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Overlearning hyperstabilizes a skill by rapidly making neurochemical processing inhibitory-dominant

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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 in humans abruptly changes neurochemical processing, to hyperstabilize 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 until after passive stabilization occurs hours later. However, overlearning so rapidly and strongly stabilizes the learning state that it not only becomes resilient against, but also disrupts, subsequent new learning. Such hyperstabilization is associated with an abrupt shift from glutamate-dominant excitatory to GABA-dominant inhibitory processing in early visual areas. Hyperstabilization contrasts with passive and slower stabilization, which is associated with a mere reduction of excitatory dominance to baseline levels. Using hyperstabilization may lead to efficient learning paradigms.

Continuous training conducted after performance improvement has been maximized is called overlearning. Musicians, for example, believe that it is crucial to continue to practice the same piece of music over and over again even after they have mastered a particular piece. What is the benefit of overlearning? As early as 1885, Hermann Ebbinghaus, who pioneered experimental studies of memory, pointed out that overlearning allowed subjects to retain what had been learned for a longer period of time[1]. However, whether overlearning improves the retention in a way that is truly beneficial has been controversial. Some researchers think that overlearning is an unnecessary and useless practice[2].

Here we demonstrate a previously unreported and crucial role for overlearning. We found that less than 20 min of overlearning stabilized a post-training plastic state of learning by drastically changing excitatory- to inhibitory-dominant neurochemical brain environments. When human subjects underwent training on a visual task without overlearning, visual perceptual learning (VPL, defined as improvement of a visual task as a result of visual experience[3]) of the task was disrupted, or interfered with, by subsequent training on a new task. This result is indeed consistent with the well-established view that soon after standard training, learning status is so plastic that it is vulnerable to disruption by subsequent new learning[4–9]. However, if training continued for as little as 20 min after performance improvement reached a plateau, VPL of the first task survived training of the new task by superseding and disrupting, or interfering with, the latter. To investigate how overlearning changes neural processing, we used magnetic resonance spectroscopy (MRS) and measured the concentrations of glutamate, a key excitatory neurotransmitter, and GABA, a key inhibitory neurotransmitter[10], in the early visual areas, which have been found to be involved in some types of VPL[11–18]. We found that after training without overlearning, the ratio of the concentrations of glutamate for excitation to GABA for inhibition (E/I ratio) was significantly higher than a pre-training baseline. In contrast, 20 min of overlearning decreased the E/I ratio below the pre-training baseline. Moreover, the amount of E/I ratio reduction due to overlearning was significantly correlated with the amount of interference from learning of the first task on learning the second task, which is indicative of the degree of stability. These results suggest that just a short period of overlearning drastically changes a post-training plastic and unstable VPL state in the early visual areas to a hyperstabilized state that is resilient against, and even disrupts, new learning. These results indicate that hyperstabilization due to overlearning is associated with rapid and drastic changes in the early visual areas from a more excitatory and plastic state to a more inhibitory and stable state than the pre-training baseline (**Supplementary Fig. 1**).

Notably, hyperstabilization is qualitatively and quantitatively different from typical stabilization[4–9], which takes hours to complete after training and causes no disruption between the first-trained task and new learning[4–9]. Our results also indicate that typical stabilization is a slow, passive process, in which a post-training excitatory state returns to the pre-training baseline level. No disruption between encoded learning and new learning occurs after typical stabilization. In contrast, hyperstabilization can occur due to as little as

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20 min of active overlearning and disrupts subsequent new learning to protect the previously learned task. Hyperstabilization is associated with abrupt changes of neurochemical environments from an excitatory signal-dominant state to an inhibitory signal-dominant state (**Supplementary Fig. 1**).

RESULTS

We conducted a series of experiments whose designs and results are summarized in **Supplementary Table 1**. In Experiment 1, we behaviorally examined the interference effects between the first and second training sessions using a two-interval forced-choice orientation detection task (**Supplementary Fig. 2a**). The complete experiment consisted of three stages: pre-test, training with two sessions and post-test (**Fig. 1a**,**b**). A preliminary experiment ($n = 60$) found that performance improvement for the orientation detection task plateaued around the eighth block of training (**Supplementary Fig. 2b**) and that there was no significant performance improvement between the eighth and sixteenth blocks. Thus, we used eight blocks in the first training session 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 two groups: the two training sessions were separated by a 30-min interval and there were eight blocks in the second training session. In each training session, a different orientation was used for the 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 of the task and stimuli.

