GABA$_A$ RECEPTOR MATURATION IN RELATION TO EYE OPENING IN THE RAT VISUAL CORTEX

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Abstract—Changes in subunit composition of *N*-methyl-D-aspartate (NMDA) receptors have been reported to be affected by visual experience and may therefore form a major aspect of neuronal plasticity in the CNS during development. In contrast, putative alterations in the expression and functioning of the inhibitory GABA$_A$ receptor around eye opening have not been well defined yet. Here we describe the timing of changes in GABA$_A$ receptor subunit expression and the related synaptic functioning in the neonatal rat visual cortex and the influence of visual experience on this process.

Quantitative analysis of all GABA$_A$ receptor subunit transcripts revealed a marked $\alpha 3$ to $\alpha 1$ subunit switch, in addition to a change in $\alpha 4$ and $\alpha 5$ expression. The changes were correlated with an acceleration of the decay of spontaneous inhibitory postsynaptic currents (sIPSCs). Both changes in receptor expression and synaptic functioning were initiated well before eye opening. Moreover, dark rearing could not prevent the robust upregulation of $\alpha 1$ or the change in sIPSC kinetics, indicating that this is not dependent of sensory (visual) input.

Upon eye opening a positive correlation was observed between a faster decay of the sIPSCs and an increase in sIPSC frequency, which was absent in dark-reared animals. Thus, lack of extrinsic input to the cortex does not affect overall developmental regulation of synaptic functioning of GABA$_A$ receptors. However, we cannot exclude the possibility that visual experience is involved in proper shaping of the inhibitory network of the primary visual cortex. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: ACSF, artificial cerebrospinal fluid; BDNF, brain-derived neurotrophic factor; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IPSC, inhibitory postsynaptic current; IQR, interquartile range; K-S, Kolmogorov-Smirnov; LGN, lateral geniculate nucleus; NMDA, *N*-methyl-D-aspartate; pn, postnatal day; τ_{decay}, decay time constant.

During development of the visual cortex, a combination of spontaneously generated and experience-dependent neuronal activity critically influences the cellular connectivity of this brain area (Katz and Shatz, 1996). For instance, ocular dominance columns initially come about in an endogenous, activity-independent manner (Crowley and Katz, 1999; Crowley and Katz, 2000), but are subsequently strongly modulated by sensory input during the so-called critical period, starting 1 week after eye opening. During this period, interference with visual experience by means of monocular deprivation will shift the neuronal response toward the undeprived eye (Berardi and Maffei, 1999; Crair et al., 1998; Gordon and Stryker, 1996; Katz and Shatz, 1996). Correlating in time, glutamatergic synapses display enhanced plasticity (Kirkwood et al., 1995) and experience-dependent changes in *N*-methyl-D-aspartate (NMDA) receptor subunit expression have been suggested to be a possible mechanism underlying the developmental decline of this plasticity (Monyer et al., 1994). In addition, GABAergic inhibition has been implicated both in the onset and the decline of the critical period (Fagiolini and Hensch, 2000; Hensch et al., 1998a).

Like the NMDA receptor, the GABA$_A$ receptor displays changes in expression during neonatal development (Araki et al., 1992; Fritschy et al., 1994; Laurie et al., 1992). Changes in subunit composition of the GABA$_A$ receptor may affect receptor properties such as synaptic current decay (Brussaard, 1997; Okada et al., 2000), affinity for GABA or allosteric modulating agents (Ebert et al., 1994, 1997) and receptor targeting (Gao et al., 1993; Nusser et al., 1996, 1998).

It is reasonable to assume that alterations in the postsynaptic receptor properties of fast excitatory and inhibitory synaptic transmission affect the balance between these systems and therefore also the pattern of overall firing activity in the visual cortex. This, in turn, may affect activity-dependent plasticity processes that take place in particular after eye opening. However, the precise timing of alterations in GABA$_A$ receptor subunit expression and their functional consequences for synaptic receptor functioning in relation to the moment of eye opening and the course of the critical period have not been well defined.

Therefore, we studied the developmental mRNA expression profile of GABA$_A$ receptor subunits in the rat visual cortex and the corresponding postsynaptic receptor current kinetics. Our aim was to quantify the temporal changes in the superficial layers (L.I–IV) that process in-

coming information from among others the lateral genicu-late nucleus (LGN). The measurements were performed at all major stages of neuronal development, starting at post-natal day 6 (pn6), when cortical layer formation has been completed, and ending at pn35, at the closure of the critical period. In addition, we investigated whether these changes were dependent of visual experience in dark-reared animals.

EXPERIMENTAL PROCEDURES

Animals

Young Wistar rats (6–35 days old; Harlan, Horst, Netherlands) were used for all experiments. They were either housed under normal light-dark conditions (12-h light/dark cycle) or in total dark-ness for the dark rearing experiments. In the latter case rats were born in darkened boxes and kept in the dark until they were either killed or transferred to a normal environment. During dark rearing, the animals were only once a week shortly exposed to red light, when they were transferred to a clean box. For electrophysiolog-ical recordings, each experimental group consisted of at least six cells coming from at least three different preparations. The follow-ing age groups were investigated when using acute slices for biochemical or electrophysiological analysis: pn6–7, pn11, pn14, pn20–24 and pn34–35. All experimental methods were approved by the Animal Welfare Committee of our institute which judged our experiments conform with European standards. All efforts were made to minimize the number of animals used and their suffering.

Preparation of slices

Non-anesthetized rats were decapitated, the brains were quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF; in mM: 125 NaCl, 25 NaHCO$_3$, 3 KCl, 1.2 NaH$_2$PO$_4$, 2.4 CaCl$_2$, 1.3 MgSO$_4$, 10 D-(+)-glucose carboxygenated with 5% CO$_2$/95% O$_2$; 304 mosml; pH 7.4). Coronal sections (400 μm thick) of the visual cortex were cut using a Leica VT1000S vi-bratome. Tissue was either immediately stored at -80 °C for subsequent RNA isolation or kept up to 8 h in continuously car-boxygenated ACSF at room temperature before mounting the slices in the recording chamber for electrophysiological record-ings. Organotypical visual cortex slice cultures were obtained by culturing cortical explants for a period of maximal 28 DIV as described previously (Romijn et al., 1984). The explants were incubated pair-wise, with their ventral sides touching each other, allowing the formation of functional connections (Baker and Van Pelt, 1997).

