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## Overlearning hyper-stabilizes a skill by rapidly making neurochemical processing inhibitory-dominant

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### Abstract

Overlearning refers to the continued training of a skill after performance improvement has plateaued. Whether overlearning is beneficial is a question in our daily lives that has never been clearly answered. Here, we report a new important role: Overlearning abruptly changes neurochemical processing to hyper-stabilize and protect trained perceptual learning from subsequent new learning. Usually, learning immediately after training is so unstable that it can be disrupted by subsequent new learning, unless waiting for passive stabilization, which takes hours. However, overlearning so rapidly and strongly stabilizes the learning state that it not only becomes resilient against, but disrupts, subsequent new learning. Such hyper-stabilization is associated with an abrupt shift from glutamate-dominant excitatory to gamma-aminobutyric-acid-dominant inhibitory processing in early visual areas. Hyper-stabilization contrasts with passive and slower stabilization, which is associated with a mere reduction of an excitatory dominance to baseline levels. Utilizing hyper-stabilization may lead to efficient learning paradigms.

### Introduction

Continuous training conducted after performance improvement has been maximized is called overlearning. Musicians intuitively know that it is crucial to continue to practice the same music over and over again even after they have mastered the music. What is the benefit of overlearning? As early as 1885, Hermann Ebbinghaus, who pioneered experimental

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#### Author Contributions

K.S., Y.S., E.G.W., M.G.M., M.T., and T.W. designed the experiments. K.S., J.B., M.G.M., M.T., and L.C. conducted the experiments. K.S., E.G.W., M.T., and M.G.M. analyzed data. K.S., Y.S., J.B., E.G.W., and T.W. wrote the manuscript.

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effects between the first and new learning using a 2-interval-forced-choice orientation detection task (**Supplementary Fig. 2a**). The complete experiment consisted of 3 stages: pre-test, training including 2 phases, and post-test (**Fig. 1a, b**). A preliminary experiment ( $N=60$ ) had found that performance improvement for the orientation detection task plateaued around the 8th block of training (see **Supplementary Fig. 2b**) and that there was no significant performance improvement between the 8th and 16th blocks. Thus, we used 8 blocks in the first-training phase of Experiment 1 for the no-overlearning group ( $N=12$ , **Fig. 1a**) and 16 for the overlearning group ( $N=12$ , **Fig. 1b**). All other aspects of the experimental procedures were identical between the 2 groups: 2 training phases were separated by a 30-min interval and there were 8 blocks in the second-training phase. In each of these 2 training phases, a different orientation was used for the above-mentioned detection task. During the pre- and post-test stages, the signal-to-noise (S/N) ratio threshold was measured for the first-trained, second-trained, and untrained orientations. Performance improvement for each orientation was calculated as percent reduction in the S/N ratio threshold in the post-test relative to the pre-test stages. See Online Methods for more details about the task and stimuli.

If overlearning stabilizes the learning state, no interference with the first-trained learning by the second learning should occur with the overlearning group, whereas the interference should be observed with no-overlearning group. Results showed that this prediction was correct: overlearning greatly stabilized the first learning. Although with no overlearning the first learning was disrupted by the second learning, with overlearning the first learning disrupted the second learning (**Fig. 1c, d**). To test how overlearning in the first training influenced the magnitude of first and second learning, a two-way mixed-model ANOVA on performance improvement with factors of orientation (first-trained, second-trained, vs. untrained orientations) and group (no-overlearning vs. overlearning groups) was conducted. A significant main effect of orientation ( $F_{2,44}=6.106$ ,  $P=0.005$ ) and a significant interaction between orientation and group ( $F_{2,44}=5.736$ ,  $P=0.006$ ) were found. No significant main effect of group was observed ( $F_{1,22}=0.390$ ,  $P=0.539$ ). The significant interaction between orientation and group indicates that overlearning influenced the amounts of learning of the tested orientations in a different way than training with no overlearning.

Then, to test which orientation was learned in the 2 groups, a t-test was conducted on the amount of improvement in each orientation and each group. With the no-overlearning group (**Fig. 1c**), a significant performance improvement was found for the second-trained orientation (one-sample t-test,  $t_{11}=3.845$ ,  $P=0.016$  after Bonferroni correction for 6 comparisons), but not for the first-trained ( $t_{11}=1.120$ ,  $P=0.256$  without Bonferroni correction) or untrained orientation ( $t_{11}=0.987$ ,  $P=0.345$  without Bonferroni correction). Note that the preliminary experiment showed that 8 and 16 blocks of training on an orientation led to the similar amplitudes of performance improvements of the orientation (**Supplementary Fig. 2b**), indicating that learning occurs in these conditions unless a second training on a different orientation occurs. These results altogether are consistent with classical retrograde interference where the first learning is disrupted by the second learning<sup>4-9</sup>. That is, training with no overlearning rendered the learning state so plastic and unstable that it was vulnerable to interference by subsequent new learning. In contrast, with

the overlearning group (**Fig. 1d**), a significant performance improvement was found for the first-trained orientation ( $t_{11}=3.612$ ,  $P=0.025$  after Bonferroni correction for 6 comparisons), and not for the second-trained ( $t_{11}=1.432$ ,  $P=0.178$  without Bonferroni correction) or untrained orientation ( $t_{11}=1.277$ ,  $P=0.228$  without Bonferroni correction). These results are consistent with anterograde interference where the first learning disrupts the second<sup>5,8,19-21</sup>. Overlearning made the first learning so stable that it prevented interference from the second learning, and, to our surprise, instead interfered with the latter. Since 8 blocks of training took approximately 20 min and 16 blocks took about 40 min, we observed dramatic qualitative changes in the learning state which occurred as little as 20 min after the 8 blocks of training.

The results of Experiment 1 are consistent with the hypothesis that overlearning induces a learning mechanism that drastically changes neuronal plastic states to hyper-stabilized states (**Supplementary Fig. 1**). To protect existing learning from being replaced with new learning, hyper-stabilization may make the existing mechanism of learning so strongly stabilized that the learning supersedes new learning, that is, not only protects itself from being interfered with by, but also prevents the new learning from being established.

However, there are several alternative explanations. First, the anterograde interference observed in the overlearning group could be simply a byproduct of more training given initially compared to the second round of training (16 vs. 8 blocks). If so, then no anterograde interference should be observed when both orientations are trained for 16 blocks. However, the results of Control Experiment 1 ( $N=12$ , **Supplementary Fig. 3a**) demonstrated that anterograde interference still occurred when both orientations were trained for 16 blocks (**Supplementary Fig. 3c**). These results indicate that anterograde interference occurred due to overlearning in the first training and not because of more initial training.