If overlearning stabilizes the learning state, no interference with the first-trained orientation by the second should occur in the overlearning group, whereas the interference should be observed in the no-overlearning group. Results showed that this prediction was correct: overlearning greatly stabilized learning of the first-trained task. Although with no overlearning learning of the first-trained task was disrupted by the second, with overlearning, learning of the first-trained task disrupted learning of the second-trained task (**Fig. 1c**,**d**). To test how overlearning in the first training session influenced the magnitudes by which subjects learned in the first and second training sessions, a two-way mixed-model ANOVA on performance improvement with factors of orientation (first-trained and second-trained orientations versus untrained orientations) and group (no-overlearning versus 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 how well subjects learned the tested orientations in a different way than training with no overlearning.

Then, to test which orientation was learned in the two groups, a t-test was conducted on the amount of improvement in each orientation and each group. In the no-overlearning group (**Fig. 1c**) we found a significant performance improvement for the second-trained orientation (one-sample t-test, $t_{11} = 3.845$, $P = 0.016$ after Bonferroni correction for six comparisons) but not for the first-trained ($t_{11} = 1.120$, $P = 0.256$ without Bonferroni correction) or untrained orientations ($t_{11} = 0.987$, $P = 0.345$ without Bonferroni correction). Note that the preliminary experiment showed that 8 and 16 blocks of training on an orientation led to similar amplitudes of performance improvements for the orientation (**Supplementary Fig. 2b**), indicating that learning occurs in these conditions unless training on a different, second orientation occurs. Taken together, these results are consistent with classical retrograde interference, in which the learning of one task is disrupted by learning a second[4–9]. 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, in the overlearning group (**Fig. 1d**) we found a significant performance improvement for the first-trained orientation ($t_{11} = 3.612$, $P = 0.025$ after Bonferroni correction for six comparisons) and not for the second-trained orientation ($t_{11} = 1.432$, $P = 0.178$ without Bonferroni correction) or untrained orientation ($t_{11} = 1.277$, $P = 0.228$ without Bonferroni correction). These results are consistent with anterograde interference, in which learning the first task disrupts learning the second[5,8,19–21]. Overlearning made learning of the first-trained orientation so stable that it prevented interference from learning of the second-trained orientation and, to our surprise, instead interfered with the latter. Since eight blocks of training took approximately 20 min and 16 blocks took about 40 min, we observed dramatic qualitative changes in the learning state that occurred in as little as 20 min after the initial eight blocks of training.

The results of Experiment 1 are consistent with the hypothesis that overlearning induces a learning mechanism that drastically changes

