiting period, we examined not only the M1 but also the rostral forelimb area (RFA) for three reasons. First, the RFA has strong connections with the M1 (Figure 1F; Rouiller et al., 1993). Second, electrical stimulation of the RFA can evoke forepaw movements (data not shown; Neafsey et al., 1986). Third, both the M1 and RFA (or premotor area in primates) are considered to be involved in motor preparation in rodents and primates (Murakami et al., 2014; Riehle and Requin, 1989; Smith et al., 2010; Tanji and Evarts, 1976).

We investigated the relationship between preparation and local circuit activity in discrete populations of M1 and RFA neurons using in vivo two-photon calcium imaging (Hira et al., 2013; Komiyama et al., 2010; Masamizu et al., 2014; Peters et al., 2014; Sato et al., 2007; Stosiek et al., 2003). We expressed a genetically encoded calcium indicator (GCaMP6m) in a large population of neurons using adeno-associated virus (AAV) vectors (AAV2/1-syn-GCaMP6m) (Figure 2A, left) to image neural activity. The majority of imaging sites were in layer II/III of the M1 and RFA (n = 48 imaging planes for the M1 from 11 mice and n = 14 for the RFA from three mice, 100–300 μm in depth), and the rest were in layer Va of the M1 (450–600 μm in depth, n = 19 from five mice). The waiting period evoked increased neuronal activity (Figure 2A, right) in a substantial percentage of both M1 and RFA neurons. We classified these neurons into three groups based on their temporal activity patterns: "instruction-responsive neurons," which responded to the onset of the instruction cue and maintained or decreased neuronal activity during the waiting period (Figures 2B–2D, top); "build-up neurons," which increased neuronal activity during the waiting period (Figures 2B–2D, bottom); and "other neurons," which did not show a consistent increase in activity during the waiting period. The RFA contained a larger percentage of instruction-responsive neurons compared with the M1 (Figure 2E; M1 L2/3 17.0%, RFA 29.7%, M1 L5 18.3%; $p < 0.001$ for both M1 L2/3 versus RFA and M1 L5 versus RFA, Pearson chi-square test). The percentage of build-up neurons was similar in the RFA and M1 (M1 L2/3 13.7%, RFA 16.1%, M1 L5 17.3%; $p > 0.05$, Pearson chi-square test).

## Circuit Activity during Motor Preparation

Increased activity during the waiting period is considered to represent preparation for intended movements (Dorris et al., 1997; Riehle and Requin, 1989; Wise, 1985). Because we observed such an increase in build-up neurons, we first examined how this activity would change when mice were well prepared for a movement. Our analysis was based on the assumption that trials with shorter RTs are indicative of more preparation versus trials with longer RTs. There are two scenarios in which build-up neurons might enhance the signal-to-noise ratio when an upcoming movement is well prepared. In the first scenario, build-up neurons would show higher activity when the RT is shorter, correlating directly with how well the mice are prepared (Dorris et al., 1997; Hanes and Schall, 1996). In the second scenario, the activity pattern of build-up neurons would be unaffected by the preparation, but the activity of the remaining neurons (instruction-responsive and other neurons) would be

**Figure 2. Identification of Instruction-Responsive and Build-up Neurons in the M1 and RFA**

(A) Left: a representative two-photon image of neurons in the motor cortex. Right: responses of an instruction-responsive neuron (Cell 1) and two build-up neurons (Cells 2 and 3) in ten trials. Solid lines indicate the time of instruction cue (white triangles) and execution cue (black triangles), and dashed lines indicate the time when the mouse released the hold bar. The right two columns indicate the average responses aligned to the instruction cue onset (left) and the execution cue onset (right).

(B–D) Normalized average activity (*Z* score) from all instruction-responsive (top) and build-up (bottom) neurons in M1 layer II/III (621 instruction-responsive neurons and 500 build-up neurons of 3,650 neurons in 11 mice) (B), RFA layer II/III (177 instruction-responsive neurons and 96 build-up neurons of 596 neurons in three mice) (C), and M1 layer Va (151 instruction-responsive neurons and 142 build-up neurons of 823 neurons in five mice) (D). The colors are in *Z* score. The cells were sorted based on the onset of the activity.

(E) Proportions of instruction-responsive (blue) and build-up (magenta) neurons in M1 layer II/III, RFA layer II/III, and M1 layer Va. The RFA contained more instruction-responsive neurons than M1 layer II/III or layer Va ($p < 0.001$). The proportion of build-up neurons was similar between the cortical areas ($p > 0.05$).