Another possibility is that the mere amount of time passage during the 16 blocks in the first-training phase somehow made learning of the first-trained orientation hyper-stabilized and led to the anterograde interference. However, the results of Control Experiment 2 ( $N=12$ ) rejected this possibility. In this experiment, between the end of the first-training phase and the beginning of the second-training phase, no training was conducted for 50 min which was the same time interval as was taken for the 8 blocks of training and 30-min interval between the 2 training phases in Experiment 1 (**Supplementary Fig. 3b**). If the mere passage of time was responsible for the shift from plasticity to hyper-stabilization, then anterograde interference should have occurred. However, retrograde interference occurred (**Supplementary Fig. 3d**), which was inconsistent with the possibility.

The results of these experiments indicate that it is likely that the process of overlearning causes the dramatic shift from a post-training plastic state to a hyper-stabilized state, where prior VPL supersedes and inhibits subsequent VPL. To our knowledge, this is a novel role of overlearning, and it indicates that even after behavioral performance improvements are maximized and no more performance improvement is observed, overlearning can be beneficial to learning in the sense that it rapidly becomes stabilized and resilient against being interfered with another learning.

It takes hours for typical stabilization of learning to occur and that once this stabilization occurs the learning neither interferes nor is interfered with by subsequent new learning<sup>4,6,9</sup>. However, hyper-stabilization shown in Experiment 1 due to overlearning interferes with new learning. Such differential functions of typical stabilization and hyper-stabilization raise the following question: do these 2 types of stabilization include different functional mechanisms and occur separately? To address this possibility, in Experiment 2 the time interval between the first- and second-training phases was set to 3.5 hours (**Fig. 2a, b**), a lengthy amount of time which has experimentally been found to be sufficient for the first learning to stabilize<sup>4,6,9</sup>. There were no-overlearning ( $N=12$ ) and overlearning ( $N=12$ ) groups. All other aspects of the procedure were identical to those of Experiment 1. If neither retrograde nor anterograde interference occurred in either group, it would suggest that during the 3.5-hour interval after the first-training phase, typical stabilization occurred irrespectively of whether overlearning had occurred or not in the first-training phase. The results showed that this is the case. The patterns of results with the no-overlearning and overlearning groups (**Fig. 2c, d**) were similar to each other. To test whether similar patterns would be obtained between the 2 groups, a two-way mixed-model ANOVA on performance improvement with factors of orientation (first-trained, second-trained, vs. untrained orientations) and group (no-overlearning vs. overlearning groups) was conducted. A significant main effect of orientation ( $F_{2,44}=15.474$ ,  $P<10^{-4}$ ) was found. However, neither a significant effect of group ( $F_{1,22}=1.822$ ,  $P=0.191$ ) nor any interaction between the 2 factors ( $F_{2,44}=0.103$ ,  $P=0.902$ ) was observed. In the no-overlearning group, significant performance improvements were found for the first-trained (one-sample t-test,  $t_{11}=5.046$ ,  $P=0.002$  after Bonferroni correction for 6 comparisons) and second-trained ( $t_{11}=4.090$ ,  $P=0.011$  after Bonferroni correction for 6 comparisons) orientations, but not for the untrained orientation ( $t_{11}=0.334$ ,  $P=0.744$  without Bonferroni correction). Similarly, in the overlearning group performance improvements were significant for the first-trained ( $t_{11}=5.652$ ,  $P<10^{-4}$  after Bonferroni correction for 6 comparisons) and second-trained ( $t_{11}=4.566$ ,  $P=0.005$  after Bonferroni correction for 6 comparisons) orientations, but not for the untrained orientation ( $t_{11}=1.248$ ,  $P=0.238$  without Bonferroni correction). These results show that during the 3.5-hour time interval after the first-training phase, typical stabilization occurred for the first learning, irrespectively of overlearning. These results suggest that hyper-stabilization due to overlearning has different temporal properties compared to typical stabilization, as illustrated in **Supplementary Figure 1**.

What is the underlying neural mechanism of hyper-stabilization due to overlearning? The results so far suggest that the underlying neural mechanisms of the 3 post-training states, such as those after no-overlearning, overlearning, and 3.5 hours of time passage, are all different. It has been suggested that plasticity and stability depend on the amounts of excitatory and inhibitory signals and/or their ratio in brain regions involved in learning<sup>22-30</sup>. These studies raise the possibility that the above-mentioned 3 post-training states are associated with different ratios of excitatory to inhibitory signals. In Experiment 3, we tested this possibility by measuring the concentrations of glutamate and GABA in the early visual areas, which have been suggested to be involved in a number of types of VPL<sup>11-18</sup> using MRS (see Online Methods). The procedure with the no-overlearning ( $N=12$ ) and overlearning ( $N=12$ ) groups was the same in that MRS measurements were conducted (1)

before training for the pre-training baseline, (2) 30 min after training, and (3) 3.5 hours after training for typical stabilization. The only difference between the groups was that the number of blocks for training (8 vs. 16 blocks; **Fig. 3a, b**). Note that in Experiment 3, changes in the ratios of excitatory to inhibitory signals in the early visual areas (**Fig. 3c**) were measured to examine the effect of the first learning. Therefore, training of the second learning was not necessary. We calculated changes in the concentration of glutamate divided by changes in the concentration of GABA relative to the pre-training baseline as E/I ratio changes in the 3 different post-training states.

**Figure 3d** shows E/I ratio changes relative to the pre-training baseline for the no-overlearning and the overlearning groups. A higher E/I ratio than the pre-training baseline should indicate that the early visual areas became more excitatory than the baseline. A lower E/I ratio than the baseline should indicate that the early visual areas became more inhibitory than the baseline. First, to test whether there is any difference in the E/I ratio changes between the MRS measurement times or between the groups, a two-way mixed-model ANOVA was conducted on E/I ratio change with factors of time (30 min after vs. 3.5 hours after training) and group (no-overlearning vs. overlearning groups). A significant main effect of group ( $F_{1,22}=20.092$ ,  $P<10^{-3}$ ) and a significant interaction between time and group ( $F_{1,22}=4.620$ ,  $P=0.043$ ) were found. No significant main effect of time was observed ( $F_{1,22}=0.076$ ,  $P=0.785$ ). Given the significant interaction between the 2 factors, we further examined how the E/I ratios were changed in the time-course with and without overlearning separately. For the no-overlearning group (**Fig. 3d**, red), a significant quadratic trend was observed in the time-course of the E/I ratio changes ( $F_{1,11}=5.435$ ,  $P=0.040$ ). Furthermore, the E/I ratio change 30 min after 8 blocks of training was significantly greater than zero (one-sample t-test,  $t_{11}=3.064$ ,  $P=0.043$  after Bonferroni correction for 4 comparisons). However, 3.5 hours after training the E/I ratio change was not significantly different from zero ( $t_{11}=1.167$ ,  $P=0.288$  without Bonferroni correction). That is, the E/I ratio increased 30 min after the 8-block training without overlearning and returned close to the pre-training baseline 3.5 hours after the training. For the overlearning group (**Fig. 3d**, cyan), a significant quadratic trend was also observed in the time-course of the E/I ratio changes ( $F_{1,11}=8.207$ ,  $P=0.015$ ). In contrast to the no-overlearning group, the E/I ratio significantly decreased 30 min after the 16 blocks of training that led to overlearning (one-sample t-test,  $t_{11}=3.771$ ,  $P=0.012$  after Bonferroni correction for 4 comparisons), but then rebounded close to the baseline 3.5 hours after training ( $t_{11}=1.592$ ,  $P=0.140$  without Bonferroni correction). These results also suggest that hyper-stabilization is different from typical stabilization (**Supplementary Fig. 1**), for which E/I ratios returned close to the baseline at 3.5 hours after training.