Total RNA isolation and cDNA preparation

Total RNA was isolated from a 1 mm wide, 400 μm thick acutely dissected L.I–IV visual cortex slice according to the procedure described by (Chomczynski and Sacchi, 1987), dissolved in 25 μl RNAse-free H$_2$O and subsequently treated with 10 U DNAse-I (Roche) to remove residual genomic DNA. Hexanucleotide (125 pmol) primed cDNA was generated on approximately 1 μg RNA using 250 U Superscript (Gibco BRL) in a total volume of 50 μl using the manufacturers protocol. Samples were aliquotted and stored at -20 °C.

Quantitative PCR

Specific primer combinations (Eurogentec) were designed for each GABA$_A$ receptor subunit sequence. Amplified sequences were: GABA$_A$ receptor (GABA$_A$R) α1 subunit (L08490.1; nt 1258–1338); GABA$_A$R α2 (L08491.1; nt 1461–1541); GABA$_A$R α3 (L08492.1; nt 1571–1651); GABA$_A$R α4 (L08493.1; nt

1362–1442); GABA$_A$R α5 (L08494.1; nt 1633–1757; GABA$_A$R β1 (X15466.1; nt 1706–1795); GABA$_A$R β2 (NM_012957.1; nt 1820–1900); GABA$_A$R β3 (NM_017065.1; nt 1085–1155); GABA$_A$R γ1 (X57514.1; nt 1312–1392); GABA$_A$R γ2 (L08497.1; nt 1265–1345); GABA$_A$R γ3 (M81142.1; nt 1389–1469); GABA$_A$R δ (L08496.1; nt 1333–1413); GABA$_A$R ϵ (U92284.1; nt 6826–6951); brain-derived neurotrophic factor (BDNF; AY011462.1; nt 183–286) and the housekeeping gene hypoxanthine-guanine phospho-ribosyltransferase (HPRT; M63983.1; nt 268–343). HPRT was used to normalize the expression levels from the different devel-opmental stages. 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 for the experiments. cDNA quantification was performed on Perkin-Elmer ABI PRISM 7700 sequence detection system (PE Applied) using 45 cycles (95 °C, 15 s; 59 °C, 1 min) on 0.7 μl cDNA per reaction (in 20 μl; SYBR Green Core reagent kit; PE Applied). HPRT-normalized expression (ΔX) for a given gene X could be calculated by ΔX=1/E(Ct$^{(geneX)}$$^-Ct^{(HPRT)}$) where Ct (geneX, HPRT)=number of cycles at which the PCR product reaches the set threshold value (0.3$\times\Delta Rn$; ΔRn=relative amount of fluorescent PCR product compared with internal reference dye) and E=the amplification efficiency, which was considered 2 for all primers.

Significant changes in the normalized GABA$_A$ receptor sub-unit expression at different time points during development com-pared with pn6 were examined using a one-way ANOVA.

In situ hybridization

Cryostat sections (14 μm) from 1 mm wide acutely dissected slices were thaw-mounted on gelatin-coated slides and treated for hybridization as described by (Young et al., 1990). The cDNA for the total coding sequences of the α1 and α2 GABA$_A$ receptor subunits, subcloned into pBluescript SK($-$), were kindly provided by Dr. H. Lüddens (Department of Psychiatry, University of Mainz, Germany). PCR fragments of these cDNA clones were generated using primers for the RNA polymerase promoter sites (T7 and SP6) flanking the cDNA sequences. The PCR fragments were subsequently used as templates for *in vitro* transcription to syn-thesize [^{35}S]-UTP-labeled cRNA probes, according to the protocol provided by Promega (Madison, WI, USA). After hybridization, sections were air-dried, dipped in Ilford K.5D photographic emul-sion (Polysciences, Niles, IL, USA) and exposed for 1 week. Finally, the slides were developed in Kodak D-19 and fixed in Kodak Rapidfix (Eastman-Kodak, Rochester, NY, USA). Grain-density was qualitatively analyzed by visual inspection.

Electrophysiology

In situ whole-cell voltage-clamp recordings were made from ran-domly selected neurons in layers II–III of the visual cortex using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA) and borosilicate glass (Harvard Apparatus Ltd, UK) elec-trodes with tip resistances of 2–5 MΩ. All experiments were per-formed at 33 °C using ACSF with 20 μM DNQX and 20 μM APV. The pipettes were filled with, in mM, 135 CsCl, 1 CaCl$_2$, 10 EGTA, 10 HEPES, 2 MgATP, 296 mosml, pH 7.2 (with CsOH). All spontaneous inhibitory postsynaptic currents (sIPSCs) could be blocked by the specific GABA$_A$ receptor antagonist bicuculline methiodide (10 μM; Sigma).

Experimental data were stored on digital tapes and analyzed using Computer Disk Recorder v1.3 and Whole Cell Program v2.3 (both by J. Dempster, Strathclyde University, UK). Only single events with sharp single rising phases starting from a stable base line were accepted. In this study, we detected sIPSCs instead of mIPSCs, implying that presynaptic action potential activity may have influenced the frequency of the occurrence of sIPSCs. In line with this, application of TTX did affect the frequency of the occur-

rence of sIPSCs but not the amplitude of inhibitory postsynaptic currents (IPSCs) (data not shown), which shows that the extent of multiquantal events was very limited. Dendritically filtered sIPSCs were excluded from analysis as much as possible, according to the analysis criteria described in Bosman et al. (2002). Moreover RC time constants of individual recordings were not significantly different between the distinct developmental stages ($P>0.3$; one-way ANOVA; data not shown), indicating that changes in cable attenuation may have had only minor effects on the developmental changes in $GABA_A$ receptor kinetics as discussed in this article (see also Bosman et al., 2002).

The decay time constant (τ_{decay}) of each sIPSC was calculated from a mono-exponential fit. Subsequently for each neuron, histograms were made of the peak currents, τ_{decay}s and interval times of all sIPSCs per recording, i.e. neurons with less than 50 accepted sIPSCs were rejected from further analysis. Typically, we analyzed approximately 400 sIPSCs per neuron. Peak current and τ_{decay} histograms were skewed and best fitted with lognormal curves, interval time histograms were best fitted with mono-exponential functions, as described previously (see Brussaard et al., 1996). Overall group-averages of these synaptic current parameters were not normally distributed (Kolmogorov-Smirnov [K-S] goodness of fit test) and for comparisons illustrated by plotting the median$\pm$ interquartile range (IQR) of the developmental stages we used. The level of statistical significance used was $P<0.05$.

For the correlation analysis in Fig. 6, the contribution of data obtained in individual recordings was assessed, instead of the overall group medians. To this end, for each developmental stage, the average τ_{decay} of each individual neuron was plotted versus the average interval time of that experiment. The scatter plots thus obtained were subsequently tested for linear regression lines using SigmaPlot (SPSS Inc.). In addition, significance of correlations was tested at each developmental stage using the Spearman Rank test in order to exclude false conclusions on the basis of different numbers of neurons tested per developmental stage. Similar procedures were followed for the other two possible correlations.

