An anatomical substrate for experience-dependent plasticity of the rat barrel field cortex
(synapse/γ-aminobutyric acid/stereology)

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ABSTRACT The objective of this study was to examine the influence of sensory experience on the synaptic circuitry of the cortex. For this purpose, the quantitative distribution of the overall and of the γ-aminobutyric acid (GABA) population of synaptic contacts was investigated in each layer of the somatosensory barrel field cortex of rats which were sensory deprived from birth by continuously removing rows of whiskers. Whereas there were no statistically significant changes in the quantitative distribution of the overall synaptic population, the number and proportion of GABA-immunopositive synaptic contacts were profoundly altered in layer IV of the somatosensory cortex of sensory-deprived animals. These changes were attributable to a specific loss of as many as two-thirds of the GABA contacts targeting dendritic spines. Thus, synaptic contacts made by GABA terminals in cortical layer IV and, in particular, those targeting dendritic spines represent a structural substrate of experience-dependent plasticity. Furthermore, since in this model of cortical plasticity the neuronal receptive-field properties are known to be affected, we propose that the inhibitory control of dendritic spines is essential for the elaboration of these functional properties.

The mammalian neocortex is highly plastic during development. The lack of adequate sensory experience strongly interferes with the establishment and refinement of the neuronal circuits, especially in the sensory areas (1). Denervation by nerve sectioning or peripheral receptor lesioning causes profound changes in the cytoarchitectonics, somatotopy and function of the denervated cortical areas (reviewed in refs. 2 and 3), but the effect of the loss of afferent activity is difficult to estimate because of the intervening influences of nerve degeneration, cell death, and primary afferent regeneration. On the other hand, deprivation experiments in which the sensory pathways are left intact and only the level and pattern of their activation is altered can be successfully used as a model of experience-dependent cortical plasticity (see ref. 4).

The barrel subfield region of the rodent somatosensory cortex is particularly well suited for the study of cortical plasticity subsequent to peripheral sensory deprivation. It contains an arrangement of cellular aggregates, called barrels, representing, in a one-to-one fashion, the large whiskers on the contralateral mystacial vibrissa pad (5, 6). These barrels are easily visualized with various histochemical markers, such as cytochrome oxidase (7), and, thus, the effects produced by any change in the periphery can be directly assessed at the precise location of the corresponding cortical region.

Recent findings suggest that experience-dependent developmental plasticity in the neocortex could be related to modifications of the γ-aminobutyric acidergic inhibitory transmission. For example, sensory deprivation causes profound changes in the receptive-field properties of neocortical neurons (4, 8, 9), and it is known that γ-aminobutyric acid (GABA)-mediated inhibition is involved in shaping these properties (10, 11). The objective of the present study was to examine the influence of sensory experience during postnatal development on the organization of the GABA intracortical circuitry by quantitatively estimating the GABA synaptic population. Our working hypothesis was that the number of GABA synaptic contacts in the deprived cortex would be affected, particularly in layer IV, which is the main input layer of the thalamocortical projection. To address the issue of the possible functional significance of the expected changes, the distribution of GABA contacts on their postsynaptic targets was also analyzed, as GABA can have different effects on neuronal activity depending on the site of its action (see ref. 12).

MATERIALS AND METHODS

Ten male rats (Long-Evans; Charles River Breeding Laboratories) from different litters were used in the present study. Starting on the day after birth, in 5 of the 10 animals representing the sensory-deprived group, the vibrissae from the three middle rows on the right face whiskerpad were gently pulled out three times a week, as described by Fox (4). The vibrissa follicles were not damaged by this procedure. Five other animals, which had not undergone any specific manipulation, formed the control group.

Tissue Processing. At 2 months of age, all animals were deeply anesthetized with sodium pentobarbital (Somnotol; 65 mg/kg, i.p.) and then perfused through the ascending aorta with 50 ml of 0.1 M cacodylate buffer (pH 7.4; room temperature) followed by 600 ml of fixative containing 1% paraformaldehyde, 2.5% glutaraldehyde, and 3 mM calcium chloride in 0.1 M cacodylate buffer. Coronal vibratome sections (alternating thickness of 50 and 100 μm) were cut through the postero medial barrel subfield region (the cortical representation of the large mystacial vibrissae, referred to here as the barrel field cortex) of the two hemispheres. To precisely locate the barrels, which have an average diameter of about 400 μm (13), the 50-μm sections of the series were processed for cytochrome oxidase histochemistry (14). The adjacent 100-μm-thick sections were osmicated, dehydrated in an ascending series of ethanol, and flat-embedded on slides in Durcupan resin. Large pieces of sections corresponding to the three middle rows of vibrissae were cut out from the slides and reembedded in resin. Series of semithin sections (0.5–1.0 μm) were first obtained from each block, stained with azur-methylene blue, and used to determine the cortical layers. The blocks were then retrimmed and serial ultrathin sections of gray interference color were cut and placed on Pioloform-coated, single-slot nickel grids. The ultrathin sections extended from the pia to the white matter and were situated within the three middle barrel rows. They were then treated for the