We conducted various control analyses for reliability of the results. The MRS volumes were quite consistently placed at the same location across measurements (**Supplementary Table 2**). See **Supplementary Figures 4** and **5** for examples of spectra and their line-width, *Range of frequency drift* in Online Methods for the range of frequency drift, and *Comparison of shim values* in Online Methods for the shim values. During MRS measurements, subjects' eye fixation was constant (*Fixation task* in Online Methods). **Supplementary Figure 6** shows performance improvements in Experiment 3. No effect of testing order during the pre-

test stage on learning was found (*Control behavioral analysis* in Online Methods). We used glutamate concentrations as excitatory signals but combined concentrations of glutamate and glutamine also provided the similar results (**Supplementary Fig. 7a**). Concentration of creatine, a control metabolite, was consistent across measurements (**Supplementary Fig. 7b**). See *Exclusion of subjects* in Online Methods for the criteria for data exclusion.

Is there any possibility that E/I ratio changes merely depend on the amount of training, irrespective of whether learning occurs or not? In Control Experiment 3, we tested whether the E/I ratio changes under a training paradigm where VPL should not occur. When 2 different stimulus conditions are interleaved during training blocks, learning does not occur for either condition<sup>21,31,32</sup>. Subjects ( $N=7$ ) underwent 16 blocks of the interleaved training during which 2 different orientations were presented on alternating blocks (**Supplementary Fig. 8a**). All other aspects of the procedure were identical to those of Experiment 3. No significant performance improvements were found for any of the tested orientations, nor were any E/I ratio changes observed (**Supplementary Fig. 8b, c**). These results are consistent with the hypothesis that the E/I ratio changes depend on learning, and not merely on the amount of training.

Does the reduction in the E/I ratio due to overlearning found in Experiment 3 actually correlate with anterograde interference, which is indexed as hyper-stabilization? In Experiment 4, we used 2 training stages and measured both the degree of E/I ratio reduction and the performance improvement of the second learning after overlearning ( $N=12$ ; **Supplementary Fig. 9a**). Note that the performance improvement of the second learning should be inversely proportional to the amount of anterograde interference. We found both anterograde interference and a significant reduction of the E/I ratio (**Supplementary Fig. 9b, c**) in Experiment 4, which directly replicated the results of Experiments 1 and 3. More importantly, we found a significant correlation ( $r=0.64$ ,  $P=0.024$ ) between the degree of E/I ratio reduction and the degree of anterograde interference across subjects (**Supplementary Fig. 9d**). This result confirmed that the E/I ratio reduction underlies hyper-stabilization.

## Discussion

The results of all experiments consistently suggest that overlearning abruptly changes neurochemical processing to hyper-stabilize and protect trained perceptual learning from subsequent new learning.

Are the E/I ratio changes in the early visual areas governed by changes in levels of glutamate, GABA, or both? When subjects underwent overlearning, changes in GABA concentration were more prominent than changes in glutamate (**Supplementary Fig. 10a**). However, changes in GABA concentration levels alone were not significant and therefore were not as robust as the E/I ratio changes. This tendency was consistent with the results of Experiment 4, where the correlation between the amount of interference and changes in GABA concentration levels was moderately high but not significant ( $r=0.512$ ,  $P=0.089$ ). Some studies have indicated that the E/I ratio better accounted for the behavioral results than glutamate or GABA alone. First, the degree of stability of the early visual areas during a visual critical period depends on a balance between cortical excitation and inhibition<sup>24</sup>.

Second, glutamate is a precursor of GABA and therefore its concentration may not be completely independent from that of GABA<sup>10,33,34</sup>. Thus, it is possible that glutamate and GABA act harmoniously to modulate plasticity and stability, making the E/I ratio a more sensitive proxy for plasticity than a measure of either neurotransmitter alone. However, systematic future research is necessary to address this question.

At the same time, changes in each neurotransmitter also provide insights into cellular-level mechanisms. When only a small fraction of GABA is found in synaptic vesicles as opposed to the cytoplasmic pool<sup>35</sup>, what could account for a 16.3% increase in MRS GABA signals following overlearning? **Supplementary Figure 10a** indicates that in spite of such a large GABA signal increase after overlearning, glutamate changes were close to zero. On the other hand, without overlearning, glutamate signals increased by 14.8% (**Supplementary Fig. 10b**). Since the majority of GABA is formed directly from glutamate<sup>10,33,34</sup>, overlearning may trigger and/or facilitate the transformation of glutamate to GABA. A growing body of evidence indicates that MRS detects both extra- and intra-synaptic GABA<sup>34,36,37</sup>. Glutamate is also observed outside synapses<sup>38,39</sup>. These findings raise the possibility that overlearning transforms glutamate that exists outside as well as inside synapses to GABA in these regions, detected by MRS. However, the spatial resolution of MRS is so low that it is impossible to draw any definitive conclusion about cellular-level processing.

It has been found that if performance gain is saturated in the first phase of training, performance is greater after sleep following training, whereas without saturation in the first phase, no such post-sleep performance improvement is observed<sup>40</sup>. This suggests that the saturation of performance gain in early phases of training triggers consolidation during sleep. Does this process reflect a continuous hyper-stabilization effect? Our study show that the rapidly decreased E/I ratio measured 30 min after overlearning returned to the baseline 3.5 hours after overlearning. Thus, hyper-stabilization may not last longer than 3.5 hours. On the other hand, in the previous study<sup>40</sup>, the interval between the 2 training sessions was 24 hours. Although overlearning may play important roles in both hyper-stabilization during wakefulness and consolidation during sleep, mechanisms underlying these functions may not be the same.