RESULTS

Temporal expression profile of all $GABA_A$ receptor subunits

In order to obtain a detailed description of the temporal expression pattern of all $GABA_A$ receptor subunits present during postnatal development of the visual cortex, quantitative PCR was performed. Levels of $GABA_A$ receptor subunit mRNAs were measured at different timepoints: when the cortical layers had just been formed (pn6), just before eye opening (pn11), just after eye opening (pn14), during the critical period (pn21 and pn28) and at the end of the critical period (pn35).

The expression of the α subunits is developmentally regulated (Fig. 1A). At pn6, expression of the $\alpha3$ subunit represented approximately 45% of all α subunit mRNA, and $\alpha1$, $\alpha2$ and $\alpha5$ subunit mRNA were equally expressed and each represented approximately 15% of total α subunit mRNA (Fig. 1A). Expression of $\alpha1$ and $\alpha4$ increased with time, although the increase of $\alpha4$ was only transient. In contrast, the expression of $\alpha3$ and $\alpha5$ decreased compared with pn6. Only the $\alpha2$ subunit expression remained relatively constant throughout the examined period. Expression of $\alpha6$ was not included in the measurements since this subunit is not expressed in the visual cortex (Pirker et al., 2000). Most of the changes in α subunit expression were initiated well before eye opening (pn13).

Expression of all α subunits reached a constant level at pn21 (Fig. 1A). The developmental regulation of α subunit expression was, in part, corroborated by an *in situ* hybridization approach (Fig. 2A, B). The staining for $\alpha1$ in layers I–IV was clearly stronger at pn28 compared with pn6, whereas that for $\alpha2$ (taken as internal control, given the fact that $\alpha2$ regulation is not developmentally regulated, see Fig. 1A) was equal at these two developmental stages.

Regarding the β subunits, robust expression of the $\beta3$ subunit was observed at pn6, whereas the expression of $\beta2$ was intermediate and that of $\beta1$ very low (Fig. 1B).

The $\gamma2$ subunit was downregulated early on (pn11), although it remained the predominant subunit among the γ, δ and ϵ subunits throughout the investigated period thereafter (Fig. 1C). In addition we observed an apparent upregulation of the δ subunit starting around pn14 and a downregulation of ϵ starting around pn11. The overall $GABA_A$ receptor subunit expression did not alter significantly during the studied period (total $GABA_A$ receptor subunit mRNA, normalized for HPRT, at pn6$=11.6\pm1.4$; at pn35$=10.8\pm1.1$, n.s.).

$GABA_A$ receptor subunit expression in relation to external input

To address the question whether the observed changes in $GABA_A$ receptor subunit expression levels during development are induced or affected by sensory input via the eyes, $GABA_A$ receptor subunit expression was compared between normally and dark-reared animals.

The influence of sensory input on the developmental expression pattern of the $GABA_A$ receptor α subunits was analyzed in visual cortex slices obtained from animals that were dark reared from birth until pn21 and from animals raised under a normal light/dark cycle.

The mRNA levels of the different $GABA_A$ receptor α subunits, HPRT and BDNF were determined in L.I–IV of the visual cortex. BDNF was used as a positive control since earlier studies have shown that dark rearing affect its expression (Castren et al., 1992; Lein and Shatz, 2000). Indeed, we found that after 21 days dark rearing BDNF expression was decreased by 47% compared with normally raised animals (Fig. 3A).