Abbreviations: GABA, γ-aminobutyric acid; Nv, numerical density (number in 1 mm3 of tissue).
demonstration of GABA by using the postembedding immunogold technique as described (ref. 15; see also ref. 16). No specific labeling was observed when either the primary or the secondary antibodies were replaced with the same dilution of normal serum. Similar results were obtained when the grids were incubated in the GABA antiserum previously absorbed with $10^{-6}$ M GABA.

Quantitative Analysis of the Overall GABA and Non-GABA Synaptic Populations in Electron Microscopic Sections. The ultrathin sections were viewed under a Philips EM-300 and six photographs at a magnification of 7500× (final print of 22,000×) were taken in every cortical layer. Three blocks were analyzed in each animal, and, thus, a total of 108 photographs per animal were obtained. The numerical density of the GABA-immunopositive and GABA-immunonegative synaptic contacts (hereafter referred to as GABA and non-GABA synapses, respectively) was estimated in each layer by using the "unbiased" disector method (17), following a similar protocol as described (16). A synaptic contact was defined by the presence of two apposed thickened membranes of a presynaptic and postsynaptic profile, with the presynaptic profile containing at least three synaptic vesicles in close association with the differentiated membranes. An element was judged to be immunopositive for GABA when the density of gold particles in it was several times higher than the background density (see Fig. 1).

Statistical analysis was performed by using analysis of variance and the post hoc Scheffe test. As there were no significant differences in the measured parameters between the left and the right hemispheres of the control animals, these data were pooled.

RESULTS

The Overall Synaptic Population in the Rat Barrel Field Cortex Appears Unchanged After Neonatal Sensory Deprivation. The numerical density [number in 1 mm$^3$ of tissue (Nv)] of the overall synaptic population in each layer of the barrel field cortex contralateral to the deprivation was similar to that in the ipsilateral and in control cortices (see Table 1). Also, no significant difference was observed in the distribution of synapses on the various postsynaptic targets (dendritic spines, shafts, and neuronal somata). Thus, the general quantitative organization of the cortical neuronal circuits was not significantly affected by our experimental manipulation. If a change was nevertheless occurring, it was too subtle to be detected by the present quantitative methods.

The Numerical Density and Proportion of GABA Synapses Significantly Decrease in Layer IV of the Sensory-Deprived Cortex. The above results did not exclude the possibility that a particular small synaptic population might have been affected by deprivation, this being obscured by the remaining majority of synaptic contacts. Indeed, when we separately examined the GABA-immunopositive inhibitory synapses, we observed a profound decrease in their Nv (Fig. 2) and proportion (Table 2) in layer IV of the contralateral deprived barrel field cortex. Thus, the Nv of GABA synapses was only 50% of the value obtained in controls ($67 \times 10^6$ vs. $134 \times 10^6$)