Hyper-stabilization due to overlearning may be different from sensory adaptation due to prolonged or excessive exposure to a stimulus<sup>41-43</sup>. The effect of adaptation to an orientation is highly specific, not applying beyond  $\pm 30$  degrees from the adapted orientation<sup>44</sup>, whereas hyper-stabilization of VPL interfered with the learning of a second-trained orientation that was 60 degrees away from the first-trained orientation. These results suggest that the mechanism of hyper-stabilization is different from adaption. However, the stimuli used in this study were of 100% contrast with random noise that has power distributed across all orientations. Thus, the adaptation process, when centered at the trained orientations, could be broadband. If that was the case, we do not completely rule out the possibility that hyper-stabilization is related to some adaptation process. It has been suggested that VPL is associated with adaptive task-irrelevant sensory plasticity and task-related plasticity<sup>45</sup>. This raises the possibility that the E/I ratio dynamics reflect inhibitory processing where task-irrelevant sensory plasticity could be mainly involved.



In VPL as well as other types of learning and memory, there are several different phases in the time-course of development including encoding, typical stabilization, and consolidation during sleep. In some types of VPL, the early visual areas are involved in encoding, stabilization, and consolidation during sleep<sup>46-48</sup>. Hyper-stabilization due to overlearning is also involved in the early visual areas. Thus, at least in VPL all of these phases may be involved in the early visual areas. In other types of learning and memory, typical stabilization is involved in a different brain area than the encoding<sup>8,49,50</sup>. It is interesting to examine whether hyper-stabilization of these types of learning and memory, if any, occurs in the same area as in typical stabilization or encoding.

In summary, overlearning rapidly and strongly makes encoded VPL hyper-stabilized and supersedes subsequent new learning, in association with a rapid change of an E/I neurotransmitter ratio from higher to even lower than the pre-training baseline in the early visual areas (**Supplementary Fig. 1**). We conclude that overlearning is beneficial in the sense that it strongly protects newly trained learning from being overwritten by subsequent learning and supersedes the latter. To our knowledge, this is a new role of overlearning since the effect of overlearning on retention length was pointed out more than a century ago<sup>1</sup>.

## Online Methods

### Subjects

A total of 183 naïve subjects (18 to 34 years old, 69 males and 114 females) with normal or corrected-to-normal vision participated in this study. The Institutional Review Board of Brown University approved this study. All subjects provided their demographic information and written informed consents to participate. The experiments were conducted during daytime. Five subjects were excluded from the study in the middle of the MRS experiments (see *Exclusion of subjects* for details). Thus, data from a total of 178 subjects were analyzed in this study. Each subject participated in one experiment only.

The sample size for the behavior only experiments was determined by a power analysis ( $\alpha=0.05$ ,  $\beta=0.80$ ) on data collected in a pilot experiment in which subjects ( $N=12$ ) underwent the same procedure in the preliminary experiment with 8 blocks of training on the orientation detection task (**Supplementary Fig. 2a**). Results of the power analysis indicated that 9 subjects would be enough to obtain significant VPL of a trained orientation. However, since we planned to conduct a series of the behavior only experiments we conservatively selected the number of subjects for one group as 12. The effect size for the MRS experiments was not known in advance when we started the study. Thus, the sample size for the MRS experiments was chosen to match previous MRS studies with similar designs<sup>27,29,51</sup>.

### Stimuli

Oriented Gabor patches (**Supplementary Fig. 2a**; spatial frequency=1 cycle/degree, contrast=100%, Gaussian filter sigma=2.5 degrees, random spatial phase) were presented within an annulus subtending 0.75 to 5 degrees from the center of a gray screen. Gabor patches were spatially masked by a noise pattern using a pixel substitution method<sup>17,52</sup>.

Noise fields were generated from a sinusoidal luminance distribution at a given signal-to-noise (S/N) ratio. For example, in the case of a 10% S/N ratio, 90% of the pixels of the Gabor patch were replaced with the noise pattern. The orientation of the Gabor patch was 10, 70, or 130 degrees, with each  $\pm 60$  degrees difference.

### Orientation detection task

Subjects performed a 2-interval-forced-choice orientation detection task (**Supplementary Fig. 2a**), where one stimulus interval contained a Gabor patch with a certain S/N ratio and the other stimulus interval contained only noise (0% S/N ratio). Each trial started with a 500-ms fixation interval. After two 50-ms stimulus intervals separated by a 300-ms inter-stimulus interval, subjects were asked to report which stimulus interval contained the Gabor patch, by pressing “1” or “2” button on a keyboard. The temporal order of the target interval was randomly determined in each trial. Subjects were instructed to fixate on a white bull’s eye fixation point presented against a gray disk (0.75 degree radius) throughout each trial. The next trial started immediately after subject’s response. No feedback regarding the accuracy of a subject’s response was provided.

### Threshold measurement

A S/N ratio threshold for each orientation was measured using a standard 2-down 1-up staircase rule, which converges to a 70.7% accuracy rate. The threshold was measured in a blocked fashion. The initial S/N ratio was set to 25%. The step size of the staircase was 0.05 log units<sup>53</sup>. Each block ended after 10 staircase reversals, typically about 40 trials, taking approximately 2 min. The geometric mean of the last 6 reversals was taken as the S/N ratio threshold for that block<sup>53</sup>.

### Pre- and post-test stages

The pre- and post-test stages measured a subject’s S/N ratio threshold for each orientation. There were 6 possible combinations as to which orientation is tested in each of the 3 blocks of each test stage. In each test stage of each subject, one of the 6 combinations was randomly selected. A brief break was provided after each block upon a subject’s request.

Performance improvements after the training stage were calculated as percent reduction in the S/N ratio threshold measured during the post-test stage relative to the pre-test stage for each subject. Performance improvement  $Thresh_{imp}$  for each of the 3 orientations was calculated by

$$Thresh_{imp} = 100 \times \left( 1 - \frac{Thresh_{post}}{Thresh_{pre}} \right).$$

Here,  $Thresh_{pre}$  and  $Thresh_{post}$  represent S/N ratio thresholds in the pre- and post-test stages, respectively<sup>53</sup>. Note that this formula inverts the sign of the calculated threshold metric, such that a reduction in the S/N ratio threshold, which represents a performance improvement, is a positive number for interpretive convenience.

## Behavior only experiments (preliminary experiment, Experiments 1 and 2, Control Experiments 1 and 2)

The behavior only experiments consisted of 3 stages that occurred over 2 consecutive days: pre-test, training, and post-test stages. The pre-test and training stages were conducted on the first day, and the post-test stage was conducted on the second day.