The developmental upregulation of the $GABA_A$ receptor $\alpha1$ subunit was not significantly altered due to 21 days dark rearing (Fig. 3A). In contrast, the expression of the $\alpha2$ and $\alpha3$ subunits was significantly higher in the dark reared compared with normally raised rats at pn21 (Fig. 3A). Despite the apparent sensory input-dependent nature of the changes in $\alpha2$ and $\alpha3$ expression, increased expression of these subunits at pn21 only has a minor impact on the relative expression levels of the different α subunits (as illustrated in Figs. 3B). As a consequence, no major effects are expected in subunit composition of the $GABA_A$ receptor. In line with this, no significant differences were found in sIPSC decay (see below; Fig. 5). We conclude that the developmental upregulation of the $\alpha1$ $GABA_A$ receptor subunit expression cannot be prevented by visual deprivation. In contrast, we cannot exclude that the developmental downregulation of the $\alpha3$ mRNA is mediated in part by visual experience.
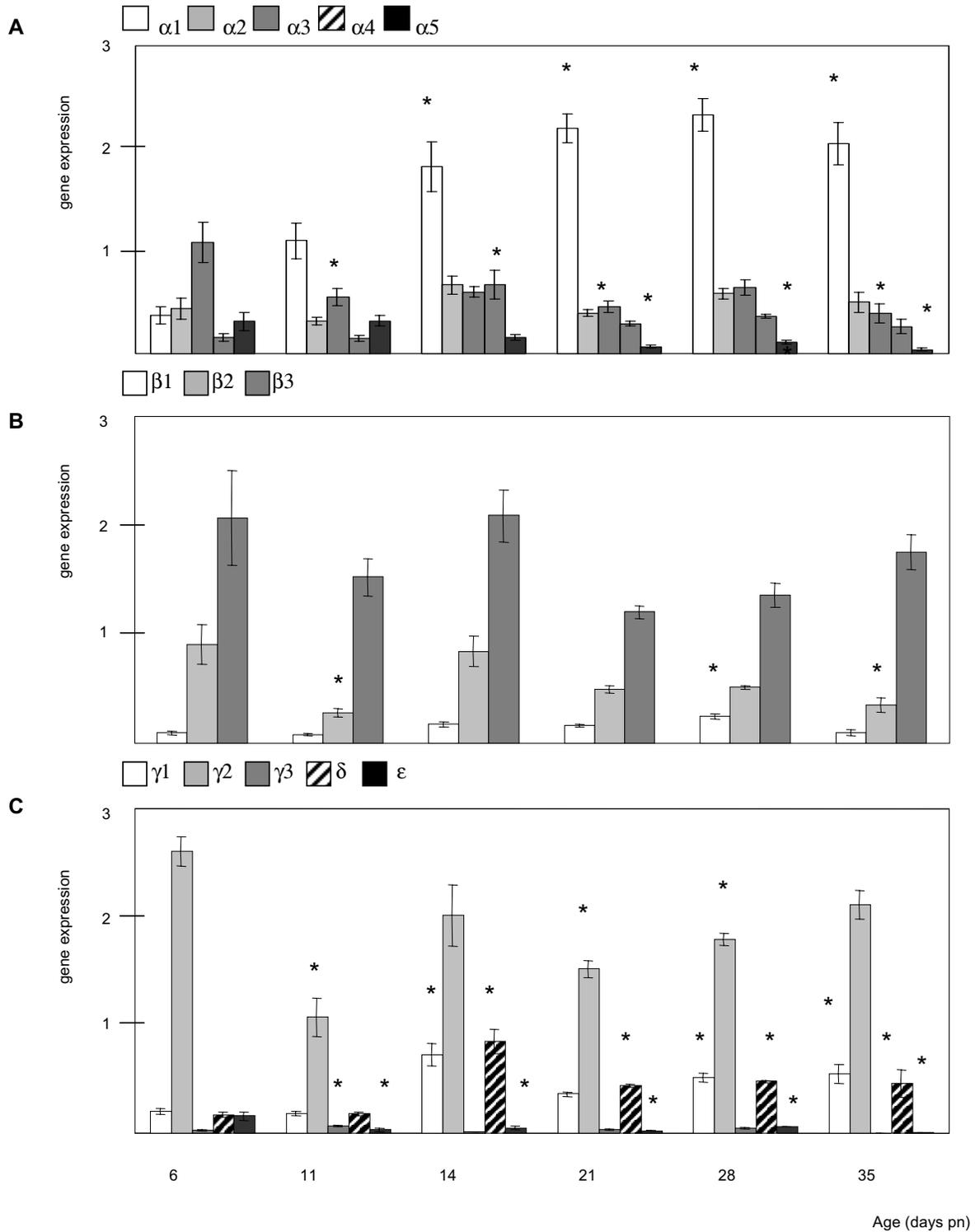
Fig. 1. Temporal expression profile of GABA$_A$ receptor α subunits (A), β subunits (B) and γ, δ and ϵ subunits (C). Quantitative analysis of GABA$_A$ subunit mRNA expression in L.I–IV visual cortex by means of quantitative PCR at different time points during *in vivo* development (pn6, pn11, pn14, pn21, pn28 and pn35). Bars represent HPRT-normalized expression levels. Asterisks indicate significant differences compared with pn6. (* $P<0.05$; one-way ANOVA; pn11, pn14, pn35: $n=4$; pn6: $n=5$; pn28: $n=8$.)
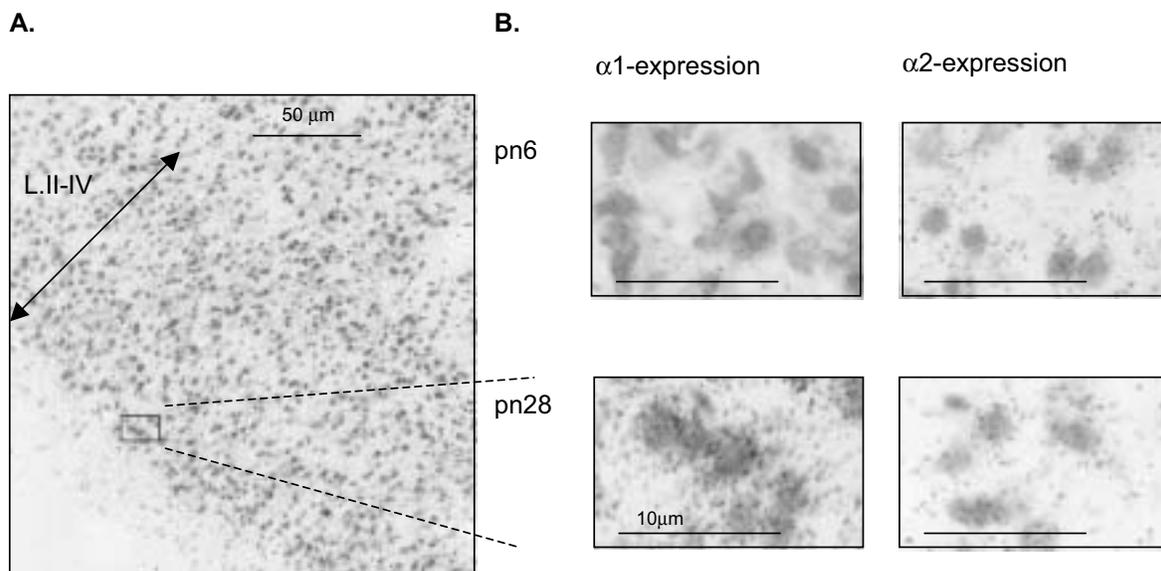
A.

B.

Fig. 2. Confirmation of qPCR by *in situ* hybridization. *In situ* hybridization for α1 and α2 expression on a visual cortex slice. Cell nuclei are visualized with Nissl-body staining (thionine). (A) Expression of α1 in a 28 day old rat. Subdivision of layers II–IV (L.II–IV) is indicated by arrows. The square indicates the enlarged area in figure B. (B) The expression of α1 and α2 subunit mRNA on the cellular level at respectively pn6 and pn28. α1 Expression is clearly increased at pn28 compared with pn6, whereas α2 expression remains constant.

Changes in the frequency and kinetics of sIPSCs

We next characterized the functional consequences of the changes in GABA$_A$ receptor subunit mRNA expression levels by making whole-cell voltage-clamp recordings in brain slices taken from rats at the same ages used for the mRNA measurements. We have measured the frequency, peak currents and τ_{decay}s of GABAergic sIPSCs to describe the functional maturation of GABAergic synapses.

The frequency of GABAergic sIPSCs depends on the number of synapses of a certain neuron and the probability of vesicular GABA release per synapse, as well as on presynaptic action potential activity (see Experimental Procedures section). In order to test whether the overall frequency of GABAergic transmission changes during postnatal development, we measured the interval times between consecutive sIPSCs at different time points during postnatal development (Fig. 4). The frequency of sIPSCs doubled between pn6 and pn11, i.e. before eye opening. This increase continues until pn21 and remains unchanged thereafter (Fig. 4G and Table 1).