<table>
<thead>
<tr>
<th>Layer(s)</th>
<th>Deprived</th>
<th>Control</th>
<th>Deprived</th>
<th>Control</th>
<th>Deprived</th>
<th>Control</th>
<th>Deprived</th>
<th>Control</th>
<th>Deprived</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>623 ± 69</td>
<td>710 ± 36</td>
<td>489 ± 53</td>
<td>587 ± 32</td>
<td>134 ± 17</td>
<td>123 ± 8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>II and III</td>
<td>581 ± 51</td>
<td>689 ± 36</td>
<td>455 ± 41</td>
<td>570 ± 33</td>
<td>117 ± 17</td>
<td>113 ± 8</td>
<td>17 ± 6</td>
<td>9 ± 2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV</td>
<td>526 ± 50</td>
<td>643 ± 34</td>
<td>407 ± 43</td>
<td>502 ± 31</td>
<td>112 ± 13</td>
<td>130 ± 12</td>
<td>10 ± 3</td>
<td>12 ± 2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>V</td>
<td>491 ± 91</td>
<td>511 ± 10</td>
<td>335 ± 59</td>
<td>355 ± 13</td>
<td>144 ± 26</td>
<td>149 ± 16</td>
<td>20 ± 8</td>
<td>12 ± 1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>VI</td>
<td>336 ± 52</td>
<td>435 ± 27</td>
<td>272 ± 40</td>
<td>339 ± 23</td>
<td>62 ± 16</td>
<td>91 ± 7</td>
<td>4 ± 0</td>
<td>6 ± 1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>482 ± 49</td>
<td>575 ± 22</td>
<td>369 ± 36</td>
<td>452 ± 17</td>
<td>107 ± 15</td>
<td>118 ± 6</td>
<td>6 ± 2</td>
<td>6 ± 1</td>
<td>—</td>
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Number of synapses in 1 mm$^3$ of tissue (mean ± SEM, $10^{-6}$) determined with the disector method. Statistical significance was calculated by using an analysis of variance and the Scheffe test. No significant difference was found for the above parameters between the deprived ($n = 5$) and control ($n = 5$) rats.
FIG. 2. Histograms of the $N_v$ of GABA and non-GABA synapses in the somatosensory cortex of deprived (contralateral hemisphere; $n = 5$; hatched bars) and control (both hemispheres, $n = 5$; solid bars) rats. Means and standard errors are given for each cortical layer. The only significant difference ($P < 0.01$, Scheffe test) was found in layer IV, where the numerical density of GABA synapses in the deprived cortex represented half of the control value. Note that in control animals there is a peak of the $N_v$ of GABA synapses in layer IV, as was reported for the rat visual cortex (16). After deprivation, this specific characteristic of layer IV is lost, and the interlaminar distribution of GABA synapses becomes uniform.

synapses per mm$^3$; $P < 0.01$). Accordingly, GABA synapses represented 12.6% ± 4.9% in the deprived compared with 21.1% ± 4.3% in the normal cortex ($P < 0.01$; Table 2). Meanwhile, no differences in these parameters were found in any other cortical layer of the contralateral hemisphere, nor in any layer, including layer IV, of the ipsilateral hemisphere.

GABA Synapses Targeting Various Postsynaptic Elements Are Differentially Affected by Sensory Deprivation. When the postsynaptic targets of inhibition were analyzed separately, it was found that the reduction of the population of GABA synapses was attributable to a specific loss of as many as two-thirds of the GABA synapses targeting dendritic spines in layer IV of the deprived animals ($19 \times 10^6$ vs. $55 \times 10^6$ GABA synapses per mm$^3$ in the deprived and control cortices, respectively; $P < 0.01$; Table 3).

Not Only the Density But the Total Number of GABA Synapses in Layer IV of the Barrel Field Cortex Changes After Sensory Deprivation. The drop in the density of GABA synapses in the deprived cortex could have been theoretically due to an increase in cortical volume without actual changes in the number of synapses. For this reason, we also calculated the total number of GABA synapses in layer IV of the barrel field cortex by multiplying its volume by the $N_v$ of GABA synapses. No difference in volume was found between the deprived and control cortex ($1.48$ mm$^3$ and $1.51$ mm$^3$, respectively, as previously determined by us in the same animals; ref. 19). Thus, there were a total of $99 \times 10^6$ GABA synapses in layer IV of the deprived rat barrel field cortex, of which $28 \times 10^6$ were on spines. These values were significantly less than those in control animals, where the numbers reached $203 \times 10^6$ and $83 \times 10^6$, respectively.

Is the Overall Number of Spines Affected by Deprivation? It is well known that the great majority if not all of the spines receive an asymmetrical synapse, while only a few also receive a symmetrical GABA synapse. Since >95% of the non-GABA synapses are glutamatergic—i.e., asymmetrical (see Discussion)—then the number of spines can be inferred from the number of non-GABA synapses on spines (Fig. 3). In layer IV contralateral to the deprivation, there is a 19% drop in the $N_v$

![Histograms of the postsynaptic targets of GABA and non-GABA synapses in the deprived (contralateral hemisphere; $n = 5$; hatched bars) and control (both hemispheres; $n = 5$; solid bars) somatosensory cortex. Means and standard errors are given for each cortical layer. Layer I is not represented in the histograms of synapses targeting somata, as their number was too small for a statistical analysis. Again, the only statistically significant difference ($P < 0.01$, Scheffe test) was found for the GABA synapses on spines in layer IV. The $N_v$ of GABA synapses on dendritic shafts also showed a tendency to decrease, but the values for the deprived and control animals were not significantly different ($P = 0.13$, Scheffe test). A decrease in the $N_v$ of GABA synapses on dendrites can indeed be expected to accompany the observed loss of GABA synapses on spines as GABA terminals synapsing on spine necks often form a second synapse directly on the parent dendrite. The decrease in the $N_v$ of non-GABA synapses on spines in layers I, II and III, and IV was not statistically significant ($P = 0.28, 0.27$, and 0.28, respectively, Scheffe test).]