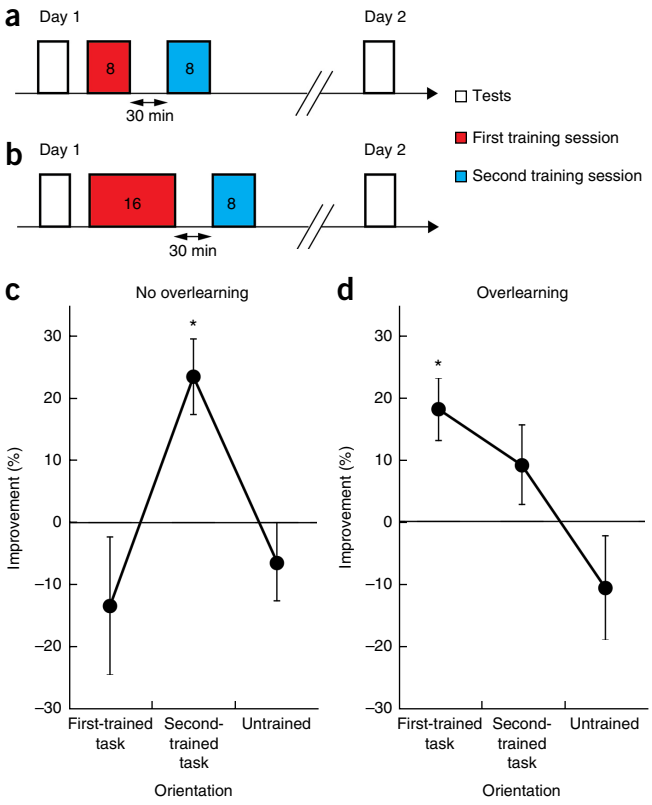

Figure 1 Procedures and results of Experiment 1. (**a**) Procedures for the no-overlearning group ($n = 12$). The white boxes represent the pre- and post-test stages. The red box represents the first training session and the cyan box the second training session. The number of training blocks is shown in each box. (**b**) Procedures for the overlearning group ($n = 12$). (**c**) Mean (± 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 (± s.e.m.) performance improvements for the overlearning group. $^*P < 0.05$ after Bonferroni correction.

neuronal plastic states to hyperstabilized states (**Supplementary Fig. 1**). To protect existing learning from being replaced with new learning, hyperstabilization may make the existing mechanism of learning so strongly stabilized that it supersedes new learning; that is, it not only protects itself from being interfered with by learning new tasks but also prevents the new learning from becoming 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 versus 8 blocks). If so, then no anterograde interference should be observed when subjects were trained on both orientations for 16 blocks. However, the results of Control Experiment 1, in which subjects were trained for 16 blocks on each orientation ($n = 12$, **Supplementary Fig. 3a**), demonstrated that anterograde interference still occurred when subjects were trained on both orientations for 16 blocks (**Supplementary Fig. 3c**). These results indicate that anterograde interference occurred due to overlearning in the first training session and not because of more initial training.

Another possibility is that the amount of time passing during the 16 blocks in the first training session somehow made learning of the first-trained orientation hyperstabilized and led to the anterograde interference. However, the results of Control Experiment 2 ($n = 12$) refute this possibility. In this experiment, between the end of the first training session and the beginning of the second, no training was conducted for 50 min, which matched the time interval for the eight blocks of training plus 30-min intersession interval between the two training sessions in Experiment 1 (**Supplementary Fig. 3b**). If the mere passage of time was responsible for the shift from plasticity to hyperstabilization, then anterograde interference should have occurred. However, retrograde interference occurred (**Supplementary Fig. 3d**), which was inconsistent with this possibility.

The results of these experiments indicate that it is likely the process of overlearning that caused the dramatic shift from a post-training plastic state to a hyperstabilized state, where prior VPL superseded and inhibited 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 by subsequent learning.

It takes hours for typical stabilization of learning to occur, and once this stabilization occurs, the learned skill neither interferes with nor is interfered with by subsequent new learning[4,6,9]. However, hyperstabilization due to overlearning, as shown in Experiment 1, interferes with new learning. Such different functions of typical stabilization and hyperstabilization raise the following question: do these two 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 sessions was set to 3.5 h (**Fig. 2a**,**b**), an amount of time that has experimentally been found to be sufficient for learning of the first-trained task to stabilize[4,6,9]. 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-h interval after the first training session, typical stabilization occurred, irrespective of whether overlearning had occurred or not in the first training session. The results showed that this was 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 two groups, a two-way mixed-model