We next measured the peak currents and τ_{decay}s of the GABAergic sIPSCs at different time points during development (Fig. 4H, I). The average peak current of monophasic events (i.e. putative monoquantal events; see Experimental Procedures) is likely to be determined by the average number of GABA$_A$ receptors being active per synapse and their single channel properties. This parameter showed an initial, highly significant decline during the second postnatal week. In the third to fifth week, however, a significant increase in peak current was observed (Fig. 4H and Table 1). Neonatal neurons display relatively long-lasting synaptic events. Later on, starting well in advance of eye opening, the τ_{decay} shortens drastically. Between pn21 and pn35 there is no further change in τ_{decay} (Fig. 4I).
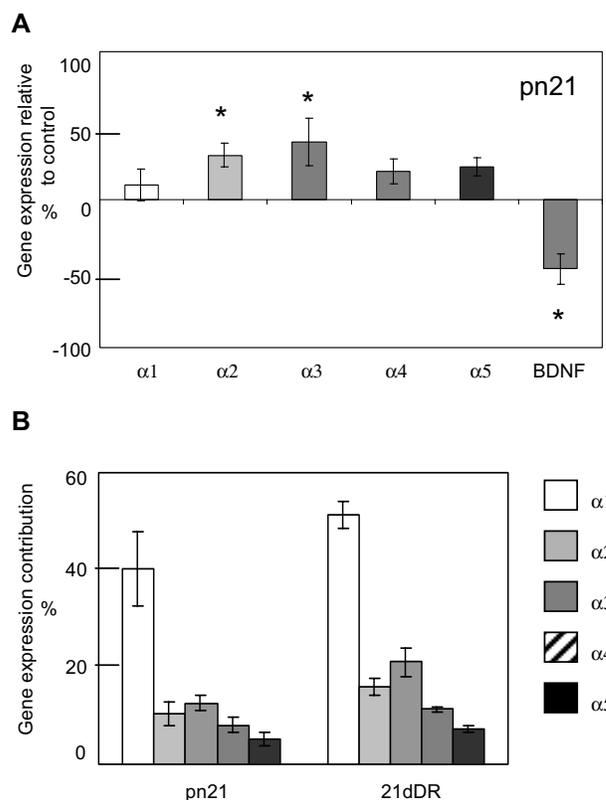
A

B

Fig. 3. Expression profile of GABA$_A$ α subunits and BDNF in visual cortex after dark rearing from birth. (A) Bars represent the regulation of the different α subunits and BDNF induced by 21 days' dark rearing compared with the control situation (21 days' normal light rearing. (* $P<0.05$, Student's t-test, $n=4$ for each group.) (B) Relative contribution of expression levels of the different α subunits at 21 days' normal dark-light rearing and 21 days' dark rearing.
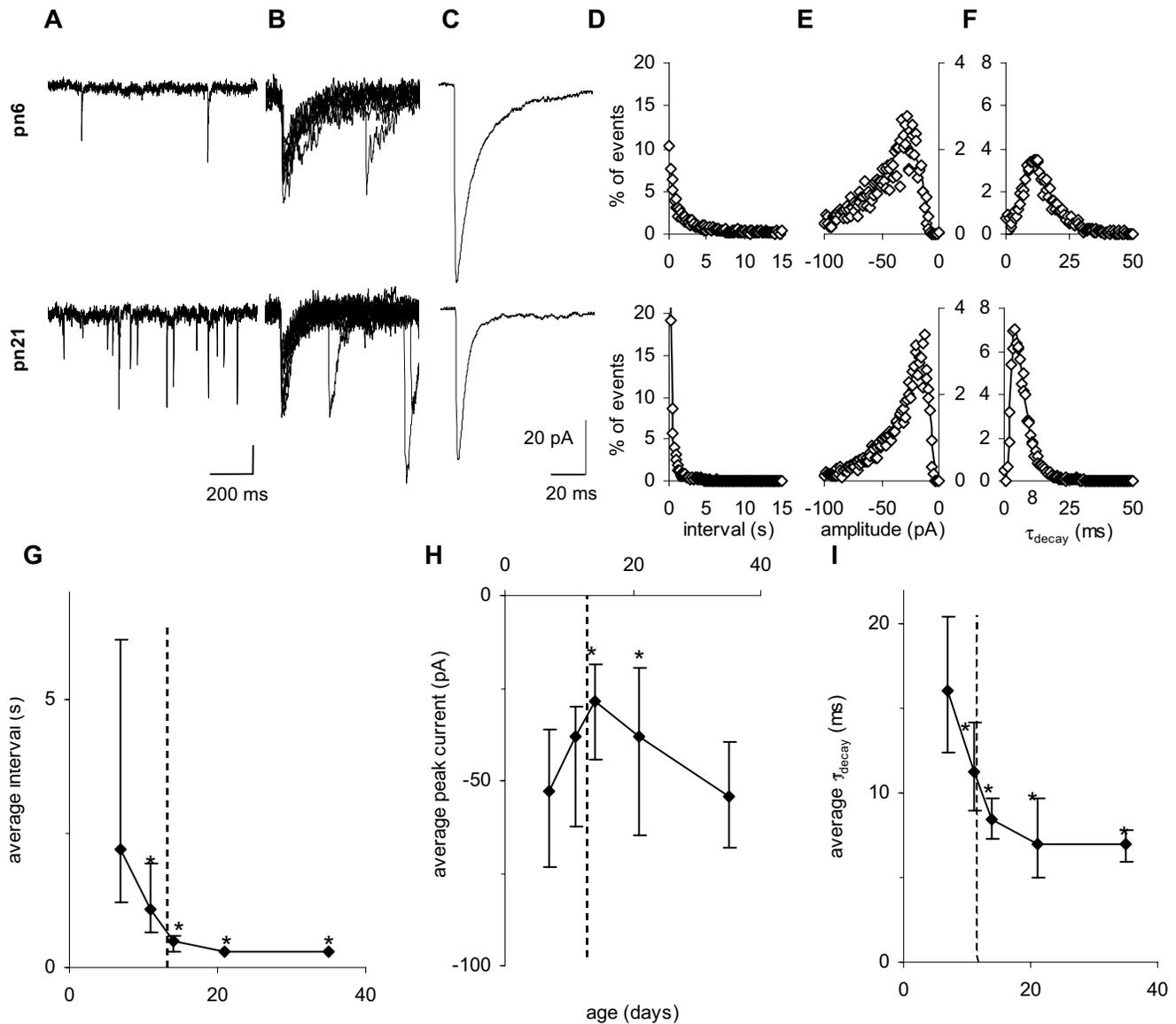
Fig. 4. Changes in GABAergic neurotransmission during neonatal development. (A) Example current traces recorded from a neuron in acutely prepared visual cortex slices from pn6 and pn21 animals. (B) Ten superimposed example sIPSCs. (C) Averaged sIPSCs from two individual experiments. Histograms of interval time (D), peak current (E) and τ_{decay} (F) made of equal numbers of sIPSCs of all experiments. Changes in interval time (G), peak current (H) and τ_{decay} (I) during neonatal development are displayed as median$\pm$IQR. The average day of eye opening (pn13) is indicated by a vertical line. Asterisks indicate statistical significant deviations from average interval times at pn6 (Kruskall-Wallis test). The number of cells used is mentioned in Table 1.

In conclusion, we observed a seven-fold increase in the occurrence of sIPSCs, accompanied with a shortening of the average τ_{decay} of 56%. Both processes start well in advance of eye opening and are completed at pn21. These changes correlate well in time with alterations in the pattern of mRNA levels.