**Preliminary experiment**—The purpose of the preliminary experiment was to estimate the amount of training that induces the saturation of performance improvements of the orientation detection task, so that effects of overlearning on stability of VPL would be tested in the main experiments (**Supplementary Fig. 2b**). Sixty subjects were randomly assigned to one of the 4-block, 7-block, 8-block, 9-block, and 16-block training groups ( $N=12$  each). During the training stage, subjects in the 4-block, 7-block, 8-block, 9-block, and 16-block training groups performed the orientation detection task with one orientation (trained orientation) for 4, 7, 8, 9, and 16 blocks, respectively. The trained orientation was selected from the 3 orientations and counter-balanced across subjects. The remaining 2 orientations served as untrained orientations.

**Experiment 1**—Twenty-four subjects were randomly assigned to either the no-overlearning ( $N=12$ ) or overlearning ( $N=12$ ) group (**Fig. 1**). During the training stage, subjects underwent 2 training phases (first- and second-training phases), which were separated by a 30-min time interval. In the first-training phase, subjects performed the orientation detection task for one orientation (first-trained orientation) for 8 blocks (no-overlearning group) or 16 blocks (overlearning group). In the second-training phase, subjects in both groups performed the task with another orientation (second-trained orientation) for 8 blocks. Two different orientations for the first- and second-training phases were selected from the 3 orientations and counter-balanced across subjects. The remaining orientation served as an untrained orientation.

In Experiment 1, we found no significant difference in the S/N ratio thresholds among the 3 orientations in the pre-test stage for the no-overlearning (one-way ANOVA with repeated measures,  $F_{2,22}=0.130$ ,  $P=0.878$ ) or overlearning ( $F_{2,22}=0.560$ ,  $P=0.579$ ) group. This result indicates no performance bias among the 3 orientations before training. This tendency was also found in all the other experiments.

**Experiment 2**—The procedures were identical to those of Experiment 1, except that the 2 training phases were separated by a 3.5-hour time interval (**Fig. 2**). Twenty-four new subjects were randomly assigned to either the no-overlearning ( $N=12$ ) or overlearning ( $N=12$ ) group.

**Control Experiment 1**—The procedures (**Supplementary Fig. 3a**) were identical to those of the overlearning group in Experiment 1, except that in the second-training phase subjects performed the orientation detection task for 16 blocks ( $N=12$ ).

**Control Experiment 2**—The procedures (**Supplementary Fig. 3b**) were identical to those of the no-overlearning group in Experiment 1, except that the 2 training phases were separated by a 50-min time interval ( $N=12$ ).

## Magnetic resonance spectroscopy (MRS) experiments (Experiments 3 and 4 and Control Experiments 3 and 4)

**Experiment 3**—There were 6 stages over consecutive 2 days (**Fig. 3**): pre-MRS, pre-test, training, post-MRS 1, post-MRS 2, and post-test stages. The first 5 stages were conducted on the first day, and the post-test stage was conducted on the second day. There were no-overlearning ( $N=12$ ) and overlearning ( $N=12$ ) groups. The procedures of the training stage were identical to those for the 8-block (no-overlearning) and 16-block (overlearning) training groups in the preliminary experiment. Only the first-training phase was conducted in the training stage of Experiment 3. See *MRS Stages* below for details of MRS procedures.

**Experiment 4**—There were 6 stages over consecutive 2 days (**Supplementary Fig. 9a**): pre-MRS, pre-test, first-training, post-MRS 1, second-training, and post-test stages. The first 5 stages were conducted on the first day, and the post-test stage was conducted on the second day ( $N=12$ ). The procedures of the first- and second-training stages were identical to those of the first- and second-training phases for the overlearning group in Experiment 1, except that the 2 training stages in Experiment 4 were separated by the post-MRS 1 stage, which took one hour.

**Control Experiment 3**—As in Experiment 3, there were 6 stages over consecutive 2 days (**Supplementary Fig. 8a**): pre-MRS, pre-test, training, post-MRS 1, post-MRS 2, and post-test stages ( $N=7$ ). The first 5 stages were conducted on the first day, and the post-test stage was conducted on the second day. In the training stage, there were 16 blocks. In each block, the presented orientation alternated between 2 different orientations (trained orientations 1 and 2). These 2 different trained orientations were randomly selected from the 3 orientations. The remaining orientation served as an untrained orientation.

**Control Experiment 4**—To estimate the range of the frequency drift for spectra obtained in the GABA scans, we employed 3 subjects for Control Experiment 4 in which the procedures were identical to those in Experiment 3, except that subjects did not undergo the pre-test, training, or post-test stage for behavioral measurements.

**MRS stages**—The MRS stages were conducted to measure the concentrations of glutamate and GABA in the early visual areas. Identical procedures were used during the pre-MRS, post-MRS 1, and post-MRS 2 stages. First, we measured a subject's high-resolution  $T_1$ -weighted anatomical brain structure. Second, based on the measured anatomical structure a voxel ( $2 \times 2 \times 2 \text{ cm}^3$ ) was manually placed on the most posterior part of the occipital lobe, to ensure that the voxel would cover the Calcarine sulci, which define the primary visual areas<sup>54</sup> bilaterally, but would minimize contamination from unnecessary tissues containing lipids. This voxel position was carefully replicated during the post-MRS 1 and post-MRS 2 stages by referring to the picture that shows both the anatomical brain structure and the voxel position in the pre-MRS stage. Overall, the voxels were overlapped by more than 90% in volume across the MRS stages (**Supplementary Table 2**). Third, shimming was performed using a vendor-provided automated shim tool. Automated shimming was followed by manual shimming to further improve the shim value (defined by a full width at half maximum of the water peak). See *Comparison of shim values* for

statistical comparisons of shim values. These first 3 steps (anatomical structure measurement, voxel placement, and shimming) took approximately 30 min. Fourth, a 774-sec GABA and a 384-sec glutamate scans were conducted to quantify the concentration of GABA and glutamate within the voxel (see *MRI data acquisition* and *MRS analysis* for details). The ( $\pm$ s.e.m.) mean line-width of N-acetylaspartate (NAA) across the MRS stages, scans, and subjects was  $8.757\pm 0.161$  Hz in Experiment 3 (**Supplementary Fig. 5**). Throughout the scans, subjects conducted a fixation task (see *Fixation task* for details). A brief break was provided between the scans upon a subject's request.

