Abstract—In this study we investigated the functional implications of GABA\textsubscript{A} receptor \(\alpha1\) subunit deletion on dendritic arborization and spine maturation in the visual cortex. This subunit is normally strongly upregulated during early postnatal development. Our main finding is that mice lacking the GABA\textsubscript{A} receptor \(\alpha1\) subunit displayed an increased density of dendritic filopodia during the second and third postnatal weeks. However, there was a concomitant decreased density of mature mushroom-shaped spines, which became more pronounced in adults. In contrast, dendritic arborization was not altered in these mice. We propose that an increased efficacy of the inhibitory synaptic transmission in the \(\alpha1\) knockout mice may lead to an enhancement of the outgrowth of filopodia around eye opening, but to a failure in spine maturation at later stages. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: visual cortex, development, synaptic transmission, morphology, critical period.
dark overnight at room temperature. The brains were briefly rinsed in distilled water and placed in 2% potassium dichromate–1% osmium tetroxide (4 parts to 1, respectively) for 3–6 days in the dark at room temperature. The pieces were washed with 0.75% silver nitrate until the red precipitate could no longer be seen in the wash and then transferred to fresh silver nitrate and kept for 3–6 days in the dark at room temperature. Subsequently, the brains were transferred to 70% ethanol and 160 μm sections were cut from the visual cortex on a vibratome. The sections were placed on subbed slides, dehydrated, cleared in Histoclear (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and mounted in Histomount (Sigma-Aldrich Chemie B.V.) and assigned codes that did not reveal group identity.

**Dendritic morphology and spine density quantification**

After Golgi staining (see Fig. 1A), pyramidal (Fig. 1B) and non-pyramidal cells (Fig. 1C) in layers II, III or IV of the visual cortex were analyzed. These superficial layers were chosen because incoming information from the lateral geniculate nucleus is processed here. Cells to be analyzed were chosen in the middle of the section to avoid the encountering of cut endings. Cells with damaged dendrites were not included in the measurements. Apical dendrites were defined as single, thick dendrites that extended from the dorsal aspect of the neuronal soma toward the pial surface; basal dendrites were defined as those extending radially from the soma (see Fig. 1B).

Golgi-stained neurons were morphologically reconstructed using a semi-automated dendrite measuring system (Overdijk et al., 1978; Uylings et al., 1986). We have quantitatively measured the following parameters: (i) total dendritic length per neuron; (ii) total apical dendritic length; (iii) total basal dendritic length. Total dendritic length measurements were further analyzed as follows: (a) path length (length of dendritic path from soma to a terminal tip); (b) intermediate segment length (segment lengths between two consecutive branch points); (c) terminal segment length (segment length between a final branch point and terminal tip); (d) number of dendritic segments per neuron; and (e) number of tips per dendrite. Each morphological parameter was statistically tested for possible differences between the α1 KO and the WT group using the non-parametric Mann-Whitney test.

In order to quantify spine density, the numbers of spines along a distance of 10 μm at the first intermediate segment length from the cell soma on both basal and apical dendrites were counted. A discrimination was made between filopodia-like protrusions and mushroom-shaped spines. Uniform thin protrusions were counted as filopodia, whereas thin-necked and thick-headed protrusions were counted as mushroom-shaped spines (Harris and Kater, 1994). In those cases where the total length of the spine could not be adequately seen, protrusions were excluded from analysis. Possible differences between the α1 KO and the WT group were statistically tested using the non-parametric Kruskal-Wallis test. All data collection and analysis was performed by an observer blinded to the genotype of the mice.

**Quantitative PCR**

Specific primer combinations (Eurogentec) were designed for each transcript. Amplified sequences were: brain-derived growth factor (BDNF; forward primer: GCTGGATGAGGACCAA; reverse primer: GAAGGT; reverse primer: GAGGCTCCAAAGGCA) (nucleotide [nt] 466–562); GABA receptor type A (GABA AR) (forward primer: GAAGCTGGAGCAGGAGTTCAC; reverse primer: GAAAGTGCCAAACAAGATGGG), postsynaptic density protein 95 (forward primer: GAAGCTGAGCAGGAGTTCAC; reverse primer: CCAGGGCAGGAATCGAGTCT), the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; gi 20984453 (nt 30–116)), which was used for normalization of the expression levels for the total RNA input. Amplicons were chosen within a range of 80–120 bp. Each primer-combination was tested on amplification efficiency and only those that had a value between 1.8 and 2 were accepted. Copy DNA (cDNA) quantification was performed on Perkin-Elmer ABI PRISM 7700 sequence de-
tection system (PE Applied) using 45 cycles (95 °C, 15 s; 59 °C, 1 min) on 0.3 μl cDNA per 20 μl reaction (SYBR green II mix; PE Applied). Regulation of transcription in the α1 KO mice compared with the WT mice was calculated by:

\[
\text{regulation} = \frac{E(\text{Ct geneX} \text{ WT} - \text{Ct GAPDH WT}) - E(\text{Ct geneX} \text{ α1KO} - \text{Ct GAPDH α1KO})}{\Delta Rn/\Delta Rn \text{ relative amount of fluorescent PCR product compared with internal reference dye}}
\]

where \(E\) is the amplification efficiency, which was considered 2 for all primers. Significant regulation between the α1 KO and the WT group was examined by using an unpaired t-test on the GAPDH subtracted data.

**RESULTS**

**Similar dendritic arborization in α1 KO and WT mice**

Analysis of the dendritic morphology revealed no significant differences between adult α1 KO and WT mice in total dendritic length of pyramidal or non-pyramidal cells (Fig. 2), nor in intermediate or terminal segment lengths (Table 1). This indicated that overall dendritic outgrowth was not changed. In addition, no differences were observed in the number of dendritic segments per neuron or the number of tips per dendrite (Table 1). Thus, the degree of branching of the dendrites was not affected either.

**Spine density in α1 KO mice compared with WT mice**

In order to investigate spine maturation, we quantified spine densities on the first intersegment length of pyramidal dendrites (Fig. 3A) in α1 KO and WT mice at different stages of postnatal visual cortex development. This was done at pn14 (when α1 upregulation has just started in WT mice), at pn21 (when α1 upregulation reaches a plateau level) and at the adult stage.

In the WT mice total spine density doubled during the third postnatal week and continued to increase thereafter (Fig. 3B). Total spine density was increased in the α1 KO mice at pn14 when compared with WT mice (Fig. 3B). However, it hardly increased thereafter. At pn21, total spine density was equal to that seen in the WT, whereas in adult mice it was significantly decreased.

Since basal and apical dendrites develop differently (Baker et al., 1997; McAllister et al., 1995) we also discriminated between spines that were located on basal dendrites and those on apical dendrites. This showed that the increased spine density in α1 KO mice at pn14 is mainly due to a change in basal dendritic spine number (Fig. 3B, right graph). In contrast, the decreased spine density in the adult mice was due to a decrease in apical dendritic spine number.

**Spine maturation in α1 KO mice compared with WT mice**

Spines appear in diverse morphologies, especially during development (Harris, 1999). We have, therefore, discriminated between filiform-shaped protrusions (filopodia) and mushroom-shaped spines (see Fig. 3A and Experimental Procedures section for selection criteria). At pn14 filopodia were present in WT mice, but they decreased in number during the following week to completely disappear at the adult age (Fig. 3C). In contrast, the filopodia density was relatively high in the α1 KO mice at pn14 compared with WT mice (3.2 per 10 μm (α1 KO) versus 1.4 per 10 μm (WT; \(P<0.05\)). This difference was almost exclusively due to an increase in filopodia density on basal dendrites (Fig. 3C, right graph). During the following week, filopodia density also decreased in the α1KO mice, but at a reduced
Table 1. Total, intermediate and terminal segment length, number of dendritic segments and number of tips per dendrite of basal and apical dendrites in pyramidal cells and in non-pyramidal cells are presented.