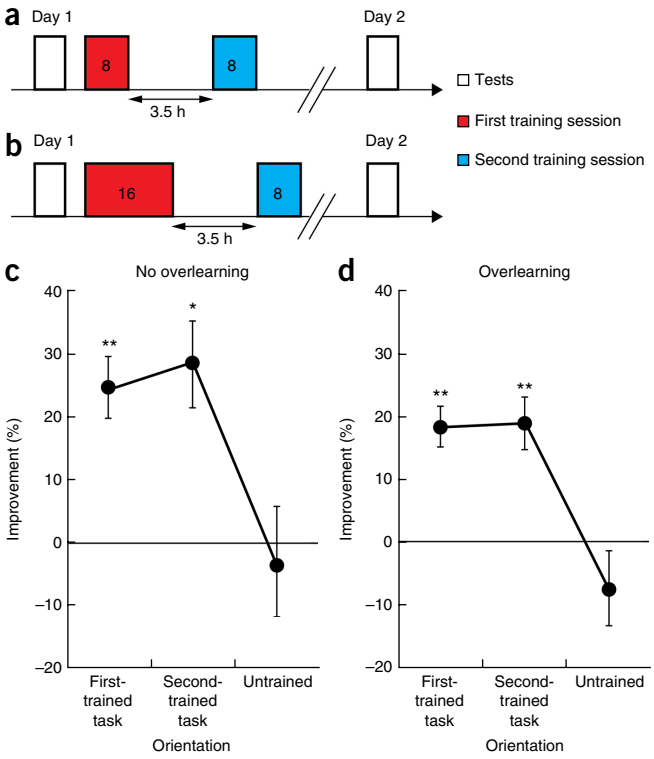

Figure 2 Procedures and results of Experiment 2. (**a**) Procedures for the no-overlearning group ($n = 12$). (**b**) Procedures for the overlearning group ($n = 12$). (**c**) Mean (± s.e.m.) performance improvements in the no-overlearning group for the first-trained, second-trained and untrained orientations.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

ANOVA on performance improvement with factors of orientation (first-trained and second-trained versus untrained orientations) and group (no-overlearning versus 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 two 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 six comparisons) and second-trained ($t_{11} = 4.090$, $P = 0.011$ after Bonferroni correction for six 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 six comparisons) and second-trained ($t_{11} = 4.566$, $P = 0.005$ after Bonferroni correction for six 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-h time interval after the first training session, typical stabilization occurred for the first-trained orientation, irrespective of overlearning. These results suggest that hyperstabilization due to overlearning has different temporal properties compared to typical stabilization, as illustrated in **Supplementary Figure 1**.

What is the underlying neural mechanism of overlearning-induced hyperstabilization? The results so far suggest that the underlying neural mechanisms of the three post-training states, such as those after no-overlearning, overlearning and 3.5-h intersession interval, 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[22–30]. These studies raise the possibility that the abovementioned three post-training states were 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[11–18] using MRS (Online Methods). The procedure with the no-overlearning ($n = 12$) and overlearning ($n = 12$) groups was the same in that MRS measurements were conducted (i) before training to acquire a pre-training baseline, (ii) 30 min after training and (iii) 3.5 h after training for typical stabilization. The only difference between the groups was the number of blocks for training (8 versus 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-trained orientation. Therefore, training of the second orientation 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 three 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. An E/I ratio higher than the pre-training baseline should indicate that the early visual areas became more excitatory than the baseline. An E/I ratio lower than the baseline should indicate that the early visual areas became more inhibitory than the baseline. First, to test whether there was 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 versus 3.5 h after training) and group (no-overlearning versus 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 two factors, we further examined how the E/I ratios were changed over time with and without overlearning separately. For the no-overlearning group (**Fig. 3d**), a significant quadratic trend was observed in the timecourse of the E/I ratio changes ($F_{1,11} = 5.435$, $P = 0.040$). Furthermore, the E/I ratio change 30 min after eight blocks of training was significantly greater than zero (one-sample t-test, $t_{11} = 3.064$, $P = 0.043$ after Bonferroni correction for four comparisons). However, 3.5 h 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 eight-block training without overlearning and returned to near the pre-training baseline 3.5 h after the training. For the overlearning group (**Fig. 3d**), a significant quadratic trend was also observed in the timecourse 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 four comparisons) but then rebounded to near-baseline 3.5 h after training ($t_{11} = 1.592$, $P = 0.140$ without Bonferroni correction). These results also suggest that hyperstabilization is different from typical stabilization (**Supplementary Fig. 1**), for which E/I ratios returned to near-baseline at 3.5 h after training.

We conducted various control analyses on the 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 (Online Methods). **Supplementary Figure 6** shows performance improvements in Experiment 3. We found no effect of testing order on learning during the pre-test stage (Online Methods). We used glutamate concentrations as excitatory signals but combined concentrations of glutamate and glutamine also provided similar results (**Supplementary Fig. 7a**). Concentrations of creatine, a control metabolite, were consistent across measurements (**Supplementary Fig. 7b**). See "Exclusion of subjects" in Online Methods for the data exclusion criteria.

