Skilled motor learning does not enhance long-term depression in the motor cortex in vivo

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ABSTRACT

Learning of motor skills may occur as a consequence of changes in the efficacy of synaptic connections in the primary motor cortex. We investigated if learning in a reaching task affects the excitability, short-term plasticity and long-term plasticity of horizontal connections in layers II-III of the motor cortex. Because training in this task requires animals to be food-deprived, we compared the trained animals with similarly food deprived untrained animals and normal controls. The results show that the excitability, short-term plasticity and long-term plasticity of the studied horizontal connections was unaffected by motor learning. However, stress-related effects produced by food deprivation and handling significantly enhanced the expression of LTD in these pathways. These results are compatible with the hypothesis that the acquisition of a complex motor skill produces bi-directional changes in synaptic strength that are distributed throughout the complex neural networks of motor cortex, which remains synaptically balanced during learning. The results are incompatible with the idea that learning causes large unidirectional changes in the population response of these neural networks, which may occur instead during certain behavioral states, such as stress.
**Introduction**

Skilled motor movements rely heavily upon the primary motor cortex for their accurate expression (Lawrence and Kuypers, 1968; Schieber and Poliakov, 1998). Lesions of the primary motor cortex of rodents dramatically impair the performance of a forelimb reaching task that requires rodents to reach and grasp food pellets (Castro, 1977; Castro-Alamancos and Borrell, 1993; Whishaw et al., 1991). Also, reaching in this task correlates with cell activity in the primary motor cortex contralateral to the trained forelimb (Dolbaky an et al., 1977; Kargo and Nitz, 2004).

A long held hypothesis is that skilled motor learning occurs as a consequence of changes in the efficacy of synaptic connections in the primary motor cortex (Ramon y Cajal, 1904; Hebb, 1949). Indeed, Cajal noted around a century ago that pianists surely have more complex synaptic networks in motor cortex as a result of activity-dependent mechanisms. In agreement with this hypothesis, studies have shown, for example, that learning of a forelimb reaching task modifies the dendritic density in the motor cortex (Greenough et al., 1985; Withers and Greenough, 1989; Bury and Jones, 2002), synaptogenesis and the size of the forelimb region in primary motor cortex derived using microstimulation mapping techniques (Kleim et al., 2004), and the signal-to-noise ratios of motor cortex cell activity (Kargo and Nitz, 2004). In addition, this hypothesis has propelled a parallel line of studies devoted to the characterization of the different forms of synaptic plasticity (Nicoll and Malenka, 1995; Bliss and Collingridge, 1993; Bear and Malenka, 1994) that are expressed in the primary motor cortex. Those studies have found that both long-term potentiation (LTP) and long-term depression (LTD) can be generated in pathways of the motor cortex under the appropriate conditions, both in vivo and in

This large body of evidence strongly suggests that the forelimb region within the primary motor cortex undergoes changes associated with the acquisition of a forelimb motor skill, such as the forelimb reaching task. A particularly intriguing possibility is that the extensive horizontal connections in the upper layers of the motor cortex can undergo changes in synaptic efficacy that underlie the acquisition of a skilled motor behavior (Asanuma and Pavlides, 1997; Keller, 1993; Hess and Donoghue, 1994). Therefore, if learning occurs through changes in synaptic strength of these connections, then learning of a motor skill should change the efficacy of motor cortex horizontal fibers. Indeed, recent work in the slice preparation supports this hypothesis (Rioult-Pedotti et al., 2000; Rioult-Pedotti et al., 1998). These papers presented evidence that learning of a reaching task was associated with a striking global increase in the amplitude of field potential population responses evoked in layers II-III by stimulating horizontal fibers as determined using input/output (I/O) curves, and this increase occurred only in the forelimb motor cortex contralateral to the trained forelimb. Moreover, the selective increase in response amplitude in the trained hemisphere was accompanied by a reduction in the amount of LTP that could be induced by theta-burst stimulation (Rioult-Pedotti et al., 1998) as well as a corresponding increase in the expression of LTD (Rioult-Pedotti et al., 2000). This data supports the idea that learning of a skilled motor behavior involving the primary motor cortex occurs by an increase in the efficacy of the population of horizontal connections in layers II-III of the motor cortex. However, the large
unidirectional change in synaptic efficacy of the population response reported in these studies is difficult to interpret because it could collapse the uneasy balance of excitation in cortical networks and perhaps lead to hyperexcitation (e.g., seizures); something that is not expected to occur with motor learning. Instead, the acquisition of a complex motor skill might be expected to produce both increases and decreases in synaptic strength that are distributed throughout the complex neural networks within layers II-III. These bidirectional distributed changes would be unlikely to cause large unidirectional changes in the population response of these neural networks. Indeed, an intrinsic property of neural networks in modeling studies is that the global state (i.e., sum of total synaptic weights) of the network must remain balanced, and is thereby stable during learning (Song et al., 2000; Buhmann and Schulten, 1986).

In the present study, conducted in vivo, we sought to determine if learning in the unilateral reaching task indeed affects the excitability, short-term plasticity and long-term plasticity of horizontal connections in layers II-III of the motor cortex. Because training in this task requires animals to be food-deprived, we compared the trained animals with similarly food deprived untrained animals and normal controls. The results show that the excitability, short-term plasticity and long-term plasticity of the studied horizontal connections was unaffected by motor learning. However, food deprivation and handling significantly enhanced the expression of LTD in these pathways.
Materials and Methods

Behavioral training

Fifty-five adult male Sprague-Dawley rats (225-300 gm) were used in this study and cared for in accordance with National Institutes of Health guidelines for laboratory animal welfare. All experiments were approved by the Drexel University Institutional Animal Care and Use Committee.