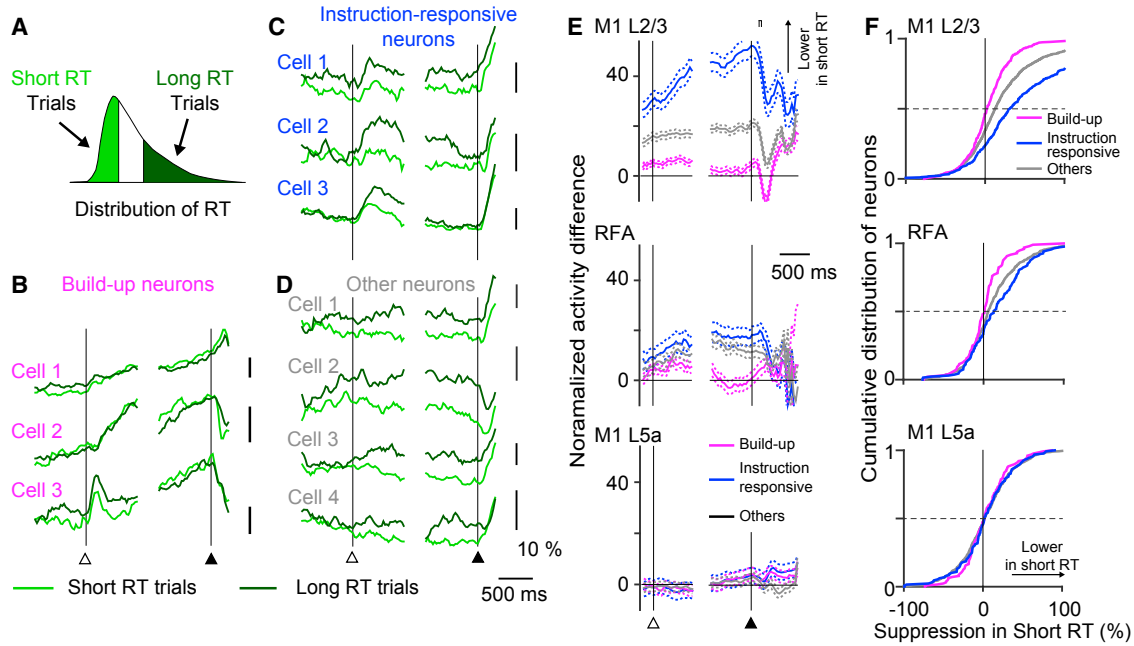


**Figure 3. Selective Suppression of Neural Activity during Motor Preparation**

(A) Schematic distribution of RTs in one imaging session. Based on the RT, the top 33% of the trials were defined as short RT trials (light green) and the bottom 33% as long RT (dark green) trials. The mean activities were computed for each of these two groups as in (B)–(D).

(B) Averaged neural activities for short and long RT trials for three representative build-up neurons in the M1.

(C) Same as (B) for three instruction-responsive neurons in the M1.

(D) Same as (B) for neurons that were neither build-up nor instruction-responsive neurons in the M1.

(E) Time courses of normalized activity difference between short and long RT trials (activity in long RT – activity in short RT). Note that up indicates lower activity in short RT trials. The activity was aligned to either the instruction cue (white triangle, left) or the execution cue (black triangle, right). Magenta lines represent build-up neurons, blue lines represent instruction-responsive neurons, and gray lines represent other cells. Solid and dashed lines indicate mean ± SEM. The tick mark at the top indicates the distribution of the RTs for short RT trials.

(F) Cumulative distribution of the normalized differences in the activity between short and long RT trials for each response type. Top: neurons imaged from M1 layer II/III (n = 390 build-up neurons, 394 instruction-responsive neurons, and 1,315 other cells). Center: neurons from RFA layer II/III (n = 96 build-up neurons, 135 instruction-responsive neurons, and 249 other cells). Bottom: neurons from M1 layer Va (n = 127 build-up neurons, 129 instruction-responsive neurons, and 470 other cells).

suppressed when the mice are more prepared. To distinguish between these two possibilities, we compared the activity of build-up, instruction-responsive, and other neurons between short RT (top 33%) and long RT (bottom 33%) trials (Sato et al., 2001; Sato and Schall, 2003; Figure 3). At the time of the execution cue, the activity of the build-up neurons was slightly reduced in short compared with long RT trials (M1 L2/3, 5.2% ± 1.6%, $p < 0.001$; RFA, 1.0% ± 2.7%, $p > 0.3$) (Figures 3B, 3E, and 3F), whereas instruction-responsive and other neurons exhibited stronger suppression compared with build-up neurons for both the M1 and RFA (M1, instruction-responsive, 51.2% ± 4.1%, $p < 0.001$ and other, 20.0% ± 1.4%, $p < 0.001$; RFA, instruction-responsive, 18.2% ± 3.4%, $p < 0.002$ and other, 11.4% ± 2.3%, $p < 0.02$; Figures 3C–3F). Such selective suppression was specific to layer II/III and was not observed in layer Va of the M1 (build-up, 3.8% ± 2.5%; instruction-responsive, 3.6% ± 3.0%; other, 2.4% ± 1.7%; $p > 0.3$; Figures 3E and 3F; a similar trend was observed until the initiation of movement; Figure S2). The selective suppression was unlikely to be mediated by parvalbumin (PV)- or somatostatin (SOM)-positive neurons because neither increased their activity in short RT trials (Figure S3C). The suppression in the instruction-responsive neurons depended on the strength of sustained activity following the instruction cue during the waiting period, with the neurons exhibiting weaker sustained activity more suppressed during short RT trials (Figure S4). Although the selective suppression we observed was based on the comparison between two groups of trials (well prepared and less prepared), preparation for the movement may not be all or none (binary). In fact, the relationship between neural activity and RT was rather gradual (Figure 4).