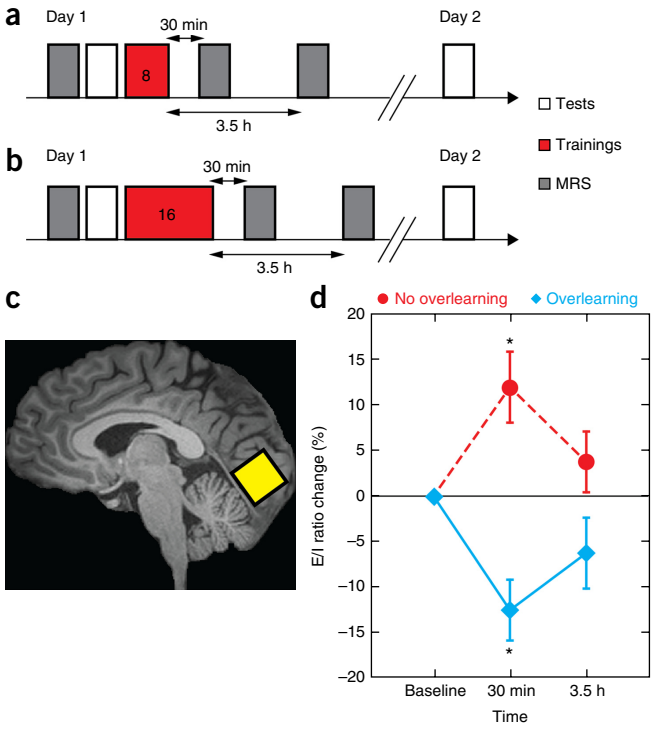

Figure 3 Procedures and results of Experiment 3. (**a**) Procedures for the no-overlearning group ($n = 12$). The gray boxes represent approximately 20-min periods for measurements of glutamate and GABA. (**b**) Procedures for the overlearning group ($n = 12$). (**c**) An example of a voxel location for MRS measurements. (**d**) Mean (± 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.

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 changed under a training scheme in which VPL should not occur. When two different stimulus conditions are interleaved during training blocks, learning does not occur for either condition[21,31,32]. Subjects ($n = 7$) underwent 16 blocks of interleaved training, during which two 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 depended 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 hyperstabilization? In Experiment 4, we used two training sessions and measured both the degree of E/I ratio reduction and the performance improvement of the second-trained orientation after overlearning (n = 12; **Supplementary Fig. 9a**). Note that the performance improvement of the second-trained orientation should be inversely proportional to the amount of anterograde interference. We found both anterograde interference and a significant reduction in 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 hyperstabilization.

DISCUSSION

The results of all experiments consistently suggest that overlearning abruptly changed neurochemical processing to hyperstabilize and protect trained perceptual learning from subsequent new learning.

Were 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, in which 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 accounts for 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[24]. Second, glutamate is a precursor of GABA, and therefore, its concentration may not be completely independent from that of GABA[10,33,34]. 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[35], what could account for a 16.3% increase in MRS GABA signals following overlearning? As shown in **Supplementary Figure 10a**, 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[10,33,34], overlearning may trigger and/or facilitate the transformation of glutamate to GABA. A growing body of evidence indicates that MRS detects both extra- and intrasynaptic GABA[34,36,37]. Glutamate is also observed outside synapses[38,39]. 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.

A prior study found that if performance gain is saturated in the first training session, performance is greater after sleep following training, whereas without saturation in the first session, no such post-sleep performance improvement is observed[40]. This suggests that the saturation of performance gain in early training sessions triggers consolidation during sleep. Does this process reflect a continuous hyperstabilization effect? Our study showed that the rapidly decreased E/I ratio measured 30 min after overlearning returned to the baseline 3.5 h after overlearning. Thus, hyperstabilization may not last longer than 3.5 h. On the other hand, in the previous study[40], the interval between the two training sessions was 24 h. Although overlearning may play important roles in both hyperstabilization during wakefulness and consolidation during sleep, mechanisms underlying these functions may not be the same.

Hyperstabilization due to overlearning may be different from sensory adaptation due to prolonged or excessive exposure to a stimulus[41–43]. The effect of adaptation to an orientation is highly specific, not applying beyond ±30° from the adapted orientation[44], whereas hyperstabilization of VPL interfered with the learning of a second orientation that was 60° away from the first-trained orientation. These results suggest that the mechanism of hyperstabilization is different from that of adaption. However, the stimuli used in this study were 100% contrast, with random noise with a power distribution 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 hyperstabilization is related to some adaptation process. It has been suggested that VPL is associated with adaptive task-irrelevant sensory plasticity and task-related plasticity[45]. This raises the possibility that the E/I ratio dynamics largely reflect inhibitory processing where task-irrelevant sensory plasticity occurs.