Three groups of rats were used in this study: control, trained and deprived. Animals in the control group were group housed and were not food-deprived or handled. The trained group was food deprived and trained in a skilled reaching task. The deprived group was treated as the trained group but these animals were not trained in the skilled reaching task. That is, although they were food deprived and placed in the training box there were no food pellets available and no skill was learned. Thus, the deprived group serves to control for stress-related variables such as food deprivation and handling.

Food deprivation consisted of placing the animals on a food-restricted diet sufficient in maintaining their body weight at 85% free-feeding weight. Water was provided ad libitum. During training in the skilled reaching task, animals were placed in a clear Plexiglas box (30 x 15 x 12 cm) with a 1 cm slit in one of its walls. The animals learned to reach through the slit with one forelimb and retrieve a small food pellet (45 mg, Noyes Precision Food Pellets) located on a platform outside the box. Only animals that solely used one forelimb during the training process were selected for further experimentation. Two groups of trained animals were studied. One group was trained 1 hr/day for 5-7 days and another group was trained 30 min/day for 11-13 days. Performance in the reaching task was evaluated by counting misses and successful
reaches for all the attempts made by the animal during the entire session. A successful reach consisted of the animal reaching with the limb through the slit of the box, grasping the food pellet and consuming it. A miss consisted in reaching through the slit of the box and failing to grasp the food pellet or to consume it. If the animal reached and failed to grasp the pellet with the initial movement but eventually managed to grasp the food pellet by a subsequent movement (without retracting the limb), this was considered a miss. Percent success rate was the number of successes divided by the number of attempts. The day after the last training or handling session the animals were subjected to surgical procedures.

**Surgical procedures**

All the animals from the control, deprived and trained groups were henceforth subjected to the same procedures. Animals were anesthetized with urethane (1.5 gm/kg, i.p.) and placed in a stereotaxic frame. All skin incisions and frame contacts with the skin were injected with lidocaine (2%). Body temperature was automatically maintained constant with a heating pad. The level of anesthesia was carefully monitored by continuously recording field potentials from the cortical recording electrodes and by testing limb-withdrawal reflexes. The cortical field potential activity was also displayed online as power spectrums derived by calculating fast-Fourier transforms (FFT) and this activity was stored for subsequent reference during data analyses. The anesthetic level was kept constant at approximately stage III/3 using supplemental doses of urethane (Friedberg et al., 1999). Small bilateral craniotomies were made over the forelimb primary motor cortex based on coordinates derived previously with microstimulation
mapping (Castro-Alamancos and Borrell, 1993). Although the extent of these representations can change between animals, the location of forelimb motor representations is constant and centered at around 2.5 mm lateral and 1 mm anterior to bregma. Small incisions were made in the dura to insert the electrodes and the cortical surface was covered with small pieces of gelfoam soaked in saline solution. At the end of the experiments, animals were euthanized with an overdose of sodium pentobarbitone (intraperitoneally).

**Electrophysiological procedures**

All electrophysiological procedures were conducted blind as to the experimental group. That is, the experimenter conducting the electrophysiology was unaware of the experimental group that the animal belonged to. A total of four electrodes were used simultaneously per animal; two stimulating electrodes and two recording electrodes. In each hemisphere, a recording electrode and a stimulating electrode were inserted into layers II-III of the forelimb primary motor cortex representation, as shown in figure 1A. Each electrode was lowered 500-µm below the pial surface at a 60° angle. The tips of the stimulating and recording electrodes faced each other at the pial surface and began their insertion at about 1 mm apart so that they would be separated about 500-µm tip-to-tip when they reached layers II-III. The mid-point coordinate between the stimulating and recording electrodes was approximately 2.5 mm lateral and 1 mm anterior to bregma. Minor adjustments were made in the depth of the stimulating and recording electrodes to produce the largest evoked response. The electrode arrangement allowed monitoring the
efficacy of horizontal pathways in the upper layers of primary forelimb motor cortex simultaneously from each hemisphere.

Extracellular field potential recordings were performed using glass micropipettes (500 kΩ) filled with saline solution. Stimulating electrodes consisted of concentric bipolar electrodes (200-µm diameter ultra-small concentric bipolar electrode; Frederick Haer Co., Bowdoinham, ME). As previously described in vivo and in slices, a 200-µsec current pulse in layers II-III produced a negative going field potential response peaking between 3-5 msec which is correlated with excitatory postsynaptic potentials recorded intracellularly and with a local current sink in layers II-III (Oldford and Castro-Alamancos, 2003; Castro-Alamancos et al., 1995). In a few experiments (n=2), the synaptic nature of the field potential evoked response was confirmed pharmacologically by applying the glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 250-µM) into the neocortex using a microdialysis probe (250-µm diameter, 2 mm long) placed adjacent to the recording electrode, as previously described (Castro-Alamancos, 2000; Oldford and Castro-Alamancos, 2003). ACSF was continuously infused through the probe at 4-µl/min and then CNQX was applied followed by a washout period. Application of CNQX completely abolished the negative field potential response that peaks between 3-5 msec post-stimulus and revealed the fiber volley (i.e., antidromic and orthodromic) component in the field potential recording, which peaks between 2-3 msec (Fig. 1B). Thus, the peak amplitude of the negative field potential response measured between 3 and 5 msec post-stimulus was taken as an index of synaptic efficacy.
During baseline recordings, stimulation alternated between each hemisphere at 0.088 Hz. After a ~1 hr stable baseline recording period was established, I/O curves were derived by averaging 12 responses at each of 7 current intensities: 0.5, 1, 2, 3, 4, 5 and 6 times the current required to elicit a field potential response of 1 mV in amplitude (maximum stimulation intensity did not exceed 300 µA). Thereafter, a minimum 30-min stable baseline was established at an intensity which elicited a half-maximal evoked response (the average intensity used was 50 µA; range 25-110 µA). LTD was induced by low-frequency stimulation (LFS) consisting of 1800 pulses at 2 Hz at twice the baseline stimulation intensity and was applied at 30-min intervals until the intracortical pathway appeared saturated (Rioult-Pedotti et al., 2000). Saturation was defined as no significant changes in field potential amplitude after two successive LTD stimulations. This typically occurred after the LTD stimulation was delivered 4-5 times. Following the last LTD stimulation, responses were recorded for a minimum of 30 min. Short-term plasticity (i.e., pair-pulse stimulation) was also monitored before and after LTD stimulation by using 3 pulses delivered at different interstimulus intervals (ISI) of 50, 150, 250 and 350-msec. Finally, LTP induction was attempted after LTD induction by using a standard theta-burst stimulation (TBS) protocol at twice the baseline stimulation intensity and consisted of 10 bursts (burst = 5 pulses at 100 Hz) delivered at 5 Hz. A total of 5 TBS were delivered at 10 sec intervals.