<table>
<thead>
<tr>
<th>Segment Type</th>
<th>WT</th>
<th>α1 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length (μm)</td>
<td>1278±122</td>
<td>1275±120</td>
</tr>
<tr>
<td>Pyramidal (basal)</td>
<td>n=17</td>
<td>n=15</td>
</tr>
<tr>
<td>Pyramidal (apical)</td>
<td>806±7.4</td>
<td>820±62</td>
</tr>
<tr>
<td>Non-pyramidal</td>
<td>1699±162</td>
<td>1850±135</td>
</tr>
<tr>
<td>Intermediate length (μm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyramidal (basal)</td>
<td>16.5±0.9</td>
<td>17.5±0.9</td>
</tr>
<tr>
<td>Pyramidal (apical)</td>
<td>25.6±1.8</td>
<td>28.9±3.0</td>
</tr>
<tr>
<td>Non-pyramidal</td>
<td>23.6±3.5</td>
<td>20.9±2.8</td>
</tr>
<tr>
<td>Terminal length (μm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyramidal (basal)</td>
<td>54.8±4.6</td>
<td>56.9±3.3</td>
</tr>
<tr>
<td>Pyramidal (apical)</td>
<td>54.7±2.7</td>
<td>53.9±3.2</td>
</tr>
<tr>
<td>Non-pyramidal</td>
<td>59.6±4.7</td>
<td>59.6±3.7</td>
</tr>
<tr>
<td># dendritic segments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyramidal (basal)</td>
<td>13.9±1.3</td>
<td>13.6±1.8</td>
</tr>
<tr>
<td>Pyramidal (apical)</td>
<td>9.5±1.0</td>
<td>9.5±0.8</td>
</tr>
<tr>
<td>Non-pyramidal</td>
<td>16.9±2.1</td>
<td>19.4±2.2</td>
</tr>
<tr>
<td># tips per dendrite</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyramidal (basal)</td>
<td>19.5±1.5</td>
<td>18.9±1.8</td>
</tr>
<tr>
<td>Pyramidal (apical)</td>
<td>10.5±1.0</td>
<td>10.5±0.8</td>
</tr>
<tr>
<td>Non-pyramidal</td>
<td>23.3±2.5</td>
<td>25.1±2.3</td>
</tr>
</tbody>
</table>

Data represent means±SEM (n=number).

rate, compared with the WT mice (2 per 10 μm [α1KO] versus 0.8 per 10 μm [WT]; P<0.05). The increased filopodia density at that age compared with the WT mice was visible both at basal and apical dendrites (Fig. 3C, right graph). Even in the adult α1KO mice, filopodia were still present both on apical and on basal dendrites, whereas these had virtually disappeared in the WT mice (0.8 per 10 μm [α1KO] versus 0.1 per 10 μm [WT]; P<0.05).

We observed a steady increase in mushroom-shaped spines in the WT mice (Fig. 3D; P<0.05): a doubling in mushroom spine density was seen between pn14 and pn21 (2.5 per 10 μm [pn14] versus 6.7 per 10 μm [pn21]; P<0.05). After pn21 mushroom spine density still increased to a density of 9.1 per 10 μm in the adult mice. Although the α1KO mice display an equal density of mushroom spines at pn14, compared with the WT mice, their density is significantly lower at later ages, both at pn21 (5.1 per 10 μm [α1KO] versus 6.7 per 10 μm [WT]; P<0.05) and in the adults (6.2 per 10 μm [α1KO] versus 9.1 per 10 μm [WT]; P<0.05). This difference was ob-

Fig. 3. Developmental changes in spine densities. (A) Dendritic spines were counted along 10 μm on the first intersegment length from the cell soma (left, bar=2 μm) and were subdivided in filopodia (middle, bar=1 μm) and mushroom-shaped spines (right, bar=1 μm). (B left graph) Number of spines per 10 μm in α1 KO mice are increased at pn14 (P<0.01; n=43, WT, four mice; n=52, α1 KO, four mice), equal at pn21 (n=58, WT, four mice; n=31, α1 KO, four mice) and decreased in the adult compared with WT mice (P<0.01; n=42, WT, three mice; n=50, α1 KO, three mice). (B right graphs) At pn14 the increased spine density is due to an increase in basal dendrites (P<0.05). In the adult the decreased spine density is noticeably observed on the apical dendrites (ns.; basal dendrites pn14: n=33, WT, four mice; n=39, α1 KO, four mice; pn21: n=46, WT, four mice; n=31, α1 KO, four mice; adult: n=27, WT, three mice; n=36, α1 KO, three mice; apical dendrites pn14: n=10, WT, four mice; n=13, α1 KO, four mice; pn21: n=12, WT, four mice; n=17, α1 KO, four mice; adult: n=15, WT, three mice; n=14, α1 KO, three mice). (C left graph) Number of filiform-shaped spines in the α1 KO mice were significantly increased both at pn14 (P<0.05) at pn21 (P<0.05) and in the adult mice (P<0.05; C right graphs). Filopodia density on basal dendrites is increased at pn14 and pn21 (P<0.05). On apical dendrites filopodia density is increased at pn21 (P<0.05). (D left graph) Number of mushroom-shaped spines in the α1 KO mice is equal at pn14 and significantly decreased both at pn21 (P<0.05) and in the adult animals (P<0.05). (D right graphs) Basal and apical dendritic mushroom spine density is decreased at pn21 and in the adult mice, however not significantly.
served both on basal and apical dendrites, though more profound on the apical dendrites (Fig. 3D, right panel).