**Fixation task**—The fixation task was conducted during the GABA and glutamate scans to maintain subjects' fixation at the center of the display and to keep their vigilance and attention level constant across the scans and the MRS stages. During the fixation task, the color of the fixation point, in a gray background, changed from white ([R,G,B] = [255,255,255]) to faint pink ([R,G,B] = [255,255- ,255- ]) in an unpredictable timing, and then returned to white after 1.5 sec. The degree of color change was initially set to 40 for each scan. We confirmed that all subjects were able to clearly see the color change with this initial value. The mean ( $\pm$ s.e.m.) number of the color changes across the MRS stages and subjects were  $156.616 (\pm 0.675)$  for the 774-sec GABA scan and  $77.919 (\pm 0.621)$  for the 384-sec glutamate scan, respectively. The difference in the numbers of the color changes between the GABA and glutamate scans depended on the difference in durations of the GABA (774 sec) and glutamate (384 sec) scans. Subjects were asked to press a button with their right hand within 1.5 sec after the onset of the color change if they could detect it. Successful response for the change (hit) was regarded as a correct response while failure of the response (miss) was regarded as a wrong response. The degree of color change was controlled according to subjects' responses using a standard 2-down 1-up staircase rule. The geometric mean of the last 6 reversals was taken as a threshold of the degree of color change for each scan.

It might be possible that the difference in the MRS results between the no-overlearning and overlearning groups in Experiment 3 (**Fig. 3d**) could be explained by differences in performance on the fixation task (differences in fixation performance may affect the concentrations of glutamate and GABA). If this is the case, color change thresholds should have differed across the MRS stages, scan types, and/or subject groups. We performed a three-way mixed model ANOVA on color change threshold with main factors of MRS stage (pre-MRS, post-MRS 1, vs. post-MRS 2 stages), scan type (GABA vs. glutamate scans), and subject group (no-overlearning vs. overlearning groups). The results showed no significant main effect of MRS stage ( $F_{2,44}=0.897$ ,  $P=0.415$ ), scan type ( $F_{1,22}=2.411$ ,  $P=0.135$ ), subject group ( $F_{1,22}=0.083$ ,  $P=0.776$ ), nor interactions between MRS stage and scan type ( $F_{2,44}=2.020$ ,  $P=0.145$ ), MRS stage and subject group ( $F_{2,44}=0.512$ ,  $P=0.603$ ), scan type and subject group ( $F_{2,44}=1.202$ ,  $P=0.285$ ), or interaction among the 3 factors ( $F_{2,44}=0.915$ ,  $P=0.408$ ). These results indicate that subjects' performance on the fixation task was kept consistent irrespective of the MRS stages, scan types, or subject groups in Experiment 3.

## MRI data acquisition

A 3T MR scanner (Siemens) was used with a 32-channel head matrix coil in the Brown University MRI Research Facility. A high-resolution T<sub>1</sub>-weighted anatomical brain structure images were obtained using a magnetization-prepared rapid gradient echo (MPRAGE; 256 slices, voxel size = 1×1×1 mm<sup>3</sup>, 0 mm slice gap, TR=2530 ms, TE=1.64 ms, flip angle=7 degrees, FoV=256 mm, matrix size =256×256, bandwidth=651 Hz/pixel) sequence. The GABA scans were conducted using a MEGA-PRESS sequence<sup>55-57</sup> (TR=1500 ms, TE=68 ms, number of average=256, scan time=774 sec) with double-banded pulses which were used to simultaneously suppress water signal and edit the  $\gamma$ -CH<sub>2</sub> resonance of GABA at 3 ppm. We obtained the final spectra by subtracting the signals from alternate scans with the selective double-banded pulse applied at 4.7 and 7.5 ppm ('Edit Off') and the selective double-banded pulse applied at 1.9 and 4.7 ppm ('Edit On').

The glutamate scans were conducted using the PRESS sequence<sup>58,59</sup> (TR=3000 ms, TE=30 ms, number of average=128, scan time=384 sec). A variable pulse power and optimized relaxation delays (VAPOR) technique<sup>60</sup> was used in both sequences to achieve water suppression.

**Comparison of shim values**—Is there any possibility that the differential MRS results obtained in Experiment 3 (**Fig. 3d**) can be attributed to differences in the quality of the MRS signals? The following results indicate that it is unlikely. The quality of the MRS signal was represented as the shim value, which was obtained once for each of the MRS stages. To compare the shim values among the MRS stages and subject groups in Experiment 3, we performed a 2-way mixed model ANOVA on shim value with factors of MRS stage (pre-MRS, post-MRS 1, vs. post-MRS 2 stages) and subject group (no-overlearning vs. overlearning groups). The results showed no significant main effect of MRS stage ( $F_{2,44}=2.534$ ,  $P=0.091$ ), subject group ( $F_{1,22}=1.861$ ,  $P=0.186$ ), nor an interaction between the 2 factors ( $F_{2,44}=0.780$ ,  $P=0.465$ ). These results rule out the possibility that the differential results obtained in Experiment 3 can be accounted for by differences in the shim values.

**Range of frequency drift**—In Control Experiment 4 (see *Control Experiment 4* for details), we calculated a range of the frequency drift<sup>61,62</sup> for the spectra obtained from the GABA scans using the MEGA-PRESS sequence. The mean ( $\pm$ s.e.m.) frequency drifts were 0.810 $\pm$ 0.034 Hz for the pre-MRS stage, 0.950 $\pm$ 0.212 Hz for the post-MRS 1 stage, and 0.854 $\pm$ 0.113 Hz for the post-MRS 2 stage. The mean value of within-subject standard deviations was 0.161 Hz. The range of the frequency drift obtained in our MRI system is similar to or even better than that reported in previous studies (e.g., refs 61 and 62).

## MRS analysis

All MRS data were analyzed using LC-model software<sup>63,64</sup>. The LC-model assumes that the obtained spectrum can be fitted in the frequency domain using a linear combination of basis functions (**Supplementary Fig. 4**). The basis functions are the complete spectra of the individual metabolites that can be detected by a given acquisition. The basis functions include models of macromolecular "spectra" to account for the baseline produced by the

numerous short  $T_2$  macromolecular and lipid components. Note that glutamate and glutamine were separately fitted by the LC-model, and that the concentration of glutamate was used for the calculation of E/I ratio changes in Experiment 3 (**Fig. 3d**). We also used a combined signal of glutamate and glutamine (typically referred to as Glx, **Supplementary Fig. 7a**) for the calculation of E/I ratio changes and confirmed that the same statistical tendency was observed as in Experiment 3.

The reliability of quantification of GABA and glutamate was indicated by the Cramer-Rao lower bounds<sup>65</sup>, and a commonly accepted Cramer-Rao lower bound criterion of 20% was chosen to reject low-quality signal. None of the GABA and glutamate scans was rejected by the criterion. The mean ( $\pm$ s.e.m.) Cramer-Rao lower bounds across the MRS stages and subjects were 5.981 ( $\pm$ 0.207)% for GABA and 4.837 ( $\pm$ 0.056)% for glutamate.