In VPL as well as other types of learning and memory, there are several different phases in the timecourse 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[46–48]. Early visual areas are also involved in hyperstabilization due to overlearning. Thus, at least in VPL, the early visual areas may be involved in all of these phases. In other types of learning and memory, typical stabilization is involved in a different brain area than the encoding[8,49,50]. It may be worthwhile to examine whether hyperstabilization 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 hyperstabilizes encoded VPL and supersedes subsequent new learning, in association with a rapid change of an E/I neurotransmitter ratio from higher than to below 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 role for overlearning has not been described since the effect of overlearning on retention length was pointed out more than a century ago[1].

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. A total of 183 naive 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 consent to participate. The experiments were conducted during daytime. Five subjects were excluded from the study in the middle of the MRS experiments (see the "Exclusion of subjects" section below 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 eight blocks of training on the orientation detection task (**Supplementary Fig. 2a**). Results of the power analysis indicated that nine subjects would be enough to obtain significant VPL of a trained orientation. However, we chose to be more conservative and made the number of subjects in each group 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[27,29,51].

Stimuli. Oriented Gabor patches (**Supplementary Fig. 2a**; spatial frequency = 1 cycle/degree, contrast = 100%, Gaussian filter sigma = 2.5°; random spatial phase) were presented within an annulus subtending 0.75° to 5° from the center of a gray screen. Gabor patches were spatially masked by a noise pattern using a pixel substitution method[17,52]. 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°, each ±60° apart.

Orientation detection task. Subjects performed a two-interval forced-choice orientation-detection task (**Supplementary Fig. 2a**), in which 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 interstimulus interval, subjects were asked to report which stimulus interval contained the Gabor patch, by pressing the '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° radius) throughout each trial. The next trial started immediately after the subject's response. No feedback regarding the accuracy of a subject's response was provided.

Threshold measurement. The S/N ratio threshold for each orientation was measured using a standard two-down-one-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 (ref. 53). Each block ended after 10 staircase reversals—typically about 40 trials—taking approximately 2 min. The geometric mean of the last six reversals was taken as the S/N ratio threshold for that block[53].

Pre- and post-test stages. The pre- and post-test stages measured a subject's S/N ratio threshold for each orientation. There were six possible combinations for which orientations were tested in each of the three blocks of each test stage. In each test stage for each subject, one of the six 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 reductions in the S/N ratio threshold measured during the post-test stage relative to the pre-test stage for each subject. Performance improvement $Thresh_{\mathrm{imp}}$ for each of the three orientations was calculated by

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

Here, $Thresh_{\mathrm{pre}}$ and $Thresh_{\mathrm{post}}$ represent S/N ratio thresholds in the pre- and post-test stages, respectively[53]. 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 three stages over two consecutive days: pre-test, training and post-test. 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 induced saturation of performance improvements in 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 three orientations and counterbalanced across subjects. The remaining two orientations served as untrained orientations.

Experiment 1. Twenty-four subjects were randomly assigned to either the no-overlearning ($n = 12$) or overlearning ($n = 12$) groups (**Fig. 1**). During the training stage, subjects underwent two training sessions (first and second training sessions), which were separated by a 30-min time interval. In the first training session, 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 session, subjects in both groups performed the task with another orientation (second-trained orientation) for eight blocks. Two different orientations for the first and second training sessions were selected from the three orientations and counterbalanced 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 three 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 ($F_{2,22} = 0.560$, $P = 0.579$) groups. This result indicated that there was no performance bias among the three orientations before training. This tendency was also found in all the other experiments.

Experiment 2. Procedures were identical to those of Experiment 1, except that the two training sessions were separated by a 3.5-h time interval (**Fig. 2**). Twenty-four new subjects were randomly assigned to either the no-overlearning ($n = 12$) or overlearning ($n = 12$) groups.

Control Experiment 1. Procedures (**Supplementary Fig. 3a**) were identical to those for the overlearning group in Experiment 1, except that in the second training session, subjects performed the orientation detection task for 16 blocks ($n = 12$).