Changes in frequency and kinetics of sIPSCs in relation to external input

To assess the functional consequences of visual deprivation on GABA$_A$ receptor development in the visual cortex, we measured the GABAergic transmission in acutely pre-pared slices from dark-reared rats at pn21. None of the biophysical parameters investigated (interval time, peak current and τ_{decay}) were affected by dark rearing (Fig. 5 and Table 1). Even in organotypical slice cultures these properties developed normally (see Table 1). This indicates that, although a slight but significant upregulation was observed in α2 and α3 gene expression following 21 days dark rearing, this is eventually not reflected in a significant functional alteration of the postsynaptic receptors.

In conclusion, light deprivation has no effect on developmental plasticity of GABAergic synapse functioning.
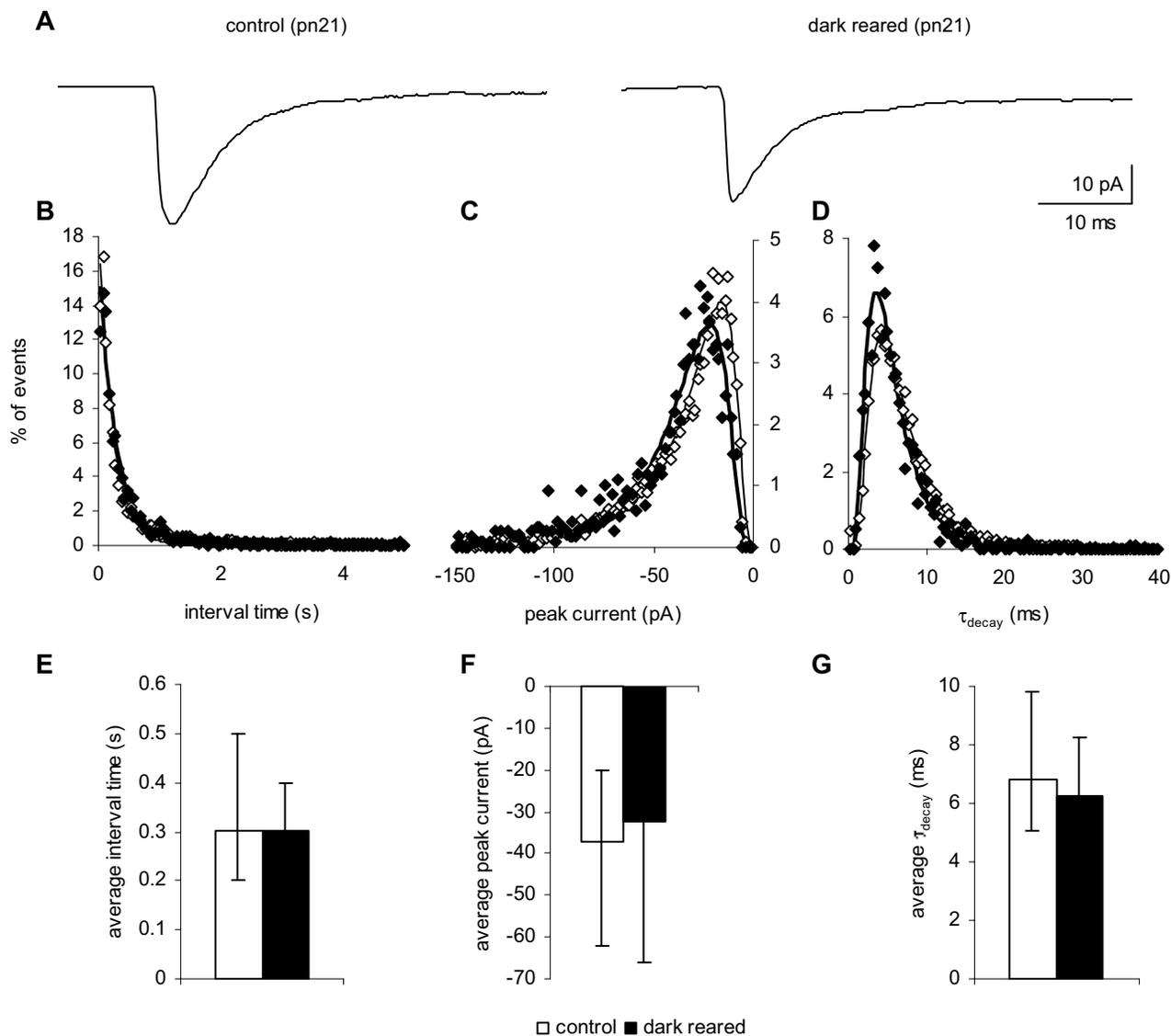
Fig. 5. The effects of dark rearing on GABAergic neurotransmission. (A) Averaged sIPSCs from two representative experiments. Histograms of equal numbers of sIPSCs from all experiments of interval time (B), peak current (C) and τ_{decay} (D) are shown. Open symbols: control; closed symbols: dark reared. Averaged interval times (E), peak currents (F) and τ_{decay}s (G) are displayed as median±IQR. None of the changes was significant (K-S test). The number of cells used is mentioned in Table 1.

Evidence for an activity dependent development

Finally, we investigated whether the extent of GABAergic innervation of any individual neuron is important for the developmental plasticity of its GABAergic synapses. This was accomplished by examining a putative correlation between the average interval time and the average τ_{decay} of sIPSCs per neuron using a large number of recordings at different developmental stages (Fig. 6; sIPSCs are also influenced by presynaptic activity, as argued above, which is relevant to the overall synaptic efficacy that native neurons receive; see also Experimental Procedures section for considerations on statistical analysis). Neurons from animals that had not yet undergone eye opening showed no correlation between average interval time and τ_{decay} (Fig. 6A and B). However, strong positive correlations were

found at pn14, just after eye opening, and at pn21 (Fig. 6C and D). This means that after eye opening, cells that receive a high frequency of GABAergic input have a greater chance of displaying fast decaying sIPSCs. The correlation between GABAergic innervation and decay maturation in individual neurons as seen under normal conditions *in vivo*, was absent in the dark-reared rats (Fig. 6E) and also in organotypical slice cultures (data not shown). This may indicate that lack of input via the eyes and/or other brain areas hampers the ability to express matured GABA$_A$ receptors at the proper synaptic locations within the neuronal network.