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**Table 2. Percentage of GABA synapses in the deprived and control rat somatosensory cortex**

<table>
<thead>
<tr>
<th>Layer(s)</th>
<th>Deprived</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>10.9 ± 1.3</td>
<td>11.3 ± 1.5</td>
</tr>
<tr>
<td>II and III</td>
<td>12.4 ± 1.5</td>
<td>11.2 ± 1.4</td>
</tr>
<tr>
<td>IV</td>
<td>12.6 ± 2.2</td>
<td>21.1 ± 1.4*</td>
</tr>
<tr>
<td>V</td>
<td>14.4 ± 1.5</td>
<td>13.8 ± 1.9</td>
</tr>
<tr>
<td>VI</td>
<td>9.9 ± 1.9</td>
<td>12.8 ± 1.2</td>
</tr>
<tr>
<td>Total</td>
<td>11.9 ± 1.0</td>
<td>13.9 ± 0.8</td>
</tr>
</tbody>
</table>

Percentage of the GABA synapses among all synapses in the deprived ($n = 5$) and control ($n = 5$) rat somatosensory cortex (mean ± SEM). The only significant difference was found in layer IV. **$P = 0.006$, Scheffe test.**
of non-GABA synapses on spines, which, however, is not statistically significant (361 × 10^6 per mm^3 vs. 447 × 10^6 per mm^3 in the control layer IV; P = 0.28). The observed decrease in layers I and II and III is not statistically significant either (layer I, P = 0.28; layers II and III, P = 0.27). Thus, the present results cannot definitively confirm a loss of dendritic spines occurring after sensory deprivation.

**DISCUSSION**

**Structural Substrates of Experience-Dependent Plasticity.**

Our main finding is that the majority of GABA synapses normally contacting dendritic spines were lost specifically in layer IV of the barrel field cortex of rats who were chronically deprived from birth of an adequate somatosensory vibrisseae input. It should be noted that the lower Nv of GABA synapses cannot be attributed to a failure to detect decreased GABA levels in axon terminals. Indeed, we never encountered a dendritic spine receiving synaptic contacts from two GABA-immunonegative terminals in the deprived cortex, which would have been the case if the GABA terminals had been present but their neurotransmitter content had dropped below immunodetectable levels.

An important question arising from our results is whether sensory deprivation affects the number of dendritic spines since the reduction of GABA synapses in layer IV could simply be due to a loss of their postsynaptic elements. Although the present study does not provide a definitive answer to this question, it is clear that the decrease in the number of GABA synapses cannot be accounted for by a general loss of spines. In layer IV contralateral to the deprivation, the Nv of spines decreases only by 19% (not statistically significant), while the Nv of GABA synapses decreases by 66% (P < 0.01). These results indicate that there is either a substantial loss of GABA synapses on spines possibly accompanied by a slight general decrease in the total number of dendritic spines or a preferential loss of spines receiving a GABA and a non-GABA synapse. We favor the first explanation for the following reasons. In the same model of cortical plasticity, sensory deprivation leads to a substantial decrease in the number of GABA neurons (19) and GABA terminals (C.B. and C. Crevier, unpublished observations) in layer IV. A diminution of these GABA elements would undoubtedly bring a decrease in the number of synaptic contacts. Furthermore, the fact that the slight decrease in the overall number of spines in the supragranular layers of the contralateral barrel cortex is not paralleled with a significant loss of GABA synapses on spines strongly suggests that the decrease of GABA synapses in layer IV represents a specific change which is not consequent to a loss of dendritic spines. It should be emphasized, however, that whatever the mechanisms, the net result of sensory deprivation is a drop of the inhibition on dendritic spines (see below).