Does the suppressed activity in prepared trials derive from a global change in the brain state? We quantified global arousal by measuring pupil size (McGinley et al., 2015; Vinck et al., 2015) and did not observe a trend relative to RT, suggesting that the short and long RT trials do not originate from global arousal changes (Figure 5). We further confirmed the absence of global change in short RT trials by imaging neural activity in a different cortical region, the hindlimb area in the motor cortex, which is not involved in this task (Figure S1B, dotted lines). In this area, build-up neurons were rare (3.7%, 30 of 810 from three mice; significantly smaller than M1 layer II/III, RFA, and M1 layer Va, $p < 0.001$, Pearson's chi-square test), and

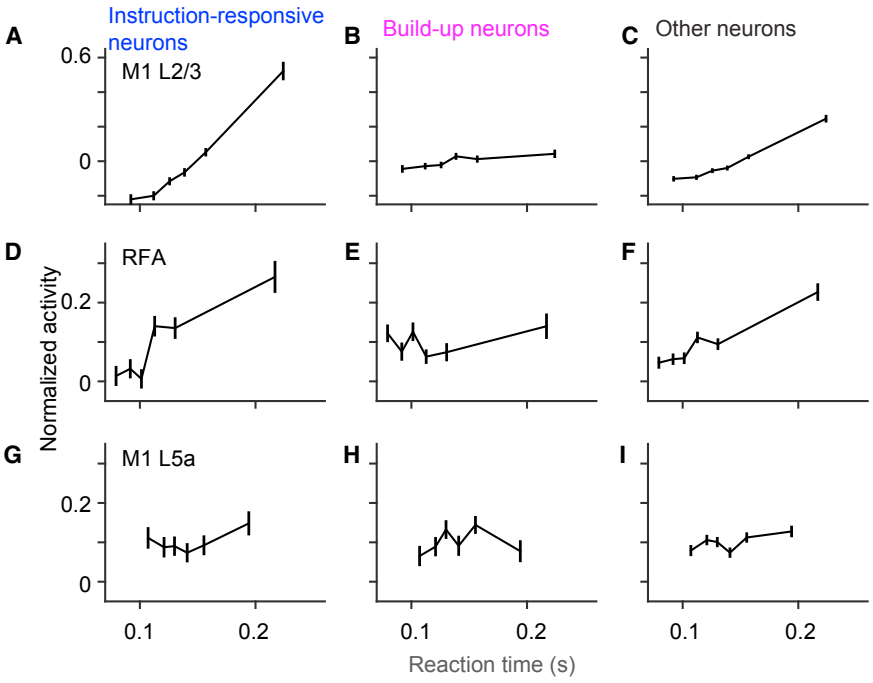


**Figure 4. Relationship between RT and Neural Activity**

(A–C). The mean normalized activity was analyzed as a function of time for instruction-responsive (A), build-up (B), and other (C) neurons in layer II/III in M1. Trials were classified into six groups based on the RT, and the mean normalized activity and RT were calculated for each group.

(D–I). Similar to (A)–(C), mean normalized activity was analyzed for the RFA (D–F) and for layer Va of M1 (G–I). The error bars indicate SEM. The activity of the second and fifth RT trial group were significantly different for M1 layer II/III instruction-responsive neurons ($p < 0.001$), M1 layer II/III other neurons ($p < 0.001$), RFA instruction-responsive neurons ($p < 0.005$), and RFA other neurons ($p < 0.05$) (Wilcoxon signed-rank test).

instruction-responsive and other neurons did not show reduced activity in short RT trials (Figure S5). These control experiments suggest that the suppression in short RT trials is specific to the forelimb area of the motor cortex and is not caused by global changes in cortical activity.

## Motor Preparation Elicited Reproducible Local Neuronal Activity

We next addressed how motor preparation modifies the reliability of local circuit activity. For this goal, we examined whether motor preparation leads to reproducible circuit activity patterns across different trials. We quantified the reproducibility of the network activity by calculating the average correlation coefficient of the population activity of neurons across trials for each imaging session (Peters et al., 2014; Figures 6A–6E). The correlation coefficient increased during the waiting period toward the execution cue in both short and long RT trials (Figures 6B and 6C), indicating that the local cortical circuits exhibited more similar patterns toward the end of the waiting period. At the time of execution cue onset, in layer II/III of M1 and RFA, correlation coefficients were higher (therefore, the circuit activities showed more reproducible patterns) in short-RT trials (M1 layer II/III, short RT 0.069 ± 0.004, long RT 0.047 ± 0.003, $n = 48$, $p < 0.001$; RFA short RT, 0.097 ± 0.016, long RT 0.064 ± 0.014, $n = 14$, $p < 0.001$) (Figures 6D and 6E; Figures S6A and S6B). In contrast, the correlation in layer Va was smaller (hence, the activity patterns were more variable) than that in layer II/III (M1 layer Va, short RT 0.025 ± 0.008, long RT 0.020 ± 0.007, $p < 0.001$ for comparisons with M1 layer II/III and RFA in both short and long RT response trials; Figure S6C). Furthermore, there was no significant difference in layer Va between short and long RT trials ($p > 0.08$). Thus, as the mice prepared for the movement, the network activity exhibited more reproducible patterns of activity in layer II/III of M1 and RFA but not in layer Va of M1.