Thus, initially at pn14, we observe an increased outgrowth of juvenile filopodia in α1 KO mice, mainly on basal dendrites. At later stages however, the spine maturation at basal but in particular at apical dendritic spines is disturbed. Whether the initial difference in the rate of development of spine density in basal versus apical dendrite is of physiological significance is currently unclear.

GABA<sub>α</sub>-subunits and BDNF expression in α1 KO mice

Altered spine maturation can be a direct or indirect consequence of the deletion of the α1 subunit in the α1 KO mice or, alternatively, be caused by compensatory regulation of other α subunits. We measured mRNA levels of the other α subunits in the α1 KO mice and found that they were not significantly changed compared with the levels in the WT mice (Table 2). Spine maturation can be directly influenced by the growth factor BDNF (Horch et al., 1999). To test whether α1 deletion had indirectly affected BDNF expression, BDNF levels were investigated. No significant changes were observed (Table 2). In line with the observed decrease in spine density however, we measured a small but significant downregulation in the postsynaptic density protein, associated with excitatory synapses, PSD-95 (Table 2; Marrs et al., 2001).

DISCUSSION

Many of the processes that are involved in the construction of the cortical network during a critical period in development (Katz and Shatz, 1996) are sculpted by neuronal activity. For instance, visual deprivation studies have shown that axonal growth and dendritic arborization are decreased in the absence of normal sensory input (Coleman and Riesen, 1968; Friedlander et al., 1982; Wiesel and Hubel, 1965). Moreover, in vitro, N-methyl-D-aspartate (NMDA) receptor blockade decreases dendritic growth and branching (Vogel and Prittie, 1995). In addition, the formation and maturation of dendritic spines (Harris et al., 1995) appears also to be modulated by activation of α-ami-

Table 2. Quantitative analysis of the different GABA<sub>α</sub> α subunits, BDNF and PSD-95 mRNA levels in L.I-IV visual cortex by means of quantitative PCR in WT and α1 KO mice (n=4, in duplo)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>α1 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>0.133±0.008</td>
<td>0.0006±0.0002**</td>
</tr>
<tr>
<td>α2</td>
<td>0.033±0.003</td>
<td>0.032±0.002</td>
</tr>
<tr>
<td>α3</td>
<td>0.033±0.002</td>
<td>0.032±0.002</td>
</tr>
<tr>
<td>α4</td>
<td>0.017±0.001</td>
<td>0.014±0.001</td>
</tr>
<tr>
<td>α5</td>
<td>0.016±0.002</td>
<td>0.029±0.005</td>
</tr>
<tr>
<td>BDNF</td>
<td>0.009±0.001</td>
<td>0.013±0.002</td>
</tr>
<tr>
<td>PSD-95</td>
<td>0.456±0.010</td>
<td>0.392±0.015*</td>
</tr>
</tbody>
</table>

Values are corrected for GAPDH expression and calculated as described in Experimental Procedures. Asterisks indicate significant difference between WT and α1 KO expression (Student’s t-test, * P<0.05; ** P<0.01).

Most studies so far have focused on the role of glutamatergic receptors as the site of modulation of dendritic outgrowth and spine formation by neuronal activity. However, GABAergic inhibition profoundly modulates glutamatergic activity by exerting control over neuronal firing and synchronization (Krnjevic, 1997; Tamas et al., 2000). Recent studies indicate that the GABAergic system may play a prominent role in regulating the onset of the critical period, during which the activity dependent outgrowth processes take place (Fa-giolini and Hensch, 2000; Hensch et al., 1998).

In this study, we observed a steady increase in total spine density in the visual cortex of WT mice during early postnatal development. This profound increase between pn14 and pn21 is likely to be induced by increased levels of activity following the moment of eye opening around pn13 (Shatz, 1996). At pn14 a significant proportion of the spines (ca. 25%) had a filopodia-like shape. Imaging studies have suggested that filopodia may act as spine precursors (Harris et al., 1995) that first protrude and then retract, after which mature mushroom-shaped spines evolve (Dailey and Smith, 1996; Fiala et al., 1998; Jontes and Smith, 2000; Ziv and Smith, 1996). Although this question has not been resolved yet (Bonhoeffer and Yuste, 2002), it is noteworthy that in the WT mice, the density of filopodia indeed gradually disappeared, while the density of mushroom-shaped spines significantly increased during the same period of development.