Control Experiment 2. Procedures (**Supplementary Fig. 3b**) were identical to those for the no-overlearning group in Experiment 1, except that the two training sessions were separated by a 50-min time interval ($n = 12$).

Magnetic resonance spectroscopy (MRS) experiments (Experiments 3 and 4, Control Experiments 3 and 4). *Experiment 3*. There were six stages over two consecutive days (**Fig. 3a**,**b**): pre-MRS, pre-test, training, post-MRS 1, post-MRS 2 and post-test stages. The first five 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 for 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 session was conducted in the training stage of Experiment 3. See "MRS stages" below for details of MRS procedures.

Experiment 4. There were six stages over two consecutive days (**Supplementary Fig. 9a**): pre-MRS, pre-test, first training stage, post-MRS 1, second training stage and post-test stages. The first five stages were conducted on the first day, and the post-test stage was conducted on the second day ($n = 12$). Procedures for the first and second training stages were identical to those of the first and second training sessions for the overlearning group in Experiment 1, except that the two training stages in Experiment 4 were separated by the post-MRS 1 stage, which took 1 h.

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Control Experiment 3. As in Experiment 3, there were six stages over two consecutive days (**Supplementary Fig. 8a**): pre-MRS, pre-test, training, post-MRS 1, post-MRS 2 and post-test stages ($n = 7$). The first five 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 two different orientations (trained orientations 1 and 2). These two different trained orientations were randomly selected from the three 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 three 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 stages 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 ($2 \times 2 \times 2$ 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[54] 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 showed both the anatomical brain structure and the voxel position in the pre-MRS stage. Overall, the voxels 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 the full width at half maximum of the water peak). See "Comparison of shim values," below, for statistical comparisons of shim values. These first three steps (anatomical structure measurement, voxel placement and shimming) took approximately 30 min. Fourth, a 774-s GABA scan and a 384-s glutamate scan were conducted to quantify the concentration of GABA and glutamate within the voxel (see "MRI data acquisition" and "MRS analysis" sections for details). The mean (± s.e.m.) line-width of *N*-acetylaspartate (NAA) across the MRS stages, scans and subjects was 8.757 ± 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 = 255,255Δ,255Δ) in an unpredictable timing pattern, and then returned to white after 1.5 s. The degree of color change (Δ) 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 (± s.e.m.) number of color changes across the MRS stages and subjects were 156.616 (± 0.675) for the 774-s GABA scan and 77.919 (± 0.621) for the 384-s glutamate scan, respectively. The differences in the numbers of color changes between the GABA and glutamate scans depended on the difference in durations of the GABA (774 s) and glutamate (384 s) scans. Subjects were asked to press a button with their right hand within 1.5 s 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 to respond (miss) was regarded as a wrong response. The degree of color change, Δ, was controlled according to subjects' responses using a standard two-down-one-up staircase rule. The geometric mean of the last six reversals was taken as a threshold of the degree of color change, Δ, for each scan.

It might be possible for the difference in the MRS results between the no-overlearning and overlearning groups in Experiment 3 (**Fig. 3d**) to 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 and post-MRS 1 versus post-MRS 2 stages), scan type (GABA versus glutamate scans) and subject group (no-overlearning versus overlearning groups). The results showed no significant main effect of MRS stage ($F_{2,44} = 0.897$, $P = 0.415$), scan type ($F_{1,22} = 2.411$, $P = 0.135$) or subject group ($F_{1,22} = 0.083$, $P = 0.776$), nor did we find any 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 three 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. High-resolution T_1-weighted anatomical brain structure images were obtained using a magnetization-prepared rapid gradient echo (MPRAGE; 256 slices, voxel size = $1 \times 1 \times 1$ mm^3, 0-mm slice gap, TR = 2,530 ms, TE = 1.64 ms, flip angle = 7°, FoV = 256 mm, matrix size = 256×256, bandwidth = 651 Hz/pixel) sequence. The GABA scans were conducted using a MEGA-PRESS sequence[55–57] (TR = 1,500 ms, TE = 68 ms, average = 256, scan time = 774 s) with double-banded pulses, which were used to simultaneously suppress water signal and edit the γ-CH2 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[58,59] (TR = 3,000 ms, TE = 30 ms, average = 128, scan time = 384 s). A variable pulse power and optimized relaxation delays (VAPOR) technique[60] 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**) could 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 shim values among the MRS stages and subject groups in Experiment 3, we performed a two-way mixed-model ANOVA on shim values with factors of MRS stage (pre-MRS and post-MRS 1 versus post-MRS 2 stages) and subject group (no-overlearning versus overlearning groups). The results showed no significant main effect of MRS stage ($F_{2,44} = 2.534$, $P = 0.091$) or subject group ($F_{1,22} = 1.861$, $P = 0.186$), nor did we find an interaction between the two 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[61,62] for the spectra obtained from the GABA scans using the MEGA-PRESS sequence. The mean (± s.e.m.) frequency drifts were 0.810 ± 0.034 Hz for the pre-MRS stage, 0.950 ± 0.212 Hz for the post-MRS 1 stage and 0.854 ± 0.113 Hz for the post-MRS 2 stage. The mean value of within-subject s.d. was 0.161 Hz. The range of the frequency drift obtained in our MRI system was similar to or better than ranges reported in previous studies (for example, refs. 61,62).

