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Formation of Dendritic Spines with GABAergic Synapses Induced by Whisker Stimulation in Adult Mice

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Summary

During development, alterations in sensory experience modify the structure of cortical neurons, particularly at the level of the dendritic spine. Are similar adaptations involved in plasticity of the adult cortex? Here we show that a 24 hr period of single whisker stimulation in freely moving adult mice increases, by 36%, the total synaptic density in the corresponding cortical barrel. This is due to an increase in both excitatory and inhibitory synapses found on spines. Four days after stimulation, the inhibitory inputs to the spines remain despite total synaptic density returning to pre-stimulation levels. Functional analysis of layer IV cells demonstrated altered response properties, immediately after stimulation, as well as four days later. These results indicate activity-dependent alterations in synaptic circuitry in adulthood, modifying the flow of sensory information into the cerebral cortex.

Introduction

Various studies have shown that modifications in sensory experience result in alterations in synapse and neuronal morphology in the central nervous system (Chang and Greenough, 1982; Greenough et al., 1985; Micheva and Beaulieu, 1995). Subsequent work demonstrated that interrupting the normal flow of sensory information into the somatosensory cortex also induces similar changes, suggesting that this plasticity was determined by changes in neuronal activity (Jones et al., 1996; Kleim et al., 1998). Investigating this idea further, recent in vitro experiments have demonstrated that the dendritic spine is central to the remodeling of neuronal circuits (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999). These small protrusions from the dendritic shaft, discovered over 100 years ago by Ramon Y Cajal (1891), form the majority of synaptic inputs in the cerebral cortex. They undergo rapid structural changes in response to alterations in sensory experience in young animals (Lendvai et al., 2000); and, in slice preparations, the induction of LTP leads to the emergence of novel spines (Engert and Bonhoeffer, 1999), accompanied by an increase in the proportion of axon terminals contacting two or more dendritic spines (Toni et al., 1999). Our current understanding, therefore, based on developmental and in vitro studies, is that the formation, and shape changes, of these highly motile structures are activity dependent, and occur within a few hours. In the adult cerebral cortex, however, do such dynamic changes in synaptic circuitry occur? We address this question in the present study, using an in vivo model that allows increased sensory stimulation of a part of the somatosensory pathway of a normally behaving mouse.

We used the whisker-to-barrel pathway (Figure 1), in which each whisker on the mystacial whiskerpad of the mouse is represented anatomically and functionally in layer IV of the somatosensory cortex by a single multineuronal assembly or barrel, clearly visible in histological preparations (Woolsey and Van der Loos, 1970). The barrelfield has several advantages for histological and electrophysiological studies as it gives the possibility of a natural and selective activation of an easily identifiable morphological representation of a peripheral receptive zone, the whisker follicle. Previous studies from this laboratory showed that single passive whisker stimulation, leaving all others intact, in a normally behaving individual caused a decreased neuronal response toward the stimulated whisker, as studied using deoxyglucose as a metabolic marker (Melzer et al., 1985; Welker et al., 1992) as well as an increase in the GAD enzymes (Welker et al., 1989) and upregulation of the mRNA for BDNF (Rocamora et al., 1996). In the present study, with the same stimulation paradigm, we have carried out an unbiased ultrastructural analysis of serial sections, using the electron microscope, to investigate the morphological alterations of neuronal ultrastructure in adult cortical barrels immediately after whisker stimulation, as well as 4 days later. The structural analysis was paralleled with single unit recordings in similarly treated mice to study the physiological effects of the whisker stimulation.

We found that immediately after 24 hr of stimulation, there was an increased density of synapses in the corresponding barrel. Serial section analysis showed that the increase was due to the appearance of excitatory synapses on dendritic spines as well as an increase in the proportion of spines with two synapses, one excitatory, the other inhibitory. Four days after stimulation was stopped, however, the inhibitory inputs to the spines remained despite total synaptic density returning to pre-stimulation levels. Along with this increased level of inhibition after whisker stimulation, measured morphologically, we also observed altered response properties of layer IV neurons to whisker deflection.

Results

EM-Analysis after 24 hr of Whisker Stimulation

For the ultrastructural analysis, we used a total of 12 mice, 6 of which had been stimulated whereas the 6 others served as controls. From each group, the brains of 3 mice were processed for postembedding GABA immunohistochemistry to classify presynaptic and postsynaptic elements into inhibitory (GABA positive) and excitatory (GABA negative). The remaining 3 mice, in the 24 hr stimulated group, were processed for conventional