**Statistical analysis**

Although three groups of animals were generated experimentally (trained, deprived and control), the statistical analyses were performed on fours groups of data
corresponding to the trained hemispheres (i.e., contralateral to the trained forelimb),
untrained hemispheres (i.e., ipsilateral to the trained forelimb), deprived hemispheres
(i.e., data from both hemispheres for each animal were grouped) and control hemispheres
(i.e., data from both hemispheres were also grouped). Grouping of the data from both
hemispheres in the deprived and control groups was performed after comparing the left
vs. right hemispheres for those groups, which revealed no significant differences (see
below).

The statistical analysis consisted primarily of repeated measures ANOVAs where
the within subjects factor was either training day, current intensity for the I/O curves,
response amplitude before and after LFS or TBS and amount of facilitation before and
after LTD. The between subjects factor consisted of the different groups of hemispheres
as outlined below. Simple effects were used to decompose significant main effects and
interactions. Post hoc comparisons were performed with the Scheffe test. Individual
comparisons between groups were performed with a one-factor ANOVA. All data are
presented as mean ± SD.

Results

Motor skill learning

Animals were trained in a skilled reaching task for either 5-7 days (n=18 animals;
n=8 left-handed, n=10 right-handed) or 11-13 days (n=7 animals; n = 3 left-handed, n=4
right-handed). Animals rapidly acquired the skilled reaching behavior during the
extensive daily training sessions. Figure 2 shows the percent success rate for both of
these groups of animals per day of training. By day 5-6 of training, the animals in both
groups reached asymptotic success rates of about 65% that were significantly higher than
the first day. Thus, a repeated measures ANOVA revealed a significant effect of training
on success rate for the group trained for 5-7 days ($F_{4,76}=230.045; p<0.001$) and for the
group trained for 11-13 days ($F_{10,70}=92.412; p<0.001$). Post hoc comparisons revealed
that success rate in the task was significantly improved after the second day of training
when compared to the first day of training ($p<0.001$). A comparison of the success rate
during the final training day for the animals trained for 5-7 days and those trained for 11-
13 days revealed no significant differences ($F_{1,26}=1.1; p=0.300$). Thus, since there were
no apparent differences in the reaching behavior success rate between the animals trained
for 5-7 days and those trained for 11-13 days, electrophysiological data for the trained
animals were grouped together unless otherwise indicated.

**I/O curves reveal no significant differences in excitability**

Twenty-four hours after the last training session, animals underwent stereotaxic
surgery to compare the excitability of horizontal pathways in layers II-III of motor cortex
by deriving I/O curves across a range of seven current intensities (*i.e.*, 0.5, 1, 2, 3, 4, 5, 6
times the intensity required to evoke a 1 mV response). The I/O curve plots the amplitude
of the field potential evoked response as a function of current intensity. Figure 3A shows
an example of this procedure in a trained animal for both the trained and the untrained
hemispheres. Note that the amplitude of the evoked response increases with current
intensity as a consequence of the recruitment of more fibers with increases in current
spread. This procedure was performed for each of the four groups of hemispheres
studied: trained hemispheres (n=23), untrained hemispheres (n=24); deprived hemispheres (n=19), and control hemispheres (n=36). Since the control hemispheres group and the deprived hemispheres group were formed by combining the left and right hemispheres of those animals, we first compared the left and right hemispheres of the control and deprived animals to assure that they were not significantly different. Note that in the statistical analysis, the 1-times intensity values were excluded because the field potential amplitudes are set at 1 mV for each group. Figure 3B shows I/O curves for the left and right hemispheres of the control and deprived animals. A two-factor repeated measures ANOVA of the amplitude of the evoked responses revealed that there were no significant differences between the left and right hemispheres for either the control animals (F1,34=0.486; p=0.491) or the deprived animals (F1,17=0.172; p=0.684). Thus, the left and right hemispheres of these animals could be combined to form the control hemispheres group and the deprived hemispheres group. An additional comparison was made between the trained and untrained hemispheres of the trained animals to test if motor skill training affected the excitability of the horizontal motor cortex pathways contralateral to the trained forelimb. Figure 3C shows the I/O curves for these animals. A two-factor repeated measures ANOVA revealed no significant differences between the trained and untrained hemispheres of the trained animals (F1,45=0.341; p=0.562) indicating that the tested motor cortex pathways ipsilateral and contralateral to the trained forelimb did not differ in excitability. Finally, to test if there were significant differences between the I/O curves derived from the four groups of hemispheres taken together (Figure 3C, right panel), we performed an analysis to compare them. A two-factor repeated measures ANOVA revealed an obvious significant effect of intensity on the
amplitude of the evoked response (within subjects factor; $F_{5,490}=2142.1; p<0.001$), but no significant differences between the four groups of hemispheres (between subjects factor; $F_{3,98}=1.8; p=0.14$) and no interaction between these two factors ($F_{15,490}=1.4; p=0.11$). This indicates that neither motor learning nor food deprivation and handling affected the excitability of primary motor cortex pathways as assessed using I/O curves.