We further analyzed how the circuit activity in layer II/III became more reproducible in short compared with long RT trials. To estimate the contribution of a specific group of neurons with particular activity patterns, the most straightforward experiment would be to manipulate the activity of the specific neurons and examine the correlates to the preparatory behavior. However, without being able to genetically identify these neurons, this approach is not practical. Instead, we exchanged the activities of a specific group of neurons between short and long RT trials and examined its effects on network activity by re-calculating correlation coefficients (Figure 6F). If a group of neurons contributed to the high reproducibility in short RT trials, then exchanging their activities would reduce the correlation coefficients. When we substituted the activity of build-up neurons between short and long RT trials while maintaining the activity of other cells (Figure 6F, top), the correlation coefficients were still higher for short RT trials (i.e., the reproducibility was still enhanced) (M1 layer II/III, short RT 0.066 ± 0.004, long RT 0.050 ± 0.003, $n = 44$, $p < 0.001$; Figure 6G, top; RFA, short RT 0.089 ± 0.016, long RT 0.070 ± 0.014, $n = 14$, $p < 0.001$). Thus, the activity of build-up neurons alone cannot be the basis for the increased reproducibility in short RT trials. Next, we exchanged the activity of instruction-responsive neurons between short and long RT trials. In this case, the correlation coefficients were no longer higher in short RT trials (M1 layer II/III, short RT 0.061 ± 0.004, long RT 0.057 ± 0.004, $n = 47$, $p > 0.20$; Figure 6G, bottom; RFA, short RT 0.085 ± 0.015, long RT 0.076 ± 0.016, $n = 13$, $p > 0.15$), suggesting that the suppression of the instruction-responsive neurons was critical for the increased reproducibility of the local network. Similar results were obtained when we exchanged other cells that showed reduced activity in short RT trials. These analyses indicate that the reproducible pattern of local circuits is not induced by the specific activity pattern of the build-up neurons but, rather, by the selective suppression of instruction-responsive and other neurons.

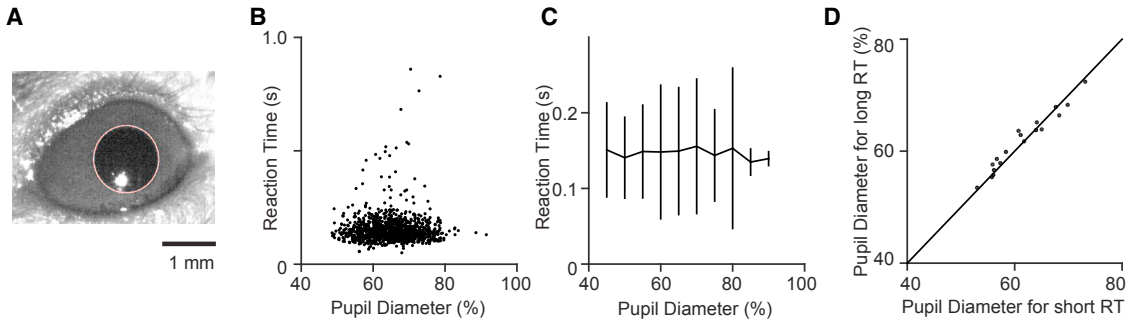


**Figure 5. The Relationship between Pupil Size and RT**

(A) The pupil was detected using a Canny edge detector followed by fitting circles (Sakatani and Isa, 2004).

(B) The relationship between pupil diameter and RT is shown for one animal ($r = 0.008$, $p > 0.75$, Pearson's correlation test, $n = 1,175$ trials). For the purpose of display, a small random fluctuation (0.7%) was imposed on the horizontal position of each data point.

(C) The relationship between pupil size and RT is shown for all three mice. The error bars indicate SDs. There was no correlation between pupil diameter and RT for any of the mice (for all the mice, $r = 0.015$, $p > 0.35$; for the individual mice, $p > 0.75$ for each of the three mice; Pearson's correlation test).

(D) The mean pupil size is shown for short and long RT trials from 18 sessions for all three mice. There was no significant difference ($p > 0.25$, Wilcoxon signed-rank test).

This finding was further supported when we analyzed the variability of activity patterns across different trials. First, at the level of individual neurons, we found that the variability of neural activity was smaller in short RT trials for instruction-responsive and other neurons compared with build-up neurons (Figure S7). Second, at the level of a population of neurons (imaged simultaneously), the network activity in two-dimensional representation (following dimensional reduction) was more clustered in short RT trials than in long RT trials (Figure 7A). This clustering was particularly prominent in instruction-responsive and other neurons (Figure 7B). These findings, together with the correlation analysis, suggest that the selective suppression of instruction-responsive and other neurons leads to reproducible network activity where the signal of build-up neurons is enhanced to prepare for the forthcoming movements (Figure 7C).

## DISCUSSION

Motor preparation can facilitate voluntary movements and make our behavior more effective. So far, it has remained unclear how motor preparation is implemented in neural network activity. Using a delayed reaching task and an in vivo calcium imaging technique, we discovered that preparation for the intended movement is associated with selective suppression of local motor circuits while maintaining the activity of specific neurons we characterized as build-up neurons (Figure 3). We observed selective suppression in layer II/III but not in layer Va, which resulted in a characteristic and reproducible activity pattern that was observed in motor circuits during motor preparation (Figure 6). Our findings on the network activity patterns that are modified by motor preparation prior to behavior provide new insights into the relationship between motor performance and local circuits (Figure 7C).