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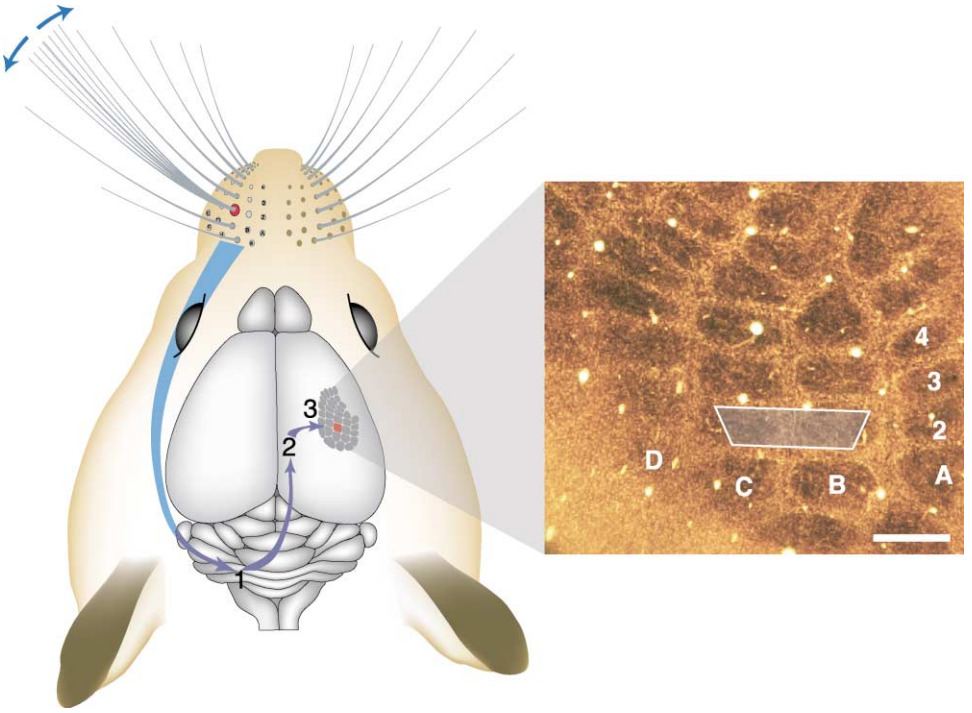

Figure 1. Illustration of the Whisker-to-Barrel Pathway in the Mouse

In this part of the somatosensory system, there exists a one-to-one relationship between each whisker follicle and its corresponding barrel in the contralateral somatosensory cortex. This topographical arrangement exists in the brainstem (1) and thalamus (2) as well as the cortex (3). The arrangment of cortical barrels is shown in the 80 μm thick tangential section (right) which has been prepared for the electron microscope and embedded in Durcapan resin. Also indicated is the exact size and orientation of the thin (60 nm) sections encompassing both the C2 (stimulated) and B2 (nonstimulated) barrels. Ribbons of between 35 and 50 serial sections were cut from blocks of this size and electron micrographs were always taken from the center of the barrel hollows. The scale bar is 200 μm.

electron microscopy with no GABA immunocytochemistry. Analyses from stacks of serial electron micrographs showed that after 24 hr of whisker stimulation, total synaptic density was significantly higher in the barrel corresponding to the stimulated C2 whisker (Figure 2). This was compared to neuropil from the neighboring B2 barrel in the stimulated mice and to the C2 barrel hollows from control (unstimulated) animals. This double comparison ruled out more general nonwhisker-specific effects on the synapse number in stimulated animals and highlights that the increase in synaptic density is specific for the barrel corresponding to the stimulated whisker. Identification of the postsynaptic structures showed that the greatest proportional increase, after stimulation, concerns synapses on spines (Figure 2), whereas the proportion on dendrites was unaltered.

Further analysis was directed toward the apparent increase in synapses found on the dendritic spines. The three stimulated and three unstimulated animals, which were all processed with GABA immunohistochemistry, had a mean spine density that was significantly increased in the 24 hr stimulated group (stimulated = 0.52 μm^{3-1} ± 0.029 μm^{3-1}; unstimulated = 0.41 ± 0.034 μm^{3-1}, $p = 0.0275$ hierarchical ANOVA). This analysis also revealed, however, a significant increase in the proportion of spines with two synapses (stimulated = 0.07 μm^{3-1} ± 0.03 μm^{3-1}; unstimulated = 0.02 ± 0.006 μm^{3-1}, $p = 0.026$ hierarchical ANOVA; Figure 3). These double synapse spines invariably had one excitatory synapse, whereas the other one was inhibitory. No spine was ever seen to have more than two synapses. Three-dimensional reconstructions of complete spines (n = 73) demonstrated the variety of spine shapes within the sampled volumes (examples shown in Figure 3). The shape and area of the asymmetric synapse varied widely, although that of the inhibitory synapse was more consistently macular. The position of this inhibitory input on the spine surface varied from being on the spine head to being on its neck or at the junction with the dendrite.

EM-Analysis Four Days after Whisker Stimulation Was Stopped

To address the question as to whether these modifications were transitory, we stimulated an additional group of three mice for the same time period, removed the metal pieces from the whiskers, without damaging the whiskers themselves, and returned the animals to their home cages for four days. In this third group of mice the total synapse density in the barrel, corresponding to the stimulated whisker, is at the unstimulated level (Figure 2), compared with the density in the B2 barrel and in C2 barrel of controls. Also, the mean density of spines (0.44 ± 0.046 μm^{3-1}) had regained the level of the control animals, as had the density of synapses on spines (0.47 ± 0.05 μm^{3-1}). However, the proportion of GABA synapses on spines remained the same (0.06