In the previous analysis, the animals trained for 5-7 days and those trained for 11-13 days were combined together since close inspection of the data revealed no apparent differences. In an effort to demonstrate that indeed there were no differences between these groups of animals, we compared the I/O curves of the following four groups of hemispheres: trained hemispheres for 5-7 days ($n=16$), untrained hemispheres for 5-7 days ($n=17$), trained hemispheres for 11-13 days ($n=7$), untrained hemispheres for 11-13 days ($n=7$). A two-factor repeated measures ANOVA revealed a significant effect of intensity on the amplitude of the evoked response ($F_{5,210}=731.329; p<0.001$), but no significant differences between the four groups of hemispheres ($F_{3,42}=2.172; p=0.108$) and no interaction between these two factors ($F_{15,210}=1.376; p=0.238$). Thus, the I/O curves were not significantly different between animals trained for 5-7 days and those trained for 11-13 days and, as mentioned above, training or food deprivation and handling had no effect on the I/O curves irrespective of the amount of time the animals spent in those conditions.
LTD is enhanced by food deprivation and handling but not by skilled motor learning

The next step was to investigate if there were differences between the different groups of hemispheres in their ability to undergo LTD. For example, one possibility is that motor skill learning may enhance the ability of horizontal pathways to undergo LTD. In order to test this hypothesis, we first established the conditions that allow to record stable baselines in urethane-anesthetized rats for extended periods of time. Thus, in a group of control animals (n=5) we found that we could maintain stable baselines for at least 3 hours as long as we kept constant the slow spontaneous oscillatory activity that occurs in neocortex under this anesthesia (Figure 4A). This activity was measured online by computing FFTs and deriving power spectrum analyses of the ongoing field potentials recorded from the electrodes implanted in each hemisphere that are used to obtain the evoked responses (Figure 4A and 4B). The FFT power spectrums were carefully monitored during the experiment and stored together with the evoked responses for further comparison off-line. Figure 4A shows an example of such a control experiment and displays both the power spectrum analysis and the amplitude of evoked responses for an extended period of time. Regardless of the group, we established that in order for an experiment to be included in the dataset the power spectrum of the slow oscillatory activity (0.1-4 Hz range) should not vary more than 40% between the baseline period and 30 minutes after the last LTD stimulation (~3 hours later). Animals that did not meet this criterion were removed from the LTD analysis unless indicated (see below). Figure 4B shows an example of the comparison of spontaneous field potential activity and the power spectrums derived using FFTs obtained during a period of baseline before LFS and
after the induction of LTD (i.e. 30 minutes after the last LFS). Although significant LTD was induced, the power spectrum activity was not significantly affected and thus the data was considered acceptable. Note that because I/O curves were generated immediately after an initial baseline period where stable stimulus-evoked responses were identified, all animals were included in the I/O curve analysis described in the previous section.

Figure 4C shows an example of LTD induced by the application of LFS four times at 30 min intervals in the trained and untrained hemispheres of a trained animal. Note a reduction in the amplitude of the evoked responses as a consequence of the LFS. The amount of LTD was measured 30 minutes after the last LFS. As done for the I/O curves, to form the group of deprived hemispheres and the group of control hemispheres, we combined the left and right hemispheres of the deprived and control animals, respectively. This was done after confirming that there were no significant differences in the amount of LTD between the left and right hemispheres for the control (F1,21=0.033; p=0.858; n=14, left; n=9, right) or deprived (F1,11=3.372; p=0.093; n=7, left; n=6, right) animals. Therefore, data from the left and right hemispheres could be combined safely to form these groups. Thus, four groups of hemispheres were studied: trained hemispheres (n=14), untrained hemispheres (n=14); deprived hemispheres (n=13), and control hemispheres (n=23). A repeated measures ANOVA of the amplitude of the evoked responses before and after the LFS revealed that there was a significant effect of the LFS stimulation on the evoked responses (F1,59=272.652; p<0.001). There was also a significant difference between the four groups of hemispheres on the evoked responses (F3,59=2.833; p<0.05) and there was a significant interaction between these two factors (F3,59=4.703; p<0.01) suggesting that the effect of LTD stimulation was different for each
Since the effect of LFS was found to be significant and to depend on the group, we compared the amount of LTD that was induced in our four groups of hemispheres. Figures 5A and 5B show group data of the amount of LTD induced in the four groups of hemispheres. Interestingly, the control hemispheres produced less LTD (17%; n=23) than the other hemispheres; i.e., deprived (27%; n=13), trained (30%; n=14) and untrained hemispheres (31%; n=14). A one-factor ANOVA of the amount of LTD induced in the four groups of hemispheres was significant (F_{3,62}=6.359; p<0.01). Moreover, posthoc comparisons revealed that the control hemispheres group produced significantly less LTD than the other three groups (p<0.01), while these three groups (deprived, trained and untrained hemispheres) did not differ significantly from each other. This indicates that food deprivation and handling which are common to the deprived, trained and untrained hemispheres significantly enhance the amount of LTD that is induced in horizontal pathways of motor cortex. Nevertheless, motor skill learning per se did not have any significant effect on the amount of LTD produced by LFS.