In each MRS stage, the amounts of GABA and glutamate were normalized by the amount of creatine obtained from the glutamate scan, and referred to as the concentrations of GABA and glutamate, respectively. Creatine is a measure of cellular integrity and a standard reference resonance<sup>66</sup>. We confirmed that concentrations of creatine were not significantly affected by MRS stages or subject groups (**Supplementary Fig. 7b**).

An E/I ratio change,  $E/I_{change}$ , during each of the MRS stages was calculated for each subject according to the formula:

$$E/I_{change}(t) = 100 \times \left( \frac{\frac{Glu(t)}{GABA(t)}}{\frac{Glu(1)}{GABA(1)}} - 1 \right).$$

Here,  $GABA(t)$  and  $Glu(t)$  represent the concentrations of GABA and glutamate, respectively, at a certain MRS stage.  $t$  of 1, 2, and 3 indicates the pre-MRS, post-MRS 1, and post-MRS 2 stages, respectively. The E/I ratio in the pre-MRS stage served as the pre-training baseline, and thus an E/I ratio change of 0% is reported for the pre-MRS stage. Note that in this study the E/I ratio does not depend on whether GABA and glutamate are normalized to creatine or NAA, which is another standard reference resonance<sup>63,64</sup>, since the value of the control metabolite is cancelled out in the computation as this value is found both in the numerator and denominator.

### Control behavioral analysis

During the pre-test stage, each of the 3 orientations including a trained orientation was presented in each of the 3 blocks in a pseudo-random order. Thus, one may wonder if learning of the trained orientation may depend on a testing order during the pre-test stage. To test this possibility, we conducted a control analysis. To achieve reliable power, we combined behavioral data collected from 4 different subject groups (the 8-block and 16-block training groups in the preliminary experiment and the no-overlearning and overlearning groups in Experiment 3;  $N=48$  in total). We used these 4 groups of subjects who underwent training on one orientation. Since training on one orientation is not subject to anterograde or retrograde interference, the behavioral data from these 4 subject groups is suitable for the examination of the effects of the testing order. During the pre-test stage, the

trained orientation was tested in the first block for 20 subjects, in the second block for 16 subjects, and in the third block for 12 subjects. Thus, we classified the trained orientation for each subject to first-tested, second-tested, or third-tested trained orientation based on the testing order during the pre-test stage. Mean ( $\pm$ s.e.m.) performance improvements were  $24.29\pm 5.28\%$  for the first-tested orientation,  $28.59\pm 4.58\%$  for the second-tested orientation, and  $24.85\pm 3.85\%$  for the third-tested trained orientation. A one-way ANOVA on performance improvement showed no significant main effect of testing order (first-tested, second-tested, vs. third-tested trained orientations;  $F_{2,45}=0.230$ ,  $P=0.795$ ). Performance improvement was significant for all orientations ( $t>4.600$ ,  $P<10^{-3}$  after Bonferroni correction for 3 comparisons). Thus, it is unlikely that learning of the trained orientation differed depending on the testing order during the pre-test stage.

### Exclusion of subjects

With 5 subjects, MRS experiments were terminated in the middle (see also *Subjects*). There were 2 criteria for the termination of the experiments: performance of the fixation task and a shape of a measured spectrum. Three of the 5 subjects were excluded due to poorer performance on the fixation task during MRS measurements than the pre-determined criterion as shown below. As mentioned above, during the fixation task (see *Fixation task* for details), the degree of color change was initially set to 40 for each scan and controlled according to subjects' responses using a standard 2-down 1-up staircase rule. It had been determined that the subjects who show a larger than 80 (twice as much as the initial value) at the end of a scan would be regarded as having excessive fatigue and be excluded from the further steps of the experiment. The remaining 2 subjects were excluded from the further steps of the experiment due to atypical shapes of measured spectra. A large movement during a scan leads to an atypical shape of a spectrum. Thus, we used the atypical shape of a spectrum as the indicator for excessive movements during the scan. For GABA scans, we determined a measured spectrum as atypical if a spectrum obtained by the MEGA-PRESS sequence had no clear peak around 2.8-3.2 ppm which is known to reflect GABA signal. For glutamate scans, if a spectrum obtained by the PRESS-sequence showed no clear bimodal peaks around 2.2-2.4 ppm which are known to reflect glutamate and glutamine signals, the spectrum was regarded as atypical.

### Statistics

Data collection and analysis were not performed blindly to the people who conducted the experiments and analyses. All tests conducted in this study were two-tailed. The alpha level threshold was set to 0.05. If corrections for multiple comparisons were necessary, we used the Bonferroni correction. Since the data collected in the pilot experiment met parametric assumptions, we used parametric tests such as t-test and ANOVA for statistical tests in the main and control experiments. However, we also used non-parametric tests on the same data just to confirm that tests with lower power would also reveal statistical significance. All effects that were significant with t-tests were also significant in the non-parametric tests such as the Wilcoxon signed rank test and rank sum test. When we conducted an ANOVA with a within factor, Mauchly's sphericity test was conducted to examine whether the assumption of sphericity had been violated. No violation was found in the present study.



A **Supplementary Methods Checklist** is available.

## Apparatus

Visual stimuli were presented on a LCD display (1024×768 resolution, 60 Hz refresh rate) during the orientation detection task and via an MRI-compatible LCD display (1024×768 resolution, 60 Hz refresh rate) during MRS measurements in a dim room. All visual stimuli were made using Matlab and Psychtoolbox 3<sup>67</sup> on Mac OS X.

## Data availability

The data that support the findings of this study are available from the corresponding author upon request.