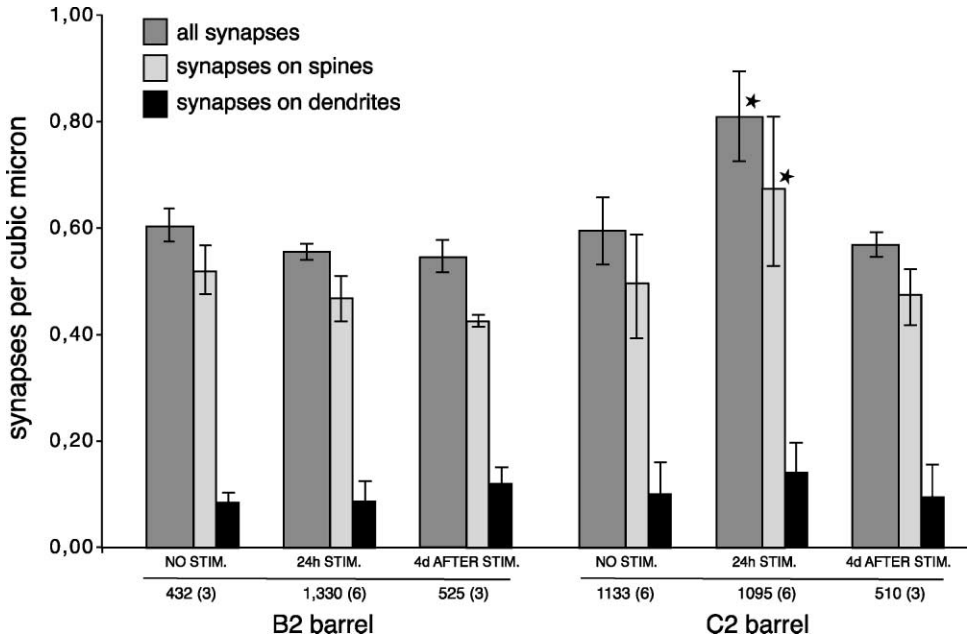

Figure 2. The Effect of Whisker Stimulation on Synaptic Density in the Corresponding Barrel

Increased synaptic density after 24 hr of whisker stimulation is mainly due to an increased number of synapses on spines. Synaptic density chart showing the mean and standard deviation of values counted from all volumes of neuropil in the B2 and C2 barrel hollows in the three groups of mice: no whisker stimulation (NO STIM), immediately after 24 hr of whisker stimulation (24h STIM), and four days after whisker stimulation (4d AFTER STIM). The mean density of all synapse types are indicated by the dark gray bars, synapses on spines by black bars, and those on dendrites, light gray. The single asterisks above the bars in the C2 barrel after 24 hr whisker stimulation indicate a statistically significant increase in the density of all synapses, and synapses on spines ($p < 0.001$ hierarchical multivariate analysis of variance). The total volume of neuropil sampled, in cubic microns, is indicated below each group and the number of mice used in parentheses.

$\mu m^{3-1} \pm 0.03\ \mu m^{3-1}$; Figure 3) as the mice analyzed immediately after the stimulation, and was significantly higher than in controls. Our results show that the synaptic density increase, seen immediately after stimulation, would appear to be transitory, but the insertion of GABA synapses onto spines is an alteration in synaptic connectivity that lasts well beyond the period of increased sensory experience.

To further interpret the morphological data in terms of a shift in balance between excitation and inhibition, we calculated the density of excitatory and inhibitory synapses on dendritic shafts and spines, as well as on spines alone, using the data of the nine mice in which GABA immunocytochemistry was carried out (Table 1). Considering firstly the change in excitatory synapses, immediately after stimulation, there was a 0.22-fold increase of these synapses on denditic spines and shafts. For those on spines alone, a 0.17-fold increase was found. The changes in inhibitory synapses, however, were more dramatic with a 0.94-fold increase in GABA synapses seen on dendritic shafts and spines at this time point, and on just the spines, a 2.84-fold increase. These changes at the level of the dendritic spine translated to a 3-fold increase in the percentage of inhibitory synapses on spines. Four days after the stimulation has been stopped, the density of excitatory synapses has returned to prestimulation levels; however, the density of GABA synapses remains significantly increased. In terms of the percentage of synapses on spines that are inhibitory, the value remains the same at both post-stimulation time points (Table 1). These differential increases in the density of excitatory and inhibitory synapses is an argument against the possibility that these structural modifications are due to a shrinkage of the neuropil.

To summarize the modifications, we calculated the overall change in the ratio between excitation and inhibition for the two sets of synapses. In the control group, for each inhibitory synapse, there are four excitatory (4.59 ± 0.5). Immediately after stimulation, however, this ratio has dropped significantly (2.89 ± 0.12, $p < 0.029$), due to a much greater increase in the total number of GABA synapses in comparison to the excitatory inputs, which increase to a much lesser degree. Four days later, this ratio is not significantly different from the control value (3.4 ± 0.87, $p > 0.2$).

Single Unit Recordings

We next performed single unit recordings to test whether these structural modifications are accompanied by changes in the response properties of laver IV cells to whisker deflection (Figure 4). The neurophysiological analysis showed a significantly decreased response of layer IV neurons toward the deflection of the stimulated whisker (77 units were recorded from a total of 20 mice), as compared to responses of neurons in control (unstimulated) mice (Figures 4 and 5; 94 units from 20 mice). This decrease was observed in the later epochs after whisker deflection (12–25 and 50–100 ms post-stimulus), while the initial, short latency response period was unaltered. The initial response of cortical neurons reflects the thalamo-cortical transmission of