We probed motor preparation in mice using a delayed reaching task with variable waiting periods. This strategy presents a fundamental advantage when investigating preparatory activity: it minimizes the contribution of internal timing to initiate movement. Although internal timing would increase hold-break errors in randomized waiting periods, the hold-break rate turned out to be extremely low in our task (Figure 1C). Moreover, even when the waiting period was unexpectedly prolonged (3.0 s versus 0.8–2.0 s), the mice successfully waited until the execution cue was presented. Thus, to initiate forepaw movements in our task, the mice relied on the execution cue and not on internal timing.

Using this task, we quantified how well the mice were prepared based on a well established behavioral performance index: the RT for the subsequent movement (Rosenbaum, 1980). We found that the RT did not reflect the arousal level, which was defined by pupil size (McGinley et al., 2015; Vinck et al., 2015; Figure 5). This result implied that the mice might reach a certain arousal range in our task after going through prior steps (touching the hold bar and maintaining the position) to proceed to the execution cue. Therefore, the most straightforward interpretation is that the variability in RT reflects the motor preparation specific for the required movement. However, motor preparation in the present task may involve multiple factors, such as the expectation of the execution cue (Niemi and Naatanen, 1981) and the internal construction of motor programs (Rosenbaum, 1980), which can be distinguished by tailoring new behavioral designs. In this study, we included these factors altogether as motor preparation and assumed that the RT reflected the extent of motor preparation.

Previous studies in primates have investigated neuronal activity patterns associated with motor preparation (Churchland et al., 2006; Crammond and Kalaska, 2000; Hanes and Schall, 1996; Riehle and Requin, 1989; Tanji and Evarts, 1976). These studies reported increased neural activity in M1 and premotor areas during the waiting period. It has been proposed that motor preparation can be associated with a specific pattern of neuronal activity in the motor cortex (Churchland et al., 2006). Our study supports this hypothesis by demonstrating that motor preparation is accompanied by the formation of reproducible activity patterns in local circuits that are mediated by selective suppression of