In the previous analysis, the animals trained for 5-7 days and those trained for 11-13 days were combined together since close inspection of the data revealed no apparent differences. In an effort to demonstrate that indeed there were no differences between these groups of animals, we performed a one factor ANOVA on the amount of LTD between the following four groups: trained hemispheres for 5-7 days (n=10), untrained hemispheres for 5-7 days (n=10), trained hemispheres for 11-13 days (n=4) and untrained hemispheres for 11-13 days (n=4). This analysis revealed no significant main effect (F_{3,24}=0.073; p=0.974) indicating that there were no differences in the amount of LTD between the animals trained for 5-7 days and those trained for 11-13 days. Furthermore,
we performed an additional analysis which included all the hemispheres of the animals trained in the skilled motor task, including those that did not meet our stability criteria described above. In this case, we still found no significant difference between the trained (23±16%; n=22) and untrained (26±17%; n=22) hemispheres in the amount of LTD (F1,42=0.158; p=0.693). These results further demonstrate that skilled motor training had no significant effect on the induction of LTD in horizontal pathways of layers II-III of the motor cortex.

**LTD is associated with a significant change in short-term plasticity**

The previous results show that significant LTD can be evoked in horizontal pathways of the motor cortex in vivo and that control animals produce significantly less LTD than the other animals. In an effort to characterize further LTD in this pathway, we evaluated the effect of LTD on short-term plasticity. In pathways where LTP and LTD are believed to be expressed by a change in neurotransmitter release probability there are consistent effects on short-term plasticity, such as facilitation and depression (Manabe and Nicoll, 1994; Weisskopf and Nicoll, 1995; Zucker, 1989). By first evaluating short-term plasticity in naïve pathways before the induction of LTD, we were able to study if motor learning or food deprivation and handling had any effect on short-term plasticity in these pathways. We found that at the 50-msec interstimulus interval horizontal pathways of the motor cortex consistently displayed facilitation (figure 6). Interestingly, before the induction of LTD, facilitation appeared to be stronger in control hemispheres than in the deprived, trained and untrained hemispheres, but this difference was not statistically significant (one-factor ANOVA; F3,57= 1.2; p=0.31). Thus, these results indicate that
although there is a tendency for food deprivation and handling to reduce the amount of short-term facilitation in horizontal pathways of layers II-III, this is not statistically significant.

Next, we tested if LTD changed the amount of facilitation in our four groups of hemispheres. A two-factor repeated measures ANOVA of the amount of facilitation at the 50-msec interval revealed a significant effect of LTD on the amount of facilitation (within subjects factor; $F_{1,57}=4.86; p<0.05$), and there was a significant interaction between the groups factor (between subjects factor) and the effect of LTD factor ($F_{3,57}=4.13; p<0.01$) indicating that the effect of LTD on facilitation depended on the particular group. Indeed, as shown in figure 6B, LTD reduced the amount of facilitation in the control group, while increasing the amount in the other three groups. Thus, after LTD, the amount of facilitation reversed in the four groups. However, just like before LTD, there was no significant difference between the four groups in the amount of facilitation after LTD (one-factor ANOVA; $F_{3,57}=1.3; p=0.27$). Because the amount of facilitation changed differently for the four groups as a consequence of LTD, we performed individual comparisons of the amount of facilitation before and after LTD for each of the four groups. The results showed that LTD significantly reduced the amount of facilitation in the control group ($F_{1,20}=5.259; p<0.05$), had no statistically significant effect in the deprived ($F_{1,14}=1.070; p=0.319$) group, but had the effect of increasing facilitation in the trained ($F_{1,11}=5.825; p<0.05$) and untrained ($F_{1,11}=6.020; p<0.05$) groups. Although this data met the assumptions of the parametric test, as determined by measuring homogeneity of variance (Levene’s test; $p>0.125$), normality (Shapiro-Wilk’s W test; $p>0.33$), kurtosis (not significant) and skewness (not significant), we also tested
the data using a non-parametric test. We found that the same comparisons conducted with the Wilcoxon T-test revealed no significant differences. Thus, it is important to realize that these differences are small and not very significant. Consequently, they should be taken with caution, but they are worth mentioning. These results taken together indicate that stress-related variables, such as food deprivation and handling, or motor learning have no significant effect on short-term facilitation in layers II-III horizontal pathways of motor cortex. However, the expression of LTD changed short-term plasticity in different ways depending on the experimental group. The expression of LTD in the control hemispheres, which produce very little LTD, is accompanied by a reduction in the amount of facilitation. In contrast, the expression of LTD in the trained and untrained hemispheres of the trained animals, which produce strong LTD, is accompanied by an increase in facilitation. This result may be taken to suggest that in the trained and untrained hemispheres of the trained animals, the expression of LTD is a consequence of a reduction in neurotransmitter release probability. However, additional studies would be required to demonstrate this directly. Finally, it was interesting that while the deprived hemispheres produce similar amounts of LTD than the trained and untrained hemispheres of the trained animals, the expression of LTD was not accompanied by a significant change in short-term plasticity. Perhaps, the two distinct mechanisms that reduced and enhanced facilitation in the control and trained animals, respectively, operated together in the deprived animals and, thus, cancelled each other.
**LTP is not induced**

Preliminary experiments conducted in naïve pathways of control animals revealed that application of theta-burst stimulation was ineffective in inducing LTP in horizontal pathways of the motor cortex in vivo. This is in agreement with studies in slices, which have shown that LTP is not generally induced in these pathways unless inhibition is suppressed (Castro-Alamancos et al., 1995; Rioult-Pedotti et al., 1998; Hess et al., 1996). We did not attempt to study LTP during the suppression of inhibition in vivo because of the dramatic consequences on spontaneous activity that disinhibition produces in the motor cortex in vivo (Castro-Alamancos, 2000). Instead, we attempted to evaluate if after the induction of LTD there was any significant difference in the ability of the different groups to undergo LTP. A two-factor repeated measures ANOVA of the amplitude of the evoked responses before and 30 min after LTP stimulation revealed no significant effect of the LTP stimulation \( (F_{1,62} = 0.04; p=0.825) \) and no significant group effect \( (F_{3,62} = 1.034; p=0.384) \) or interaction \( (F_{3,62} = 1.802; p=0.156) \). The results indicate that under these conditions (i.e., without suppressing inhibition), LTP is not induced in the horizontal pathways of layers II-III in any of the four groups of hemispheres studied.