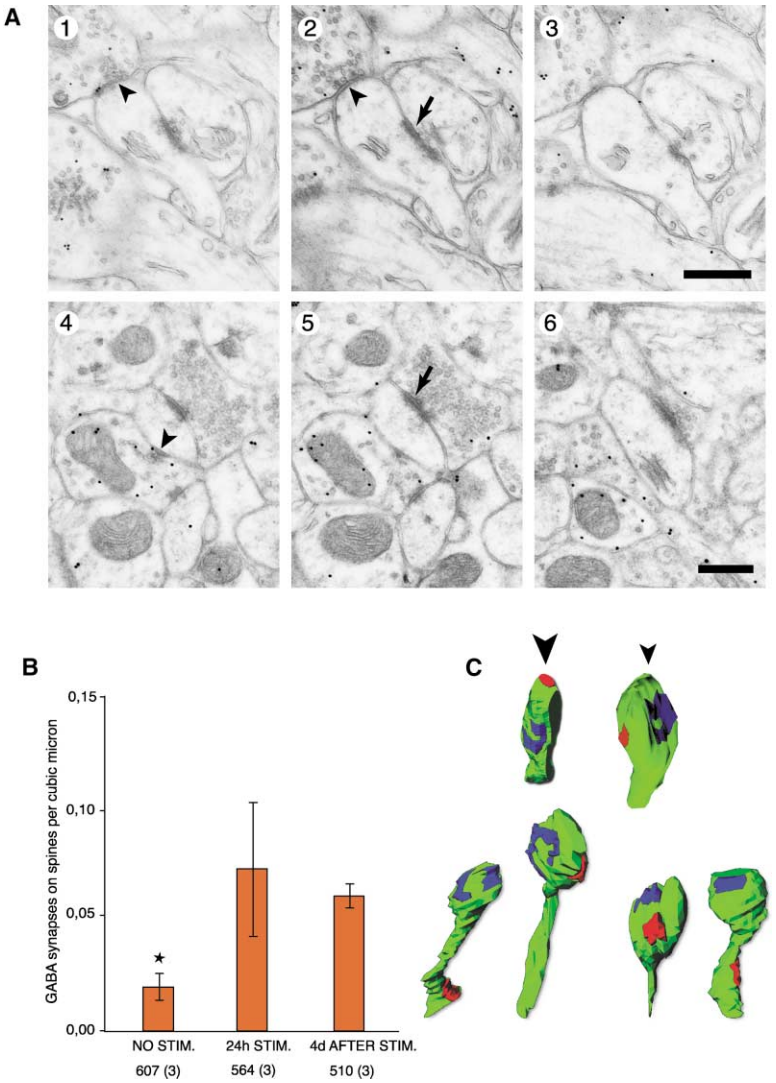

Figure 3. Insertion of GABAergic Synapses on Spines

Whisker stimulation induced a significant increase in the GABAergic innervation of spines, which remained 4 days later. Two series of three electron micrographs (1, 2, 3 one spine; 4, 5, 6 the other) each showing two synapses, one GABAergic (arrowhead), the other asymmetric (arrow). Scale bars are 0.5 μm. The three-dimensional reconstructions of these two spines are arrowed in (C) (large arrowhead corresponding to micrographs 1, 2, 3; small arrowhead, micrographs 4, 5, 6), along with four other examples, at the same scale, from a C2 barrel stimulated for 24 hr, illustrating the variety of spine shapes and their synaptic surfaces (asymmetric, blue; GABAergic, red). Further examples of reconstructed spines are also provided on our website (http://www-ibcm.unil.ch/welker/), which also includes a stack of serial sections from which the electron micrographs 4, 5, 6 were taken. The chart (B) shows the mean and standard deviation of GABAergic synapse density on spines in the C2 barrel hollow from three groups of mice receiving no whisker stimulation, immediately after 24 hr of whisker stimulation, and 4 days after whisker stimulation. The total volume of neuropil sampled, in cubic microns, is indicated below each group and the number of mice used in parentheses. The single asterisk above the no stimulation control group indicates that mice that did not receive whisker stimulation had significantly fewer GABAergic synapses on spines per unit volume compared to the two other groups ($p = 0.026$ ANOVA).

the sensory information, whereas the subsequent spikes reflect intra-cortical relay (Armstrong-James et al., 1992; Welker et al., 1993). Four days after the period of stimulation, there is an enhanced response of cortical neurons in a late post-stimulus epoch (50–100 ms; $p < 0.001$) as compared to controls (Figure 4C) and the 24 hr stimulated group.

Discussion

These results demonstrate that sensory stimulation in the adult animal is able to induce specific changes in synaptic circuitry in layer IV of the neocortex, the first relay of peripheral information arriving in the neocortical circuitry. Plasticity was induced by the passive stimula-

Table 1. Total Densities of Excitatory and Inhibitory Synapses per Cubic Micron of Neuropil

	Control	24 hr Stimulation	4 Days after Stimulation
Density of excitatory synpases (um^{-3}):			
On spines and dendrites	0.45 ± 0.03	0.55 ± 0.01**	0.44 ± 0.03
On spines	0.41 ± 0.04	0.48 ± 0.01*	0.41 ± 0.05
Density of inhibitory synapses (um^{-3})			
On spines and dendrites	0.098 ± 0.02	0.19 ± 0.012*	0.13 ± 0.03
On spines	0.019 ± 0.006	0.073 ± 0.03*	0.06 ± 0.006*
Percentage of synapses on spines that are inhibitory	4.4 ± 1.3	12.9 ± 4.5 **	12.6 ± 0.7**

Synaptic densities were calculated from the volumes of neuropil sampled from C2 barrels of the three groups of mice: control ($n = 3$), 24 hr after stimulation ($n = 3$), and 4 days after the stimulation had been stopped ($n = 3$). Values indicate the densities of all synapses (on dendritic shafts and spines together) as well as on spines alone. Also shown are the percentages of synapses on spines that are inhibitory. Levels of significance *$p < 0.05$, **$p < 0.005$.