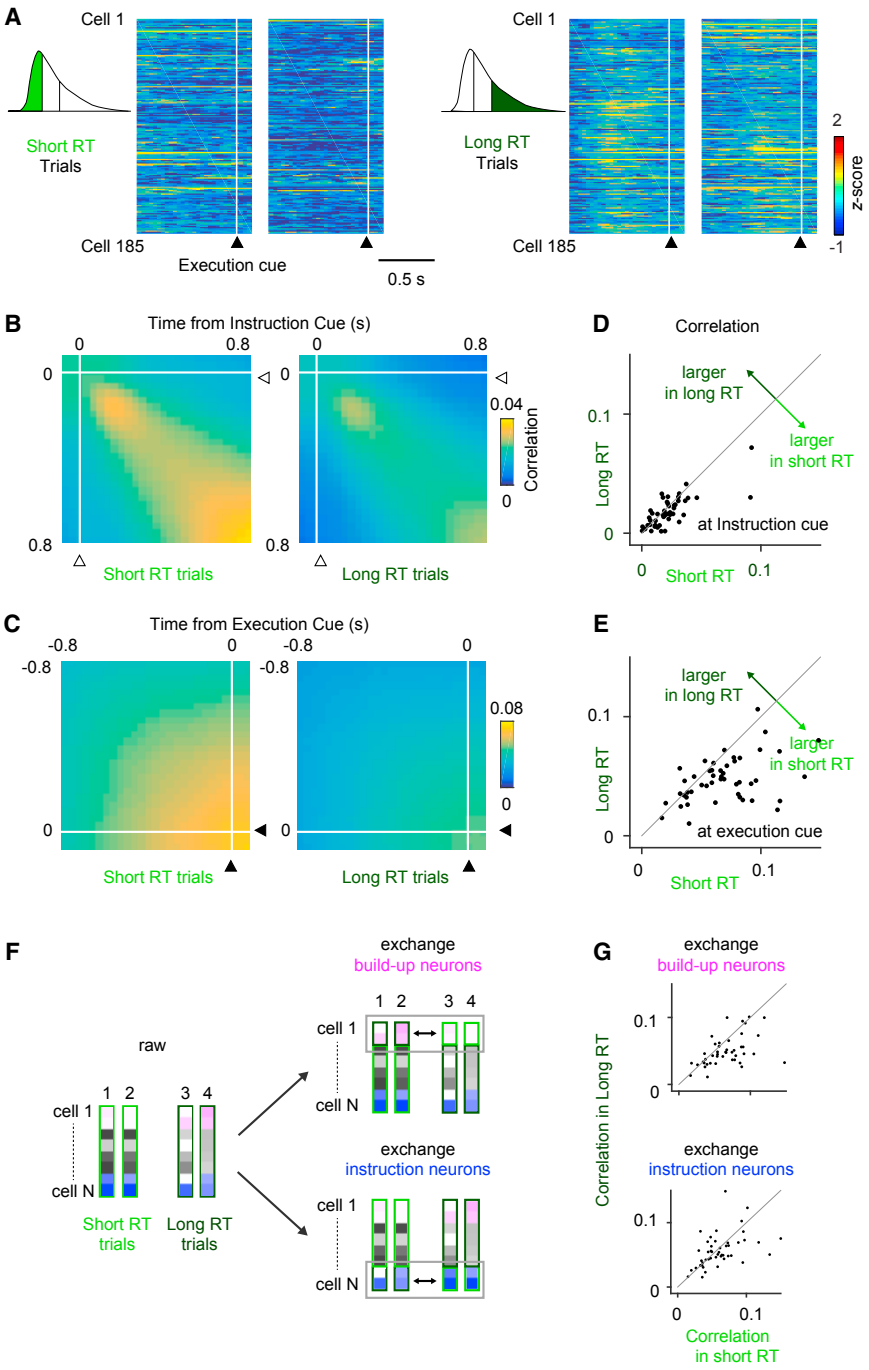


**Figure 6. Selective Suppression of Circuit Activity Enhances Local Information Processing for Motor Preparation**

(A) Activity patterns in the short and long RT trials in a single imaging session (185 neurons). Left: two representative activity patterns for the short RT trials. Right: two representative activity patterns for the long RT trials. The color scheme is in *Z* score.

(B and C) Correlation matrix of the activity states at different time points aligned on the instruction cue (B) or the execution cue (C). Within each imaging session, the correlation coefficients of population activity in each pair of trials were averaged for short and long RT trials separately. Left: correlation matrix for short RT trials. Right: correlation matrix for long RT trials.

(D and E) Correlation coefficients at the time of an instruction (D) or execution cue (E).

(F) Scheme for exchanging analysis. Left: the correlation coefficients were calculated for short and long RT trials separately as in (C). Top right: the correlation coefficients were re-calculated after the trials for build-up neurons were exchanged between short and long RT trials. In this calculation, the correlation coefficients for short RT trials were based on the activity of build-up neurons from long RT trials and other cells from short RT trials; those for long RT trials were based on the activity of build-up neurons from short RT trials and other cells from long RT trials. Bottom right: the correlation coefficients after the trials for instruction-responsive neurons were exchanged.

(G) Comparison of the correlation coefficients between short and long RT trials after trials were swapped for specific types of cells. Top: correlation coefficients were still larger in short RT trials after the trials were exchanged for build-up neurons. Bottom: when the trials were exchanged for instruction-responsive neurons, the difference between short and long RT trials was no longer seen.

specific cortical circuits. The mechanisms of the suppression are yet to be understood, but the suppression is unlikely to be induced by the two major subtypes of interneurons, PV-positive or SOM-positive interneurons (Pfeffer et al., 2013), because these neurons did not show increased activity in short RT trials (Figure S3C). The suppression might be caused by other types of interneurons (Pfeffer et al., 2013), or it might not depend on cortical inhibition (Freeman et al., 2002). In either case, the circuitry mechanisms for the selective suppression in the motor cortex appear to be different from the state-dependent sensory processing of information. For example, attentional or top-down modulation of visual information (Moran and Desimone, 1985) is known to be mediated by the activity of local interneurons, including SOM-positive interneurons (Fu et al., 2014; Makino and Komiyama, 2015; Zhang et al., 2014). Future studies of the circuitry mechanisms for motor preparation will be aided by genetic identification of build-up and other neurons and the manipulation of specific networks formed by these neurons.

The reproducible activity pattern in short RT trials was layer-specific, with selective suppression of local circuits confined to layer II/III (but not occurring in layer Va). Layers II/III and Va have been reported to modulate their circuits differently during learning of a new motor behavior, through which the activity of neurons in layer Va is changed to reflect the