**DISCUSSION**

In the present study, we assessed the effects of learning a skilled reaching behavior and the stress-related effects of food deprivation and handling on the excitability, short-term plasticity and long-term plasticity of horizontal pathways in layers II-III of motor cortex. Neither skilled motor learning nor stress-related variables affected the excitability of horizontal pathways of the motor cortex as evaluated using I/O curves.
Interestingly, stress-related variables, such as food deprivation and handling, strongly enhanced LTD in these pathways, while skilled motor learning had no effect on LTD. In addition, the ability to induce LTP, which was absent in control animals, was not affected by food deprivation and handling or by skilled motor learning. Finally, neither skilled motor learning nor stress-related variables affected the short-term plasticity of these pathways, which display slight facilitation in response to pulses delivered at 50-msec interstimulus intervals. However, the expression of LTD was accompanied by a tendency for changes in facilitation that differed between the experimental groups. While these effects were small they are worth mentioning. In control animals, the expression of LTD is accompanied by a reduction of facilitation. Food deprivation and handling eliminated this reduction of facilitation caused by LTD expression that is observed in control animals. Moreover, this effect of food deprivation and handling was augmented by motor training since the expression of LTD is accompanied by an increase of facilitation in trained animals. Notably, in the present study, we never found a difference between the trained and untrained hemispheres of trained animals indicating that skilled motor learning did not affect the excitability, short-term plasticity or long-term plasticity of population responses in horizontal pathways of layers II-III of the forelimb primary motor cortex. In contrast, stress-related variables, such as food deprivation and handling, did enhance the ability of these pathways to undergo LTD.

Data from the present study do not support the hypothesis that skilled motor learning leads to a global increase in the synaptic efficacy of a synchronously activated neuronal population in horizontal networks of layers II-III of motor cortex. Thus, the data presented here contrast with those previously reported (Rioult-Pedotti et al., 2000; Rioult-
Pedotti et al., 1998), which found that the excitability and LTD of horizontal pathways of motor cortex changed as a consequence of skilled motor learning. There are some differences between our study and the previous studies that may explain the discrepancies. For example, although the present study and the previous slice studies used adult animals, the reported weight of the animals is higher in the present study suggesting that the slice work was carried out in younger animals, which may contribute to the differences observed. Clearly, the most obvious difference is that our study was conducted in anesthetized animals in vivo, while the previous studies were done in slices. This may well have been a contributing factor, which we cannot discard. In fact, one possibility is that the effects of learning cannot be detected because they are masked by the anesthetic used in vivo. However, if the anesthetic was having a major effect it would likely have affected the induction of LTD, but we found that LTD was induced in urethane anesthetized animals at similar levels than in slices. Also, in previous studies that were conducted simultaneously in vivo using urethane anesthesia, and in vitro using slices, the response characteristics of synaptic pathways and their plasticity were found to be quite similar in vivo and in slices (Castro-Alamancos and Calcagnotto, 2001; Castro-Alamancos, 2002a; Castro-Alamancos, 2002b). Furthermore, many characteristics of the responses recorded in the present study in vivo are identical to those recorded in our lab in slices in the same pathways. For example, the shape, amplitude, excitability and short-term plasticity of field potential responses in layers II-III horizontal pathways in vivo and in slices are indistinguishable (Castro-Alamancos et al., 1995). Since LTD is expressed in vivo and in slices similarly, it would be expected that its modulation by learning should also be expressed similarly under both conditions. In fact, in the present study, LTD
induction is clearly modulated by food deprivation and handling despite the anesthesia. In any case, as indicated above, this is a major difference that can not be discarded and may explain why the previous slice studies found an effect of learning while the present study did not. Another related question is why did the slice studies not reveal an effect of food deprivation and handling on LTD? The first paper by Rioult-Pedotti et al. (1998) consisted of naïve controls (n=12) and food deprived controls (n=8), and these animals were mixed together to form one control group (n=20). Moreover, the second paper by Rioult-Pedotti et al. (2000) did not include naïve controls. In order to reveal the effect of food deprivation, it is necessary to compare naïve controls with food deprived animals, which was never done in those studies. Finally, it is important to note that the fact that we observed an effect of food deprivation and handling on LTD induction does not explain the discrepancy between the previous studies in slices and the present study on the effect of motor learning. That is, it is unclear why we found no differences between the trained and untrained hemispheres while the slice studies did. In conclusion, at least in vivo, skilled motor learning in a reaching task does not enhance the induction of LTD in motor cortex pathways, while food-deprivation and handling does.