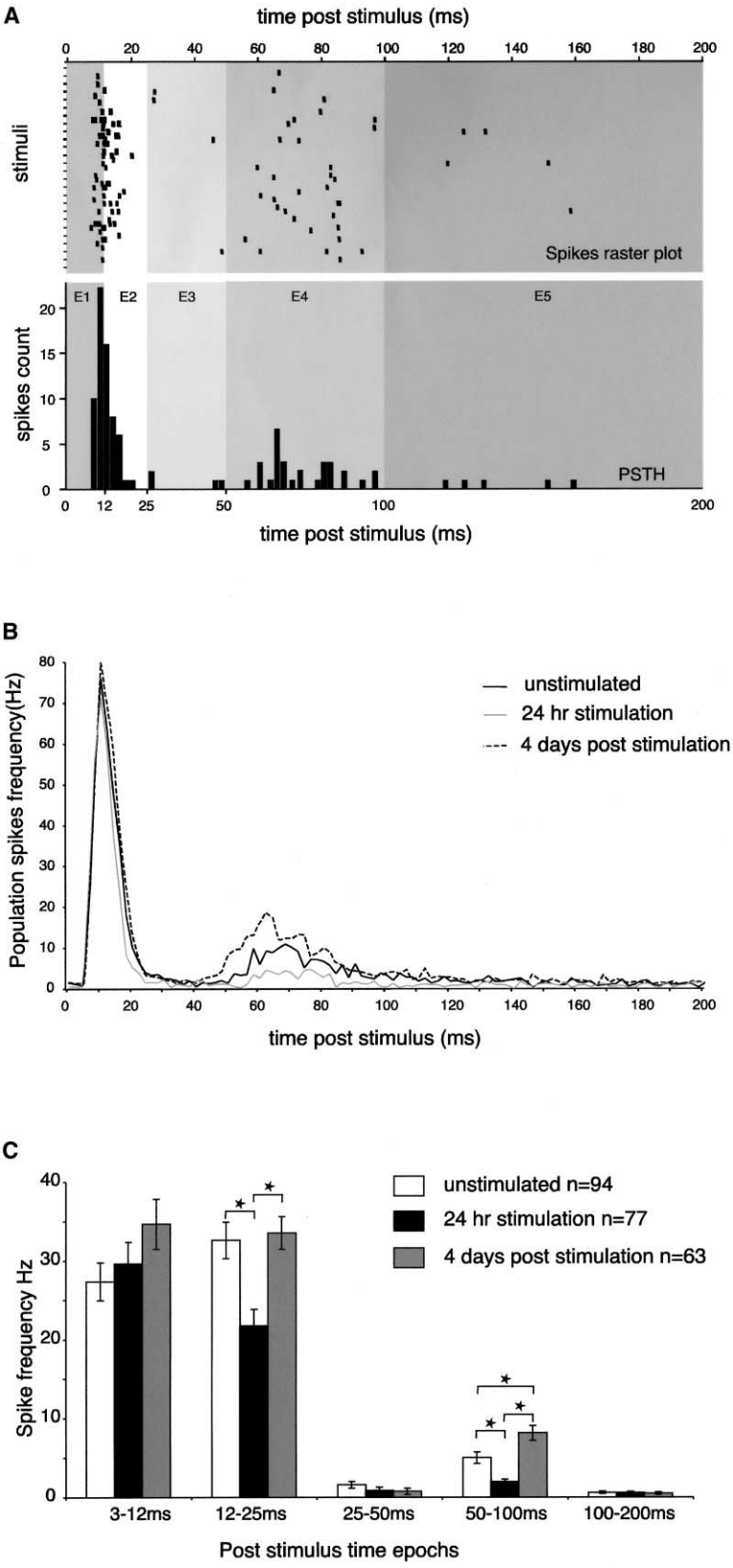

Figure 4. Whisker Stimulation Modifies Response Properties of Neurons in the Corresponding Barrel

(A) Typical raster plot and corresponding post-stimulus time histogram (PSTH) illustrating the response of a layer IV unit in barrel C2 upon deflection of the C2 whisker. In the raster display, action potentials (spikes) are represented by black dots as they were recorded during the 200 ms time period after whisker stimulation. A response was determined upon 50 stimuli and the PSTH was calculated based on 2 ms time-bins. For the epoch analysis, five post-stimulus time periods were defined, indicated as E1–E5. For each of these periods, response magnitude was calculated as the number of spikes elicited per whisker deflection minus spontaneous activity (see results displayed in [C]).

(B) Graphs showing the mean firing frequency of neurons in barrel C2 after deflection of the C2 whisker for unstimulated, stimulated, and mice which had been stimulated 4 days previously. The graph is based on the response of 94 neurons in control mice, 77 neurons in mice stimulated for 24 hr, and 63 neurons in mice stimulated 4 days previously.

(C) Altered response properties of cortical neurons induced by a period of prolonged whisker stimulation. Responses of layer IV neurons upon whisker stimulation were measured during five post-stimulus epochs (abscissa). The mean number of spikes (± SEM) were calculated for: unstimulated mice, mice analyzed immediately after the 24 hr stimulation period, and those that were recorded 4 days after the cessation of stimulation. Asterisks indicate the values that were significantly different from each other ($p < 0.001$ Kruskal-Wallis test after testing interindividual variability with multivariate anaylsis): a decreased response in the second and fourth epoch after the 24 hr of stimulation and an increased spike occurrence in the third epoch after a stimulus-free period of 4 days. Also note that the response during the earliest epoch is not significantly different between any of the groups.

tion of a single whisker for a 24 hr period at which time point we measured changes in the response properties of the corresponding cortical neurons. Structurally, we found significant changes in the synaptic densities, specifically at the level of the dendritic spine, with the insertion of GABAergic synapses. This particular synaptic