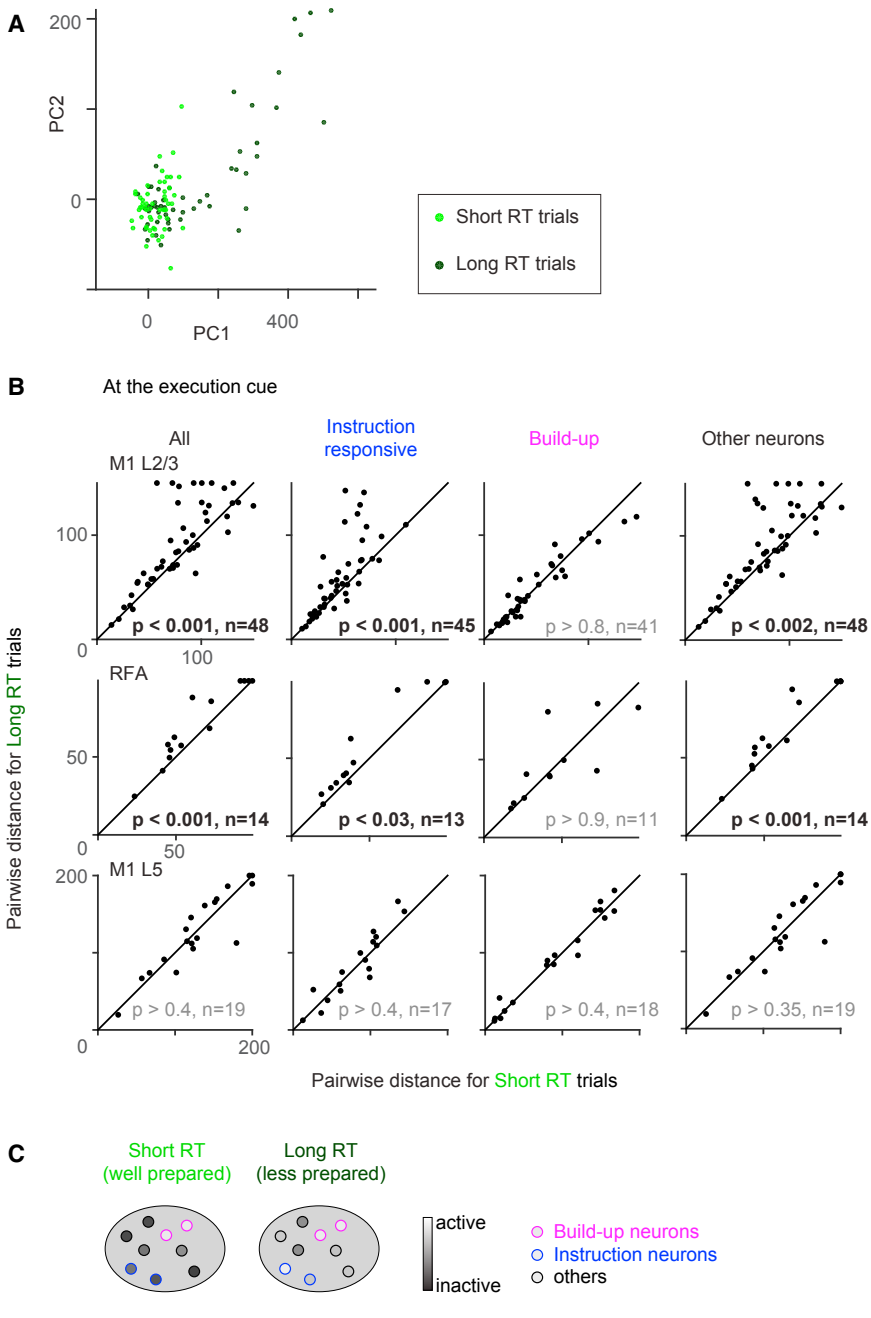


**Figure 7. Two-Dimensional Representation of Network Activity**

(A) An example of two-dimensional (2D) representation of the population activity states at the time of execution cue onset. Each dot represents one trial (light green: short RT trials, n = 54 trials; dark green: long RT trials, n = 53 trials). The abscissa and ordinate indicate the first two principle components. Note that the light green dots are more clustered than the dark green dots.

(B) Clustering in 2D representation at the time of execution cue onset was quantified by computing the mean pairwise distance among short RT (abscissa) and long RT (ordinate) trials. Each dot corresponds to one imaging session. Top row: imaging sessions in layer II/III of M1. Center row: layer II/III of RFA; Bottom row: layer Va of M1. First column: all cells in the imaging session. Second column: only instruction-responsive neurons. Third column: only build-up neurons. Fourth column: only other neurons. The pairwise distance was smaller for short RT trials for all cells and instruction-responsive neurons for layer II/III of M1 and the RFA (Wilcoxon signed-rank test) but not for layer Va of M1. p Values are shown in each plot.

(C) Summary schematic of the increased signal-to-noise ratio in short RT trials. Circles represent neurons, and the color in each circle reflects the neural activity level. The activities of build-up neurons are similar between short RT (left) and long RT (right) trials, whereas the activities of other cells are reduced in short RT trials. As a result, the relative signals of build-up neurons increased in short RT trials.

position of the forepaw (Masamizu et al., 2014). Thus, the neurons in layer Va, which mainly project to the striatum and other cortical areas, may be more involved in learning and reward rather than motor preparation. Although the circuitry mechanisms are currently unclear, the distinct activity patterns of layer II/III versus layer Va are particularly relevant when we compare our results with earlier studies that were based on extracellular unit recording; these studies tended to observe neurons from deeper layers of the cortex (Abeles, 2012).

Although we presented results based on unbiased monitoring of the activity from a large number of neurons through calcium imaging, our approach still has some limitations. First, the limited temporal resolution makes it difficult to probe the network dynamics changes between the onset of the execution cue and the initiation of the movements at a sufficiently fine timescale (Afshar et al., 2011). Second, we were not able to image neurons in layer Vb that convey direct output from M1 to the spinal cord. These two issues are of particular importance to understand how motor preparation is converted into movement execution and will be better addressed by multi-channel electrophysiological recording with optogenetic tagging.

A recent study by Peters et al. (2014) demonstrated that motor learning leads to a fewer number of active neurons with reproducible patterns. Consistent with this study, our findings also imply that the number of neurons required to prepare and execute learned movements could be small. These neurons would include build-up neurons (and some instruction-responsive neurons), which may constitute the signals of the local motor circuit. The extra activity in long RT trials in other non-essential neurons in layer II/III might contribute as noise, disturbing the precise control over concerted muscle movements, which might

be represented in downstream network activity. In contrast, when the mice were well prepared, the cortical circuit exhibited the suppression of noise or extra activity other than the essential ones that are required for signal processing. We speculate that such local suppression of distracting neurons might be one of the important processes underlying motor preparation in cortical circuits.