Taken together (Fig. 6F), this means that neurons that frequently receive GABAergic input tend to have more matured GABA$_A$ receptors per synapse. But this positive
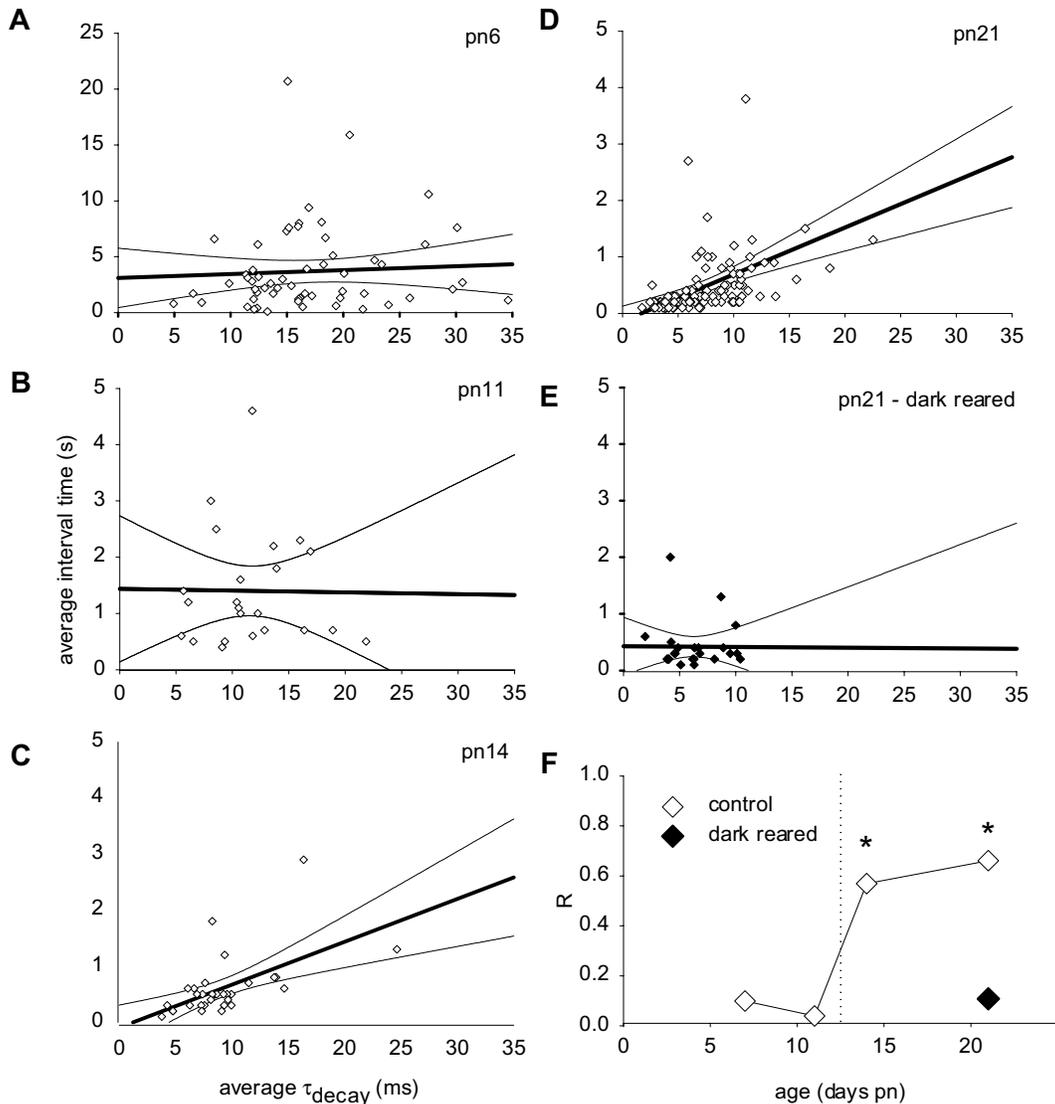
Fig. 6. Correlations between τ_{decay}s, peak currents and interval. (A–E) Every square denotes the average τ_{decay} and the average interval time of an individual neuron. Neurons are grouped per age as indicated. The straight line denotes the regression line and the curved lines the 95% confidence intervals. Note the difference in *y* axis scale between pn6 and the other groups for the average interval times versus the average peak currents (F) Summary of the regression coefficient (R) values. The correlations between τ_{decay} and interval and between amplitude and interval was absent before eye opening (pn13), but present thereafter. The correlation between peak currents and τ_{decay} was also present at pn11. In dark-reared animals, the correlation between τ_{decay} and interval was absent, but the other two were present. The average day of eye opening (pn13) is indicated by a vertical line; asterisks indicate a significant correlation (Spearman Rank test). The number of cells used is mentioned in Table 1.

correlation between overall synaptic input and postsynaptic maturation is only present in acute slices taken from animals after their eyes had opened and not in dark-reared animals. Therefore, visual input may be a prerequisite for the normal temporal and spatial pattern of GABA synapse development.

DISCUSSION

In this study we provide insight in several crucial aspects of postnatal maturation of the GABAergic system. We found that changes in mRNA expression levels of $\alpha1$, $\alpha3$ and ϵ GABA$_A$ receptor subunits occur to a large extent before eye opening (ca. pn13), whereas those of $\alpha5$, $\gamma1$ and δ

occur during or shortly after eye opening. In addition, $\alpha4$ is transiently upregulated around the day of eye opening. As a result, the dominantly expressed subunits in neonatal visual cortex (pn6) are $\alpha3$, $\beta3$ and $\gamma2$, whereas in mature rats (pn35), the GABA$_A$ receptor transcripts that occur most abundantly are $\alpha1$, $\beta3$ and $\gamma2$.

Second, changes in subunit expression are reflected in functional consequences at the level of the synapses. The τ_{decay} of sIPSCs is strongly shortened during neonatal development, which correlates with a robust seven-fold increase in the frequency of GABAergic sIPSCs. In line with the changes in subunit expression, the largest part of this increase takes place before eye opening.

Table 1. Overview of GABA$_A$ receptor properties during development of the visual cortex, after dark rearing and in organotypical tissue cultures[a]

		pn6/0 DIV	pn11/4 DIV	pn14/7 DIV	pn21/14 DIV	pn35/28 DIV
Interval time	Control	2.4±3.7 (60)	1.1±1.3 (24)	0.5±0.3 (33)	0.3±0.3 (119)	0.3±0.4 (8)
(s)	Dark reared	n.t.	n.t.	n.t.	0.3±0.2 (24)	n.t.
	Cultured	2.4±3.7 (60)	n.t.	0.9±0.4 (6)	0.4±0.4 (25)	0.2±0.1 (8)
Amplitude	Control	−51.92±33.01	−37.90±32.09	−28.42±25.59	−37.26±42.26	−54.05±28.47
(pA)	Dark reared	n.t.	n.t.	n.t.	−32.23±38.43	n.t.
	Cultured	−51.92±33.01	n.t.	−30.35±20.43	−35.88±37.53	−76.59±30.33
τ_{decay}	Control	16.02±7.58	11.24±5.19	8.47±2.37	6.82±4.74	7.03±1.86
(ms)	Dark reared	n.t.	n.t.	n.t.	6.24±4.02	n.t.
	Cultured	16.02±7.58	n.t.	13.51±1.66	12.55±3.24	4.52±1.22

[a] All data shown are the medians±IQR. Between brackets are the numbers of neurons used per group. n.t.=not tested.