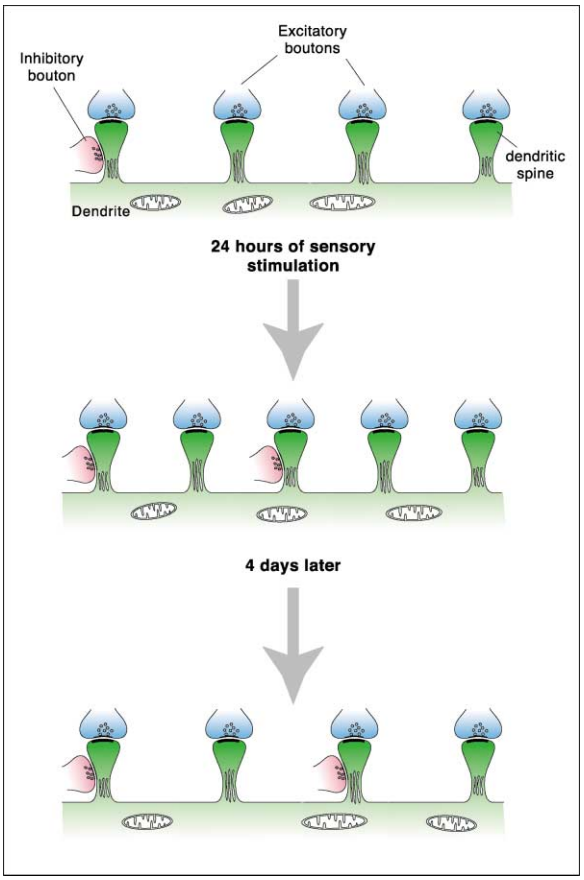

Figure 5. Summary of Structural Modifications in Cortical Circuitry after Whisker Stimulation

The density of the two types of synapses (excitatory, blue; inhibitory, red) on dendritic spines (green) approximately reflect the data presented in Table 1. Immediately after the 24 hr stimulation period, there is a 0.2-fold increase in the excitatory synapses on spines, and a 2.8-fold increase in the occurrence of inhibiotry synapses also on spines. Four days after the stimulation has been stopped, however, the total spine density has returned to prestimulation levels, but the increased density of GABAergic synapses on spines remains. The sequential progression of these morphological changes remains to be shown.

modification persisted as densities of these synapses were still raised four days after the stimulation had been stopped.

Immediately after stimulation, the volumes of neuropil sampled from stimulated barrels showed a greater density of synapses on dendritic spines as well as dendritic spines themselves. This result implicates the spine as an important component in mechanisms which alter cortical circuits as a consequence of changes in neuronal activity. It is of note that the density of spines returns to the control level once the stimulation has been stopped for a period of four days. In light of previous work showing neuronal activity increasing spine formation (Engert and Bonhoeffer, 1999), it is interesting to speculate whether the spine density increase in the stimulated barrel is a direct reflection of neuronal activity brought about by the whisker stimulation.

Whisker removal during development has been shown to alter significantly spine morphology, but not their overall density in the contralateral barrel of the adult rat (Micheva and Beaulieu, 1995; Vees et al., 1998). At around P12, however, the same deprivation technique has also been shown to significantly reduce the motility of layer II–III dendritic spine-like protrusions (Lendvai et al., 2000). Electrophysiological evidence (Stern et al., 2001) showed that receptive fields were also disrupted. The current data would appear to extend these observations and we suggest that spine dynamics are highly activity dependent, not only during development, but also during adulthood, determining the precision of cortical connectivity. Alterations in neuronal activity, therefore, could significantly increase spine production, increasing the connectivity, and changing the response properties of layer IV neurons.

The increased number of synapses in the C2 barrel is the first morphological evidence of synaptogenesis in the adult somatosensory cortex as a result of stimulation of a peripheral sensory receptor for a period of only 24 hr. LTP induces an increase in the proportion of axon terminals contacting two or more dendritic spines (Toni et al., 1999), but so far, no evidence was raised that an increased neuronal activity is altering the number of inhibitory synapses. Studies using laser optics with high temporal resolution have typically focused on postsynaptic structures, and, for the moment at least, not simultaneously localized the presynaptic elements (Engert and Bonhoeffer, 1999; Lendvai et al., 2000).

Spines receiving both an inhibitory and an excitatory input were first described by Jones and Powell (1969) in the cat somatosensory cortex, and have since been implicated in intracortical inhibitory mechanisms by which the inhibitory synapse can reduce the excitatory influence of the other synapse (Dehay et al., 1991). The density of these double innervated spines was reduced in layer IV of the barrel cortex after postnatal whisker removal (Micheva and Beaulieu, 1995). Since this deprivation paradigm is known to affect the neuronal response properties, it was suggested that the GABA axonal boutons targeting spines in layer IV represent a structural substrate of experience-dependent plasticity.

The modification of GABAergic innervation, along with the changes in response properties, points to an intracortical origin for the structural response, and leads to the question as to which cortical neurons could be involved. The arrangement of the stellate cells in layer IV barrel cortex is such that their dendritic arborizations are concentrated within the same barrel as their cell body (Woolsey et al., 1975). The apical dendrites of pyramidal cells, situated in layer V and VI, tend to ascend through layer IV in the barrel walls (White and Peters, 1993). The barrel hollow, therefore, is rich in stellate cell dendrites. As for the inhibitory component, Dehay et al. (1991) showed evidence in visual cortex indicating that inhibitory synapses on spines were more likely to veto the intracortical relay, rather than the thalamic input. These authors suggested the small basket cell as a likely candidate. This layer IV inhibitory cell (Dehay et al., 1991; Gupta et al., 2000; Kisvarday et al., 1986; Kisvarday et al., 1985; Staiger et al., 1996) shows dense local clusters of axon collaterals in the same layer (Gupta et al., 2000; Staiger et al., 1996) and synapses with dendritic spines, shafts, and somata (Somogyi and Soltesz, 1986) of spiny stellate cells (Dehay et al., 1991). However, recent com-