*How would food deprivation change the ability of horizontal pathways to undergo LTD?* An interesting finding from our study is that the food-deprived and handled animals consistently expressed more LTD than the un-manipulated control animals, and this effect was not further affected by motor learning. It is important to consider that food deprivation, and the consequent loss of ~20% of body weight, is a strong stressor comparable to, for example, electric footshocks (Carlson et al., 1987). In fact, food deprivation per se strongly enhances the activity of the hypothalamus-pituitary-adrenal
axis and also increases the levels of several neuromodulators such as norepinephrine and acetylcholine, among others (Kiss et al., 1994; Heiderstadt et al., 2000; El Fazaa et al., 2000; Savard et al., 1983; Endou et al., 2001). That food deprived animals are stressed is apparent when an investigator handles these animals; deprived animals are hyperactive and more excitable (unpublished observations; (Endou et al., 2001; Abraham and Gogate, 1989). In addition, handling animals and placing them in novel environments can also lead to stress related changes (Enrico et al., 1998; Feenstra et al., 1998; Kawahara et al., 2000; Davis et al., 2004), although these changes tend to decline with repeated exposures and handled animals are not hyperactive and as excitable as food-deprived animals. In the present study, it can not be ascertained which of these factors (i.e. food deprivation, handling) was the major contributor to the observed effects. We speculate that food deprivation was the more relevant of the two factors. Thus, the effects of food deprivation and handling are circumscribed within the realm of stress related changes. In accordance with our findings, studies that have evaluated the effects of stress on synaptic plasticity in the hippocampus have consistently demonstrated a reduced capacity for LTP and a corresponding increase in the ability to induce LTD (Xu et al., 1997; Kim et al., 1996; Manahan-Vaughan, 2000; Pavlides et al., 1996). This observation is also found in other pathways, such as the hippocampal-prefrontal cortex (Rocher et al., 2004) and amygdala-prefrontal cortex (Maroun and Richter-Levin, 2003) pathways. The generality of these effects suggest that neuromodulators and/or circulating neurohormones are the cause. Potential mediators of these modulations of plasticity are high levels of circulating hormones released by the hypothalamus-pituitary-adrenal axis such as corticotrophin releasing hormone, ACTH and corticosterone, and increases in the release of
neuromodulators, such as acetylcholine and norepinephrine, among others, which are all increased during stress (Carrasco and Van de Kar, 2003; Degroot et al., 2004; Mark et al., 1996; Valentino et al., 1993). Indeed, activation of both type I and type II glucocorticoid receptors in the hippocampus lead to an inhibition of LTP induction and enhanced LTD (Pavlides et al., 1995). Also, both norepinephrine and acetylcholine have been shown to facilitate the induction of LTD in layers II-III visual cortex synapses (Kirkwood et al., 1999) and in other brain regions (Fujii and Sumikawa, 2001; Massey et al., 2001; Scheiderer et al., 2004). Therefore, it is plausible that our findings of an increased expression of LTD in food deprived and handled animals are mediated by a stress-induced increase in one or several of these neurochemicals. Future studies could be designed to decipher which of these factors are responsible for the increase in LTD produced in motor cortex by food deprivation and/or handling. However, this was not a goal of the present study, which was instead interested in testing the effects of skilled motor learning on the excitability and synaptic plasticity of motor cortex pathways in vivo.

An additional finding in the present study is that food deprivation not only enhanced LTD but the expression of this enhanced LTD seemed to be produced via a different mechanism because facilitation (short-term plasticity) changed differently in control and food deprived animals after the induction of LTD. Moreover, this effect was exacerbated by motor training since in trained animals the expression of LTD resulted in an increase in facilitation, which contrasts with the reduction of facilitation produced by LTD in the control animals. We believe that the exacerbation of this effect by training must be considered in the context that trained animals are actually more stressed than
food deprived animals, because in addition to being hungry trained animals have to work for food. It is difficult to definitively interpret the effects observed on facilitation because these were small and also because in vivo inhibitory networks are intact making it difficult to interpret changes in facilitation. As a reference, excitatory synapses enhance facilitation as a consequence of a reduction in neurotransmitter release probability (Zucker, 1989; Manabe and Nicoll, 1994; Weisskopf and Nicoll, 1995). In a speculative note, it is possible that the neuromodulators or neurohormones increased by stress associated with food deprivation and handling enhanced LTD by affecting neurotransmitter release probability in the excitatory horizontal synapses.

*Do the present results imply that skilled motor learning does not involve changes in the efficacy of horizontal connections of the motor cortex?* One interpretation of our results is that skilled motor learning may not involve changes in the efficacy of synaptic connections in the primary motor cortex. We disagree with this assertion and believe that the present results, although compatible with this initial interpretation, can be interpreted in a different way. We believe that the changes in synaptic efficacy produced in networks of the primary motor cortex during skilled motor learning are highly distributed and bidirectional, and thus would not be apparent in any study that measures population responses that engage the whole distributed network because the synaptic efficacy of the population remains stable over the course of motor learning. This interpretation seems in agreement with neural network modeling studies of learning and memory that have long been dependent upon using synaptic weight change algorithms that implicitly maintain a stable global state (Abbott and Nelson, 2000). This serves to avoid both extremes of the network state; that of permanent inactivity and the state of epileptic hyperactivity.
(Buhmann and Schulten, 1986). Thus, studies utilizing models based on spike-timing dependent plasticity rules (Markram and Tsodyks, 1996; Bi and Poo, 1998; Magee and Johnston, 1997) also require a global balance between potentiation and depression of synaptic weights (Song et al., 2000; Fusi et al., 2000; Matsumoto and Okada, 2003; Carpenter and Milenova, 2002). These models suggest that during learning, competition for synaptic strengthening occurs through the control of timing of the post-synaptic action potential. This competitive nature of the network components automatically creates a distributed balance between potentiation and depression within the population of synaptic connections (Song et al., 2000; Zhou et al., 2003). These studies support the hypothesis that learning occurs via a distribution of synaptic strengths across the network and, thus, a unidirectional change would not be readily expressed when population responses engaging large portions of the network are simultaneously assessed. In contrast, it would be expected that changes caused by neuromodulators or neurohormones, which are more widespread than activity-driven changes, may be manifested more clearly in the population responses measured in these studies. Indeed, in agreement with this interpretation, we found that changes potentially caused by neuromodulators or neurohormones drive the population of synaptic connections in the neural network in a similar direction (i.e., enhancement of LTD), and thus are clearly expressed by the population responses measured in both hemispheres of all the food deprived animals irrespective of whether they were trained or not. In conclusion, stress-related mechanisms produced by food deprivation and handling enhance LTD in the population responses of horizontal pathways in layers II-III of the motor cortex, while skilled motor training per se has no significant effect on these responses.
Reference List


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FIGURE LEGENDS

**Figure 1.** Schematic representation of the experimental setup and synaptic nature of field potential responses.

*A,* Location of the stimulating and recording electrodes in layers II-III of the forelimb motor cortex of one hemisphere. Note that in all experiment the electrodes were implanted simultaneously in both hemispheres.