## EXPERIMENTAL PROCEDURES

### Animals and Surgery

All experimental procedures were carried out in accordance with the guidelines of the University of Tuebingen and National Center of Neurology and Psychiatry. Approval was obtained from the University of Tuebingen, the local government of Tuebingen, and the animal welfare committee of the National Center of Neurology and Psychiatry. Mice were group-housed (up to five mice per cage), and experiments were performed during the dark period of the 12-hr light/12-hr dark cycle. All mice were male and older than 8 weeks. Mice were implanted with a headpost for subsequent experiments under anesthesia (0.1 mg/g ketamine and 0.008 mg/g xylazine with a supplement of isoflurane). Dexamethasone (0.08 mg/kg) was administered as needed to reduce tissue swelling. Lidocaine was applied to the wound margins for topical anesthesia. A custom-built headpost was glued to the skull and subsequently cemented with dental acrylic (1230PNK, Lang Dental). No blinding was done in data collection and analysis.

### Behavioral Training in the Delayed Go/NoGo Task

After headpost implantation, mice were pre-trained to stay in a tube to obtain water and then trained for several weeks to perform a delayed Go/NoGo task (Figure 1). For this task, when the mice touched the hold bar, either a high- or low-frequency tone was presented as an instruction cue. The high-frequency tone (14 kHz, Go cue) instructed the mice to prepare their forepaw to reach the target bar after a waiting period, whereas the low-frequency tone (6 kHz, NoGo cue) instructed the mice not to move their forepaw. The instruction cue was followed by white noise for a random waiting period (~40 decibel [dB], 0.8–2.0 s, randomized, occasionally up to 3.0 s), after which the sound intensity of the white noise was increased to ~65–70 dB as an execution cue. Upon the execution cue, in the Go trials, the mice were required to touch the target bar within 1 s to obtain a water reward, otherwise the trial was considered an error (miss trial). The RT was defined as the time between the execution cue onset and the time when the mouse released the hold bar (Figure 1D). In the NoGo trials, the mice were required to maintain their paws at the hold bar for an additional 1 s following the execution cue. If the mice released the hold bar during this 1-s period, then the trial was considered an error (false alarm trial). The inter-trial interval was 3–5 s. The success rates of this session were compared between the trials with an 0.8–2.0 s waiting period and the trials with a 3.0-s waiting period using Pearson's chi-square test.

### In Vivo Two-Photon Calcium Imaging

After the behavioral performance reached an 80% success rate, the mice were anesthetized with isoflurane for virus injection and subsequent window implantation. A craniotomy (1.5–2 mm in a circle) was made over the M1 forelimb area (centered at anterior 0 mm, lateral 1.5 mm from the bregma), the RFA (centered at anterior 2.5 mm, lateral 1.0 mm from the bregma), or the M1 hindlimb area (centered at posterior 1.5 mm, lateral 1.5 mm from the bregma). Inside the craniotomy, the virus (AAV2/1-syn-GCaMP6m) was injected at multiple sites (20–40 nL per site, depth 200–300 μm for layer II/III or 400–500 μm for layer Va, 3–5 min per injection). In some of the PV-Cre and SOM-Cre mice, AAV2/1-CAG-Flex-tdTomato was co-injected with AAV2/1-syn-GCaMP6m to label PV+ and SOM+ interneurons, respectively.

Following virus injection, two layers of coverglass (1.0– to 1.5-mm circle for the small one, 2.0-mm square for the larger one) were implanted as an imaging window. The space between the imaging window and skull was sealed with 1.5%–2% agarose, and the window was cemented with dental acrylic. A few days after viral injection, the behavioral training was resumed, and 1–2 weeks later, calcium signals were measured with a two-photon microscope.

### Pupil Detection and Analysis

After behavioral performance reached an 80% success rate, the pupil size was monitored during the task (n = 3). The right eye was illuminated with infrared light-emitting diodes (LEDs) (850 nm) and imaged at 100 Hz with a complementary metal oxide semiconductor camera (DCC1240M, Thorlabs). An additional blue LED was positioned to provide low-intensity illumination. The precise timing of each frame was saved using custom-made software (written in Visual C++ with the Thorlabs software development kit).

The pupil was detected for each frame using a custom-written program in MATLAB in a similar way as in previous studies (McGinley et al., 2015; Sakatani and Isa, 2004; Vinck et al., 2015; Figure 5A). The pupil size was normalized to the maximum pupil size during the experiment for each animal.

### Statistical Methods

Statistical analysis was carried out using MATLAB, and the data value are shown as the mean ± SEM unless otherwise stated. The statistical significance of paired comparison was examined using Wilcoxon signed-rank test or bootstrap methods. The significance of non-paired comparison was checked using Wilcoxon rank-sum test or bootstrap methods. The performance of the mice was compared using Pearson's chi-square test (Figure 1E; Figure S1).

See the Supplemental Experimental Procedures for the full experimental procedures.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.043.

## AUTHOR CONTRIBUTIONS

T.R.S. conceived and designed the experiments. M.H., T.I., T.M., K.S., and T.R.S. performed the experiments. M.H., K.M., U.-R.A., N.C., M.I., T.K.S., Y.K., and T.R.S. analyzed the data. T.R.S., T.K.S., and M.I. wrote the paper with input from all other authors.

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