parisons, again in the visual cortex, of synaptic targets between different inhibitory interneurons have promoted a more likely candidate. The double bouquet cell preferentially targets dendritic spines, 70% on spines and the remainder on shafts and only a small percentage of spines being contacted by the basket cells (5%; Tamas et al., 1997). Notably, the characteristic "horsetail" axonal arbor of the double bouquet cell, which projects vertically through layers II/III and IV in close association with the apical dendrites, preferentially targets the shafts and spines of small dendrites in layer IV (Peters and Sethares, 1997). Yet this particular type of interneuron remains to be revealed in the barrel cortex.

How are the morphological modifications related to the altered response properties of neurons in layer IV? Immediately after stimulation, the decreased response toward peripheral stimulation in the second epoch (12–25 ms) of the post-stimulus time histogram correlates with the greater proportional increase in inhibitory synapses than excitatory. Four days after the stimulation was stopped, the response probability during the 12–25 ms epoch returned to the control level, coinciding with the re-established ratio between excitation and inhibition. At this time point, however, we detected a significant increase in the response of the neurons during the latest epoch (50–100 ms post-stimulus). This long latency spiking activity in layer IV neurons could represent a postinhibitory rebound, an intrinsic property of neurons in many parts of the brain, including neocortex. Hyperpolarization of neurons, by current injection or recruitment of local inhibition after afferent activation, is followed, at the offset of inhibition, by a rebound depolarization that can be enough to increase spiking probability (Grenier et al., 1998; Huguenard, 1996; Stafstrom et al., 1984). It was shown that the rebound amplitude is positively correlated to the degree of the preceding hyperpolarization (Dean et al., 1989; Fan et al., 2000; Stafstrom et al., 1984; Steriade and Timofeev, 1997). Although mechanisms underlying postinhibitory rebound in the cortex are only partially known, we hypothesize that the increase in rebound activity four days after cessation of the stimulation is related to the maintenance of GABAergic synapses onto spines that could be responsible for the increased hyperpolarization. Further work would need to establish for how long this alteration remains, and what role these changes play in the long-term functioning of layer IV neurons.

The form of activity-dependent plasticity in the adult cerebral cortex reported here can be considered an exaggeration of what may happen in a natural setting, where the amplitude of a neuronal response to a sensory stimulus may depend on previous experience. Layer IV would seem to be a strategically well-placed site in the sensory pathway to regulate the flow of information. The insertion of the GABA synapses onto spines in this layer possibly forms the cellular substrate of a gating mechanism by which neuronal activity in the cortex regulates its own level of excitation. We further discovered that the increase in synaptic density is a time limited response to the increased sensory experience. It is followed by a return to a level of synaptic density found in unstimulated mice. This implies removal of excitatory synapses in the adult cerebral cortex, while the GABA synapses on spines are selectively maintained for some time after the cessation of the sensory stimulation. Sensory experience in the adult mammal may therefore alter cortical circuitry leaving a lasting trace after an initial response period.

Experimental Procedures

Passive Whisker Stimulation Protocol

Twelve adult female mice (NOR, strain derived from ICR stock characterized by a standard pattern of mystacial vibrissae (Van der Loos et al., 1986)) were used in the ultrastructural analysis and six of these mice were passively stimulated for 24 hr by attaching, with cyanoacrylate glue, a small piece of ferrous metal (1.5 mm long and 0.2 mm diameter) to the C2 whisker and placing the animal in a cylindrical cage inside an electromagnetic coil which delivered a field intensity of approximately 7×10^3 A/m rms in bursts with a duration of 40 ms and interburst interval of 85 ms. This delivers a stimulation frequency of 8 Hz, and mimics the normal whisking frequency. The amplitude of the induced whisker movement varies with the position of the head; however, with the parameters used, the maximum movement of the stimulated whisker did not touch any of its surrounding neighbors. All procedures were reviewed and approved by the Office Vétérinaire Cantonal (Lausanne), in accordance with Swiss Federal Laws.

Fixation and Embedding Protocol

Immediately after removal from the stimulator, mice were anesthetized (sodium pentobarbitone, 6 mg/100 g body weight, intraperitoneal) and perfused with 20 ml of physiological saline followed by 300 ml of fixative (2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer, 0.1 M, pH 7.4, with 3 mM CaCl). Thirty minutes after perfusion, the brain was removed and left in the same fixative for 1 hr before 80 μm vibratome sections were cut (Leica VT100) tangentially from the barrel cortex. These sections were then washed in cacodylate buffer for 3 changes of 5 min each, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in alcohol, and embedded between silicon-coated glass slides in Durcapan ACM resin (Fluka). Once the resin had cured, the B2 and C2 barrels could be identified and a trapezoid prepared encompassing both the stimulated (C2) and unstimulated (B2) barrels (Figure 1). Series of between 30 and 60 silver/gray (60 nm thickness) thin sections were cut on formvar coated, single-slot, gold grids.