In juvenile α1 KO mice (pn14) the overall density of spines appeared to be increased. However, this was mainly due to an increased proportion of filopodia. At pn21 the ratio of filiform- versus mushroom-shaped spines was still increased in α1 KO, compared with WT. It is unclear at this point how this may come about. Analysis of miniature inhibitory postsynaptic currents (mIPSCs) in the α1 KO mice revealed an increased efficacy of the synaptic GABA<sub>A</sub> receptor (Bosman et al., 2002) due to the increased synaptic current decay time and an additional increase in the amplitude monoquantal synaptic currents. Moreover the frequency of mIPSCs was not affected, which implies that there was no reduction in number of synapses impinging per cell. Thus during neonatal development an enhanced inhibitory tone may have occurred within the cortical network in α1 KO mice which may suppress overall neuronal activity. This is confirmed by the observation that 40 Hz γ-band frequency oscillations are reduced in visual cortex slices of α1 KO mice (L. Bosman and H. Lodder, personal communication; unpublished data). It would be premature to conclude that there exists a causal relation between an increased inhibitory tone and an increased density of filopodia. However, in line with this hypothesis, several studies have shown that a decrease in neuronal network activity, for instance due to application of tetrodotoxin or 2-amino-5-phosphonovalerate, may lead to an increase in spine formation on immature dendrites (Dalva et al., 1994; Rocha and Sur, 1995).

At pn21 and in adult, we observed a decrease in mature mushroom spine density in α1 KO mice compared...
with WT mice. In line with this observation, we report a slight downregulation of PSD-95, a postsynaptic density protein that is associated with mature glutamatergic synapses (Marrs et al., 2001). If filopodia are the precursor stage of mushroom-shaped spines (but Bonhoeffer and Yuste, 2002 for review), it is possible that in α1 KO this transition process was impaired. In addition to a failure in filopodia-to-mushroom-spine transition, our observation could also be explained by impaired consolidation of mushroom-shaped spines, which would subsequently reverse to the filiform state. Hence ‘online’ functional imaging experiments (Yuste and Bonhoeffer, 2001) should be performed to test which stage of spine maturation is exactly affected in α1 KO mice.

Long-term potentiation (LTP) is thought to play a key role in synapse formation and consolidation and possibly also in spine maturation (Isaac et al., 1997; Kirkwood and Bear, 1994; Yuste and Bonhoeffer, 2001). GABAergic transmission can interfere with the induction of LTP (Higashima et al., 1998; Tomasulo et al., 1993), by shunting AMPA receptor-mediated synaptic currents and thereby reducing the extent of voltage dependent NMDA receptor activation. Possibly via this mechanism, the change in GABAergic synaptic efficacy may have led to the impairment in spine maturation in the α1 KO mice.

However, also other explanations should be considered. For instance steroid levels are known to affect spine maturation and density (McEwen et al., 2001). Therefore, overall changes in hormonal regulation due to altered GABAergic transmission in other brain areas might also play a role.

The implications of the differences we observed in spine densities between apical and basal dendrites remain unclear. Two-photon microscopy may elucidate whether differential subcellular expression of different types of GABA_α receptors occurs. Furthermore, the fact that no alterations were observed in dendritic arborization suggests that the process of dendritic outgrowth is less sensitive to modulation of neuronal activity, or occurs at an earlier developmental stage.

We conclude that while the density of filopodia was enhanced at an early age in α1 KO mice, formation of mature mushroom-shaped spines was impaired, possibly due to the fact that synaptic contacts are not properly established and/or consolidated. As a result, the overall spine density is decreased in the adult α1 KO mice. The data presented here indicate that, in addition to other factors, proper maturation of the GABA_α receptor (Bosman et al., 2002) at the level of individual synapses, may affect the process of spine formation. In addition to our findings, it is noteworthy that not only an enhanced GABAergic inhibition (this study) but also a decreased impact of neocortical GABA input, as is observed in glutamic acid decarboxylase 65 (GAD65) KO mice, interferes with neocortical development. These GAD65 KO mice lack a proper onset of the critical period in visual cortex in vivo (Fagiolini and Hensch, 2000; Hensch et al., 1998). Thus, maturation of the GABAergic system, both in terms of number of synapses and in terms of receptor functioning, appears to be crucial for experience-dependent fine tuning of cortical connections during the critical period.

REFERENCES


Harris BT, Costa E, Grayson DR (1995) Exposure of neuronal cultures to 5 μM L-glutamate or to N-methyl-D-aspartate increases the transcription of genes encoding the α1 and α5 GABA_α receptor subunits. Brain Res Mol Brain Res 28:338–342.


(Accepted 11 June 2003)