MRS analysis. All MRS data were analyzed using LC-model software[63,64]. 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**). Basis functions are the complete spectra of the individual metabolites that can be detected by a given acquisition. 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 fitted separately 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 glutamate and glutamine signal (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 GABA and glutamate quantifications was indicated by the Cramer-Rao lower bounds[65], 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 under the criterion. The mean (± s.e.m.) Cramer-Rao lower bounds across the MRS stages and subjects were 5.981% (± 0.207) for GABA and 4.837% (± 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[66]. 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

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

Here $GABA(t)$ and $Glu(t)$ represent the concentrations of GABA and glutamate, respectively, at a certain MRS stage. The variable t could be set to 1, 2 or 3 to indicate the pre-MRS, post-MRS 1 or post-MRS 2 stage. The E/I ratio in the pre-MRS stage served as the pre-training baseline, and thus an E/I ratio change of 0% was reported for the pre-MRS stage. Note that in this study the E/I ratio did not depend on whether GABA and glutamate were normalized to creatine or to NAA, another standard reference resonance[63,64], since the value of the control metabolite was cancelled out in the computation as this value was found both in the numerator and denominator.

Control behavioral analysis. During the pre-test stage, each of the three orientations, including a trained orientation, was presented in each of the three blocks in a pseudorandom order. Thus, one may wonder whether learning of the trained orientation depended on the 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 four 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 four groups of subjects, who each underwent training on one orientation. Since training on one orientation is not subject to anterograde or retrograde interference, the behavioral data from these four subject groups were suitable for examining 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 (± s.e.m.) performance improvements were 24.29 ± 5.28% for the first-tested orientation, 28.59 ± 4.58% for the second-tested orientation and 24.85 ± 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 and second-tested versus 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 three 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. For five subjects, MRS experiments were terminated midway through (see also "Subjects"). There were two criteria for the termination of the experiments: performance of the fixation task and a shape of a measured spectrum. Three of the five subjects were dropped from the study due to poorer performance on the fixation task during MRS measurements than the predetermined criterion, as shown below. During the fixation task (see "Fixation task" for details), the degree of color change, Δ, was initially set to 40 for each scan and controlled according to subjects' responses using a standard two-down-one-up staircase rule. We had determined that subjects who showed a $\Delta > 80$ (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 two 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 atypical spectrum shape as an indicator of 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 blinded for 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 a Bonferroni correction. Since the data collected in the pilot experiment met parametric assumptions, we used parametric tests such as *t*-tests and ANOVA for statistical tests in the main and control experiments. However, we also used nonparametric 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 nonparametric 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 violations were found in the present study.

A **Supplementary Methods Checklist** is available.

Apparatus. Visual stimuli were presented on an LCD display (1,024 × 768 resolution, 60 Hz refresh rate) during the orientation detection task and via an MRI-compatible LCD display (1,024 × 768 resolution, 60 Hz refresh rate) during MRS measurements in a dim room. All visual stimuli were made using Matlab and Psychtoolbox 3 (ref. 67) on Mac OS X.

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

Code availability. The computer code that was used to generate results central to the conclusions of this study are available from the corresponding author upon reasonable request.

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Corrigendum: Overlearning hyperstabilizes a skill by rapidly making neurochemical processing inhibitory-dominant

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