Postembedding Immunocytochemistry for GABA

Grids containing sections to be labeled for GABA were held vertically in slits cut into silicon plates. Sections were firstly pretreated with 1% periodic acid for 4 min followed by 5 min in freshly prepared 1% sodium metaperiodate. After washing in distilled water followed by 20 mM tris-buffered saline (TBS, pH 7.4) containing 0.5% bovine serum albumin (BSA) and 1% normal goat serum (NGS) in TBS (20mM, pH 7.4), they were then left overnight at 4°C in the same buffer containing anti-GABA antibody at a dilution of 1:1000 (Sigma, No. A2052). After this incubation, sections were washed again in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 0.2% BSA and 0.02% Tween 20 (Sigma) before being placed in goat anti-rabbit antibody coated in 15 nm gold (Aurion, Netherlands), at a dilution of 1:20 in the same buffer for 120 min. Sections were then washed in distilled water and immediately contrasted with uranyl acetate and alkaline lead citrate.

Series of sections were photographed at magnifications between 6000–8000 times on negative film using a Philips CM12 transmission electron microscope. Care was taken to make serial images from volumes of neuropil which did not contain any cell bodies, large dendrites (largest diameter > 1.5 μm), or large myelinated axons.

Analysis of Synapses and Dendritic Spines

Synapses were defined as a region of two apposed, thickened membranes, an identifiable cleft between, with a presynaptic element containing at least three identifiable vesicles. To calculate the synaptic density within a volume of neuropil, serial electron micrographs were first arranged in sequence and a sampling rectangle aligned and drawn on each photograph. Synapses were counted within this sampling rectangle whenever they could no longer be recognized

in subsequent images of the stack. Those that crossed one of two sides (right and lower sides) of this sampling rectangle were included in the count, but excluded if they crossed either of the opposite two sides (left and upper sides). To ensure that the counts were unbiased (blind), the observer did not know whether the serial images were taken from stimulated or unstimulated barrels.

All blocks were sectioned at the same section thickness (silver/gray), and this was calculated using the method previously described by Kirov et al. (1999). The diameters of at least 20 longitudinally sectioned mitochondria in each stack were measured and the number of sections that each occupied counted. Section thickness was, therefore, calculated as being an average of each mitochondrial diameter divided by the number of sections that it occupied.

Synapses were further classified according to whether they were located on a spine or dendrite and if either of the presynaptic boutons was GABA positive. The protocol used to reveal GABA (described above) relies on the sections being etched with periodic acid and then sodium metaperiodate to partially remove the osmium. These steps reduce the membrane contrast, so each part in the protocol was carefully monitored to maintain a precise balance between clearly defined membranes and good immunoreactivity. Boutons were identified as being GABA positive based on the analysis of all the serial sections in which the bouton was located. For boutons with a diameter smaller than 1 μm, ten gold particles in total were considered GABA positive; 30 particles for those with diameters of 1 μm or larger. The synapses associated with boutons classified using these criteria were always symmetric when the plane of section was optimal and allowed the symmetry of the synapses to be distinguished; however, this was not always the case and made the GABA staining essential.

Spines and dendrites were distinguished by their size, features of their profiles, and the presence of mitochondria and microtubules. Spines lacked mitochondria and microtubules and often contained a spine apparatus. Three-dimensional reconstructions of spines were carried out using aligned digital images of serial micrographs (Adobe Photoshope 5.5) at a resolution of 150 dpi and then transferred to a Silicon Graphics workstation (Octane 2) running the Imaris 2.7 (Bitplane, Zurich) software. Using the "DepthAnalyser" module, synapses and spine contours were traced as vectors on each image and the 3D shape visualized within this program.

The normal distribution of variables was tested using the Shapiro-Wilk test and hierarchical analysis of variance (ANOVA) used to test the effect of stimulation on the density of: all synapses, all spines, synapses on spines, and dendrites and GABAergic synapses on spines. The multivariate model was used when necessary and means were compared using the Tukey test.

A series of electron micrographs taken from within the C2 barrel hollow of a 24 hr stimulated mouse is provided on our website (http://www-ibcm.unil.ch/welker/) along with various examples of reconstructed spines and their synapses.

Single Unit Recording

Electrophysiological recordings were carried out under urethane anesthesia (10% solution in distilled water; 2 mg/g body weight, i.p.). After the urethane injection, animals received a subcutaneous dose of Lignocaine (0.1 ml; 1% w/v) above the parietal bone and were then placed in a headholder providing a continuous flow of oxygen in front of their nose. Body temperature was maintained at 37°C by a rectal thermistor-controlled heating pad. After a skin incision, a craniotomy of the right parietal bone, using a small electric drill, exposed the cortex around the posteromedial barrel subfield region. The dura mater was not removed and the exposed region was covered with 1% agar dissolved in 0.9% saline.

Using the CED-1401 interface with the Spike2 program (Cambridge Electronic design, Cambridge, UK) and carbon fiber electrodes, single unit activity was recorded from layer IV, in the C2 barrel hollow, in response to whisker deflection. Whisker deflection procedures were identical to those used in previous studies in our laboratory (Welker et al., 1993). The whisker, trimmed to approximately 1.5 cm, was inserted into a borosilicate tube attached to a piezoelectric slab. The stimulus consisted of an upward deflection of the stimulation probe of 1.2° and 3 ms duration, applied at a frequency of 0.5 Hz. During the stimulus, the probe did not touch adjacent whiskers or skin. Each tested whisker was deflected 50 times in one or two series. In the experimental (stimulated) group, 77 units were recorded from a total of 20 mice; in the control (unstimulated), 94 units were recorded from 20 mice; and 4 days after stimulation was halted, 63 were recorded from 10 mice. Response magnitude was determined to deflection of the C2 whisker and to confirm the position of the recording electrode, lesions were made at the end of each penetration using 1.7 mA negative current. The lesion site was visible in the Nissl-stained sections.

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