Previous studies have described the intracortical GABA system as being highly susceptible to a variety of manipulations of the afferent input. Sensory denervation, produced by peripheral receptor lesioning or nerve sectioning in either neonatal or adult animals, has been shown to reduce the immunoreactivity for GABA or its synthesizing enzyme GAD (20–22). Similarly, sensory deprivation, which only decreases the level and changes the pattern of afferent activity without disrupting the integrity of the pathway, results in a smaller number of GABA-immunoreactive elements in the affected cortex, and again this has been observed for both neonatal (refs. 19 and 23 and present results) and adult animals (24). Interestingly, the GABA synthesizing enzyme GAD seems to be affected by deprivation only in adult but not neonatal animals (25), which points to the dissociation between enzyme regulation and structural changes and suggests the existence of different mechanisms of experience-dependent plasticity depending on the age.

The GABA circuitry of the neocortex and particularly of layer IV, where the above effects have predominantly been reported, is ideally positioned to effectively mediate the changes occurring in the periphery and to adjust accordingly the cortical functioning. Layer IV is the main input layer for the thalamocortical axons and, therefore, the first one to be activated upon stimulation of the periphery, after which the information is relayed radially and vertically (12, 26). All GABA neurons in layer IV receive direct thalamocortical input and in turn project to other GABA and non-GABA neurons (27), and, thus, they can exert a strong influence on the barrel neuronal circuitry by means of both lateral and feedback inhibition. In fact, nearly all cortical neurons are sensitive to the strong inhibitory action of GABA (reviewed in ref. 28). GABA synapses are distributed on the somata, proximal dendrites, and axon initial segments of neurons where they can control their general responsiveness. On the other hand, GABA synapses are present on dendritic spines always together with an excitatory synapse, and, thus, they can precisely modify the gain of a particular cortical input (16, 27, 29). In this way, relatively small changes in the GABA circuitry can lead to profound and at the same time specific changes in the function of the entire cortex.

Our results cannot exclude the possibility of a modification occurring also in the excitatory connectivity of the deprived cortex, as implied by physiological studies in similar models (4, 30). The statistically nonsignificant decrease in the Nv of non-GABA synapses in layers I, II and III, and IV (see Fig. 2), might reflect a real trend, obscured by the large variability between the animals. Indeed, layers II and III have been shown to be even more plastic than layer IV in a model similar to ours (4, 30), and it has been hypothesized that a breakdown of the vertical transmission of information might be occurring. Such a hypothesis is consistent with the potential decrease in the number of non-GABA synapses in these layers, as suggested by
our results. It is important to note here that >99% of the non-GABA synapses are excitatory and glutamatergic since the synapses made by terminals containing dopamine, serotonin, noradrenaline, or acetylcholine represent <1% by the most conservative estimates (see refs. 31–34). Further anatomical studies are needed to verify the effects of sensory deprivation on the excitatory synaptic arrangement in the cortex.

**Functional Significance of the Intracortical GABA Circuitry.** The drastic loss of GABA synapses on dendritic spines in layer IV of the neonatally deprived cortex represents a highly localized change in the intracortical inhibitory circuitry. The functional significance of this anatomical rearrangement cannot be assessed directly at the level of individual dendritic spines, which, because of their small size, are not accessible for electrophysiological recordings. It is known, however, that neurons in the barrel cortex of neonatally sensory-deprived animals have altered receptive-field properties, such as increased spontaneous activity, reduced angular tuning, and enlarged receptive fields (4, 8). These plastic changes in the neuronal receptive fields could be consequent to the selective loss of GABA synapses on spines reported here, which might, for example, lead to a deficit in lateral inhibition and, thus, to a decreased vetoing of thalamocortical input.

This conclusion raises the important question of the involvement of the inhibitory circuitry in building the neuronal response selectivity. Extracellular application of GABA antagonists in the somatosensory and visual cortices profoundly affects the receptive-field properties of neurons (10, 11). In view of the possible mechanisms by which GABA can participate in building the neuronal response properties, GABA inhibition arriving on spines is in a particularly good position to selectively block the excitatory input to these spines (12, 29). However, only a small proportion of the spines in layer IV normally receive both an excitatory and an inhibitory synapse (ref. 35 and present study), and spines receiving thalamocortical synapses are not preferentially innervated by GABA contacts (18). It seems, therefore, improbable that the well-defined receptive-field properties of cortical neurons can be generated by the selective gating of such a small number of inputs. If we consider, however, a multisynaptic circuitry in which GABA inhibition successively sharpens the feedforward thalamocortical and the recurrent intracortical excitatory input of each neuron, then the additive effect of inhibition in this circuitry can indeed be a basis for the high selectivity of the neuronal responses. We, thus, propose that GABA inhibition arriving on the spines of neocortical neurons is a key component of the circuitry involved in the generation of the neuronal receptive-field properties.

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