*B,* Field potential responses and the effect of the application of a glutamate receptor antagonist (CNQX; 500 µM) with a microdialysis probe placed adjacent to the recording electrode. Application of CNQX abolished the negative field potential response that peaks between 3-5 msec post-stimulus, and reveals a fiber volley (i.e., antidromic and orthodromic) component in the field potential recording, which peaks between 2-3 msec. The abolishment of the field potential response over a 3 msec latency confirms the synaptic nature of the response measured in this study.

**Figure 2.** Effect of training in the reaching task on success rate. Percent success rate per day in a group of animals trained for 5-7 days and in a group of animals trained for 11-13 days in a reaching task. Success rate was calculated as the number of successful reaches divided by the number of attempts. A successful reach consisted in reaching, grasping a food pellet and consuming it.
Figure 3. I/O curves reveal no differences between the groups.

A, Examples of field potential responses used to derive I/O curves from the trained and untrained hemispheres of a trained animal. The I/O curves are derived across a range of seven current intensities (i.e., 0.5, 1, 2, 3, 4, 5 and 6 times the intensity required to evoke a 1 mV response). Note that the amplitudes of the evoked responses increase with intensity similarly in the trained and untrained hemisphere.

B, I/O curves for the left and right hemispheres of the control and deprived animals. The amplitudes of the left and right hemispheres did not differ significantly in either the control or deprived animals.

C, I/O curves for the trained and untrained hemispheres of the trained animals, left panel. The amplitudes of the trained and untrained hemispheres did not differ significantly, and these did not differ significantly from the deprived and control hemispheres, right panels. Statistical analyses are described in the Results.

Figure 4. Example of LTD induced by LFS in the trained and untrained hemispheres of a trained animal.

A, Example of a stable baseline maintained for 3 hours during simultaneous monitoring of the spontaneous oscillatory activity through the same recording electrode by computing FFTs and deriving a power spectrum of the ongoing activity. Increases in power for a particular frequency are represented as hot colors (red) and blue represents zero.

B, Comparison of spontaneous field potential activity and the power spectrums derived using FFTs obtained during a period of baseline before LFS and after the induction of
LTD (i.e. 30 minutes after the last LFS). Note that the power spectrums did not change significantly before and after LTD.

**C**, Example of LTD induced by the application of LFS four times at 30 min intervals in the trained and untrained hemispheres of a trained animal. Note a reduction in the amplitude of the evoked responses as a consequence of the LFS, which was similar in both hemispheres.

**Figure 5.** Population data of LTD in horizontal pathways of layers II-III in motor cortex. 

**A**, LTD induced in the control, deprived, trained and untrained hemispheres after the last LFS stimulation compared to the baseline before the first LFS. Note that the control hemispheres produced less LTD than the other hemispheres.

**B**, LTD induced 30 minutes after the last LFS in the four groups of hemispheres. Note that the control hemispheres produced significantly less LTD than the other hemispheres (* p<0.01). Data are mean ± SD.

**Figure 6.** Effect of LTD on short-term plasticity.

**A**, Ratio of the amplitude of the field potential (FP) response between the third and first responses in a train delivered at four different interstimulus intervals (ISI) for the control, deprived, trained and untrained hemispheres. Note that at 50 msec ISI the responses show facilitation, and that there were no significant differences between the groups.

**B**, Effect of LTD on facilitation measured at 50 msec ISI. Facilitation was measured as in A, before and after the induction of LTD (measured 30 min after the last LFS). Note that facilitation was significantly reduced by LTD in the control hemispheres and
significantly enhanced in the trained and untrained hemispheres (* significant difference between before and after LTD). See Results for statistical analyses. Data are mean ± SD.

C, Sample traces showing facilitation at 50 msec ISI (3 stimulus train) of an untrained hemisphere before (black) and after (light gray) the induction of LTD with LFS.

D, The traces shown in C are overlaid to reveal changes in facilitation after LTD. The left panel shows the responses to the first stimulus scaled so that they match each other in amplitude. The right panel uses the same scaling applied to the third responses, which reveals an enhancement in facilitation after LTD (light gray).
Fig. 3

A
Trained hemisphere
Untrained hemisphere

B
Control Animals: left & right hemispheres
Deprived Animals: left & right hemispheres

C
Trained Animals: trained & untrained hemispheres
Trained, Untrained, Deprived and Control hemispheres

FP Amplitude (ratio)

Intensity (x threshold)
Fig. 4

A

3 hour baseline

FFT Power (Hz)

FFT Amplitude (%)

Time (min)

B

FFT Power spectrum analysis before and after LTD

Before LTD

After LTD

0.01

0

0.004

0.008

0.012

Frequency (Hz)

0

10

20

0.6 mV

3 sec

C

Trained hemisphere

Untrained hemisphere

FP Amplitude [%]

Time (min)

0 30 60 90 120 150 180

0 30 60 90 120 150

5 msec

1 mV
Fig. 5

A. LTD after the last LFS

B. Amount of LTD in the four hemisphere groups