The third aspect of our study conveyed the possible influence of visual experience on GABA$_A$ receptor development. Visual deprivation of rats from birth until pn21 did not have a major impact on the average GABA$_A$ subunit mRNA regulation and not at all on the average synaptic functioning of the receptor. This indicates that visual input following eye opening (Katz and Shatz, 1996) is not required for the overall induction of a mature type of GABA$_A$ receptor expression and functional insertion of the receptor in the membrane. However, in control animals, that perceived normal visual experience, a positive correlation was observed between a high frequency of spontaneous GABAergic synaptic events and a shorter synaptic current τ_{decay} in individual, maturing neurons. This may imply that, due to visual experience a condition arises, where neurons that receive a higher level of spontaneous GABAergic input tend to express the mature type of GABA$_A$ receptors. In contrast, neurons that receive a lower frequency of sIPSCs, display slower decaying synaptic currents. Interestingly, this positive correlation between sIPSC decay and interval, only appears after eye opening and is absent in dark-reared animals or in organotypical slice cultures.

Subunit expression in relation to number of synapses

A minor fraction of cortical plate cells exhibit GABAergic sIPSCs on embryonic day 18 (Owens et al., 1999). The frequency with which sIPSCs occur increases gradually to 0.5 Hz at pn3–5, similar to the value we found for pn6. This indicates that GABAergic synaptogenesis already starts at an early developmental stage. However, the more than seven-fold increase in synaptic activity between pn6 and pn21 may be a result of a major second wave of synaptogenesis. This is in line with an earlier morphological and physiological study that reports that GABAergic synapse formation in the visual cortex mainly takes place during this period (Luhmann and Prince, 1991).

Subunit expression and functional implications for receptor properties

Different subunit compositions have different biophysical and pharmacological properties (Hevers and Luddens, 1998; Mody et al., 1994). The decrease in the τ_{decay} reported here during rat visual cortex development has ear-

lier been described in the thalamus (Okada et al., 2000), cerebellum (Tia et al., 1996) and also in dissociated neurons from the visual cortex (Hutcheon et al., 2000). We find that fastening of the τ_{decay} correlates in time in particular with the upregulation of $\alpha 1$, the downregulation of $\alpha 3$ subunit mRNA and also with the transient upregulation of $\alpha 4$.

Studies with $\alpha 1$ knock out mice and $\alpha 1$ antisense application indicate that the absence of $\alpha 1$ makes sIPSCs slower in the cerebellum and cortex (Bosman et al., 2002; Vicini et al., 2001). In line with this, a naturally occurring downregulation of $\alpha 1$ in the adult hypothalamus induced slower τ_{decay} (Brussaard et al., 1997). In contrast, antisense deletion of $\alpha 2$ in visual cortex cultures results in faster decay kinetics (Brussaard et al., 1997). In addition, it has been shown in recombinant receptor studies that $\alpha 2$ and $\alpha 3$ have longer single channel burst open times than $\alpha 1$ containing receptors (Lavoie et al., 1997; Verdoorn et al., 1990). The presence of the $\alpha 4$ subunit has been reported to mediate fast decaying receptors (Bosman et al., 2002). The δ subunit, upregulated during development, may also affect the τ_{decay} of IPSCs (Haas and Macdonald, 1999), but its synaptic localization is doubted (Nusser et al., 1998). The putative roles for ϵ and/or $\gamma 1$ are currently not clear.

Thus, the aggregate of data indeed supports the idea that subunit switching of GABA$_A$ receptors is responsible for the shortening of sIPSCs during development. Therefore we propose that the large fraction of $\alpha 3$ subunit, in addition to $\alpha 2$, in the neonatal brain causes IPSCs to decay slowly, whereas the expression switch to $\alpha 1$ as the predominant subunit, possibly together with the transient expression of $\alpha 4$, gives rise to GABAergic sIPSCs with a shorter decay (see Bosman et al., 2002) for causal evidence).

GABA$_A$ receptor maturation in the absence of external input

Earlier studies showed that NMDA receptor maturation is clearly affected by the sensory input following eye opening (Quinlan et al., 1999). Like the GABA$_A$ receptor, the NMDA receptor displays a switch in subunit expression during early development (Monyer et al., 1994). An increase in the NR2A:NR2B protein ratio was substantially suppressed by dark rearing (Quinlan et al., 1999). For the

GABA$_A$ receptor, we report here that visual deprivation has minor influences on mRNA expression pattern. An earlier study in cats showed stronger effects of dark rearing on α1 and α3 protein expression (Chen et al., 2001). Although we cannot exclude that in rats downregulation of the α3 subunit is affected by visual experience, our present data also indicate that overall dark rearing does not affect the developmental fastening of the τ_{decay}. This was also shown in an earlier study by Morales et al. (2002). Thus, developmental maturation of the GABA$_A$ receptor is different from that of the NMDA receptor. However, the absence of correlation between GABAergic input and fast decaying receptors may indicate that also GABA$_A$ receptor maturation is, to some extent, activity-dependent.

We observe that all major changes in GABA$_A$ receptor subunit expression and, in correlation, the biophysical changes of the receptor, occur well before (i.e. pn11) or at the latest, around eye opening (pn13–14). Thus the GABA$_A$ receptor plasticity in the visual cortex occurs amply before the onset of the critical period, which starts around pn21, whereas the developmental changes of the NMDA receptor come about during the critical period (Carmignoto and Vicini, 1992; Quinlan et al., 1999). In summary, during the onset of the critical period, GABAergic and glutamatergic transmission is determined by shorter lasting GABA$_A$ receptor and longer lasting NMDA (Monyer et al., 1994) receptor events, in addition to very short lasting AMPA receptor responses (Angulo et al., 1999). It is likely that the timing of the developmental plasticity of GABA$_A$ receptor is crucial when it comes to determining the balance between synaptic excitation and synaptic inhibition of the network at the onset of the critical period. Such an argument would be in line with previous suggestions about the role of the developmental regulation of other aspects of GABAergic transmission in determining the onset of the critical period (Fagiolini and Hensch, 2000; Hensch et al., 1998a,b).

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