## Code availability

The computer codes that were used to generate results that are central to the conclusions of this study are available from the corresponding author upon request.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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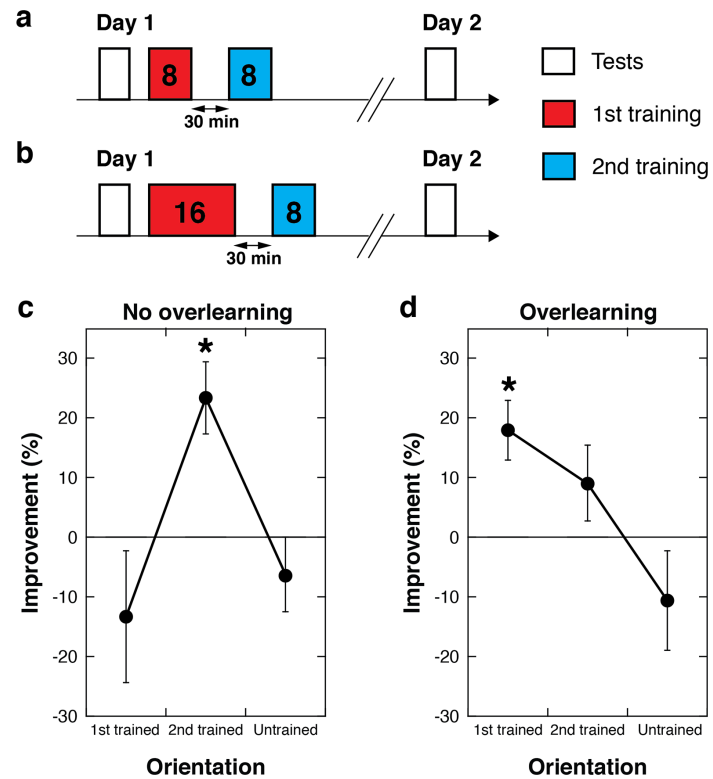
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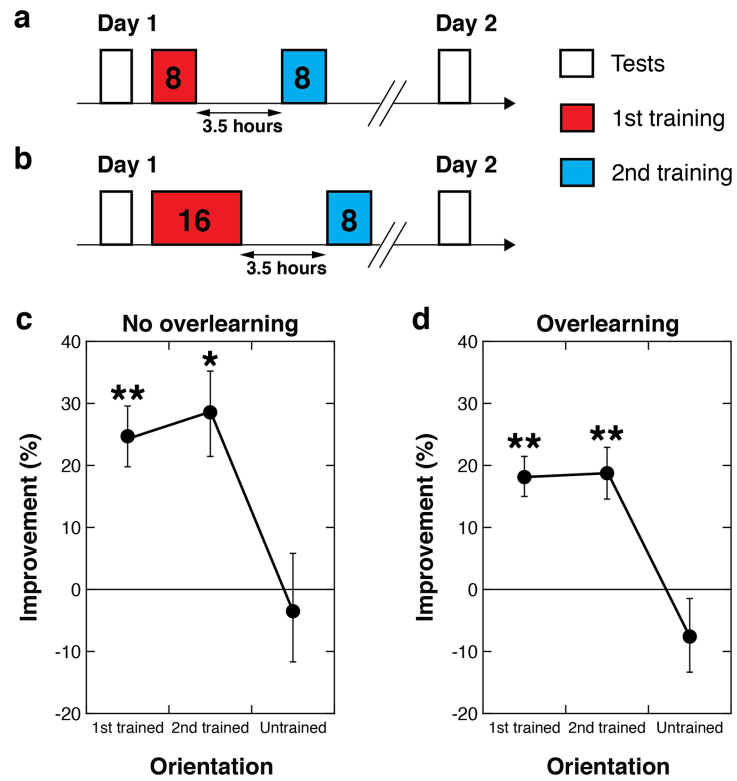
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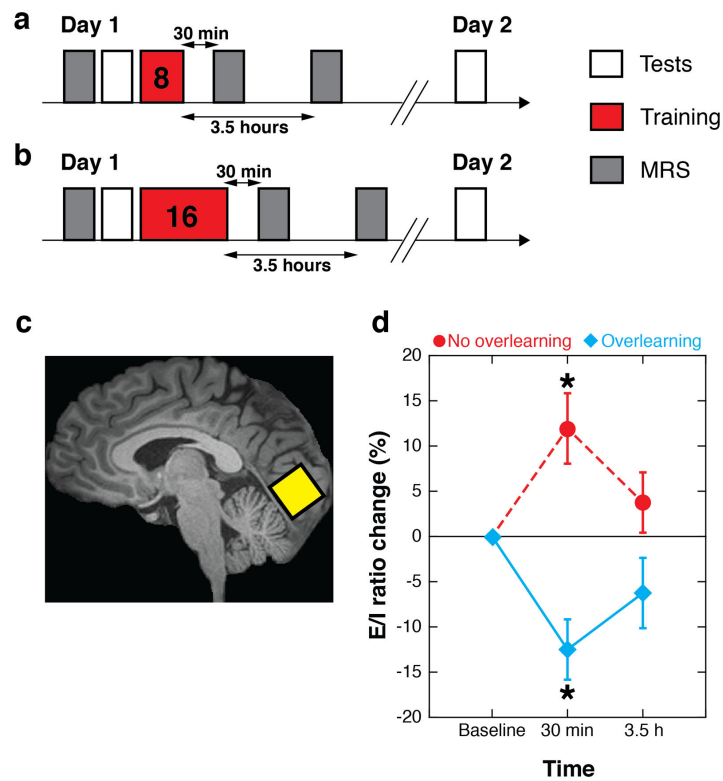


**Figure 1.**

Procedures and results of Experiment 1. **(a)** Procedures for the no-overlearning group ( $N=12$ ). The white boxes represent the pre- and post-test stages. The red box represents the first-training phase and the cyan box the second-training phase. The number of training blocks is shown in each box. **(b)** Procedures for the overlearning group ( $N=12$ ). **(c)** Mean ( $\pm$ s.e.m.) performance improvements (percent reduction in the S/N ratio threshold in the post-test relative to the pre-test stage; see *Pre- and Post-test stages* in Online Methods for details) in the no-overlearning group for the first-trained, second-trained, and untrained orientations. **(d)** Mean ( $\pm$ s.e.m.) performance improvements for the overlearning group. \*  $P<0.05$  after Bonferroni correction.



**Figure 2.** Procedures and results of Experiment 2. **(a)** Procedures for the no-overlearning group ( $N=12$ ). **(b)** Procedures for the overlearning group ( $N=12$ ). **(c)** Mean ( $\pm$ s.e.m.) performance improvements in the no-overlearning group for the first-trained, second-trained, and untrained orientations. **(d)** Mean ( $\pm$ s.e.m.) performance improvements in the overlearning group. \*  $P<0.05$ , \*\*  $P<0.01$  after Bonferroni correction.



**Figure 3.** Procedures and results of Experiment 3. **(a)** Procedures for the no-overlearning group ( $N=12$ ). The gray boxes represent periods for measurements of glutamate and GABA (approximately 20 min for each period). **(b)** Procedures for the overlearning group ( $N=12$ ). **(c)** An example of a voxel location for MRS measurements. **(d)** Mean ( $\pm$ s.e.m.) E/I ratio changes for the no-overlearning (red) and overlearning (cyan) groups. See *MRS analysis* in Online Methods for the definition of E/I ratio changes. \*  $P < 0.05$  after Bonferroni correction.