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Neonatal development of the rat visual cortex: synaptic function of $GABA_A$ receptor α subunits

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Each $GABA_A$ receptor consists of two α and three other subunits. The spatial and temporal distribution of different α subunit isomeres expressed by the CNS is highly regulated. Here we study changes in functional contribution of different α subunits during neonatal development in rat visual cortex. First, we characterized postsynaptic α subunit expression in layer II–III neurons, using subunit-specific pharmacology combined with electrophysiological recordings in acutely prepared brain slices. This showed clear developmental downregulation of the effects of bretazenil (1 μm) and marked upregulation of the effect of 100 nM of zolpidem on the decay of spontaneous inhibitory postsynaptic currents (sIPSCs). Given the concentrations used we interpret this as downregulation of the synaptic α3 and upregulation of α1 subunit. Furthermore, the effect of furosemide, being indicative of the functional contribution of α4, was increased between postnatal days 6 and 21. Our second aim was to study the effects of plasticity in α subunit expression on decay properties of GABAergic IPSCs. We found that bretazenil-sensitive IPSCs have the longest decay time constant in juvenile neurons. In mature neurons, zolpidem- and furosemide-sensitive IPSCs have relatively fast decay kinetics, whereas bretazenil-sensitive IPSCs decay relatively slowly. Analysis of α1 deficient mice and α1 antisense oligonucleotide deletion in rat explants showed similar results to those obtained by zolpidem application. Thus, distinct α subunit contributions create heterogeneity in developmental acceleration of IPSC decay in neocortex.

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Neuronal network activity is shaped by the combined action of inhibitory and excitatory synapses. Most fast inhibitory synapses in the central nervous system express the $GABA_A$ receptor, which occurs in a variety of structurally and functionally different subtypes (Hevers & Lüddens, 1998). Detailed knowledge of the spatial and temporal distribution of the individual $GABA_A$ receptor subtypes is required for a proper understanding of the impact of inhibitory neurotransmission on brain functioning.

The native $GABA_A$ receptor is a heteropentameric protein, most often consisting of two α, two β and a fifth subunit, which can be either a third β, a γ, a δ or an ϵ subunit (Farrar *et al.* 1999; Baumann *et al.* 2001; Klausberger *et al.* 2001). Different isoforms of the α, β and γ subunits have been described (Hevers & Lüddens, 1998). The $GABA_A$ receptor subunit expression differs not only between various brain areas (Wisden *et al.* 1992; Pirker *et al.* 2000), but also changes during development (Laurie *et al.* 1992; Tia *et al.* 1996). In addition, it can change under pathological conditions, like epilepsy (Brooks-Kayal *et al.* 1998; Banerjee *et al.* 1999). It has been proposed that specific α subunits are preferentially associated with the induction of certain types of behaviour, including anxiety (Rudolph *et al.* 1999; McKernan *et al.* 2000; Gulinello *et al.* 2001).

Earlier studies have indicated clear changes in $GABA_A$ receptor subunit expression during early postnatal development of the rat visual cortex (Laurie *et al.* 1992; Fritschy *et al.* 1994). If one considers the alterations in $GABA_A$ receptor subunit mRNA expression in layers I–IV of the rat visual cortex in relation to the moment of eye opening at postnatal day 13 (around pn13), it is remarkable that the alterations in subunit expression appear to be more pronounced amongst the α subunits than amongst the other $GABA_A$ receptor subunits. The clearest changes are a developmental decrease in α3 and an increase in α1 and, to a lesser extent, in α4 subunit mRNA expression (Laurie *et al.* 1992).

Here we tested the extent to which such alterations in mRNA expression are reflected in changes in the functioning of $GABA_A$ receptors in the postsynaptic membrane. To this end, whole-cell voltage-clamp recordings in acutely prepared visual cortex slices were combined with a pharmacological screening for the

function and contribution of the $GABA_A$ receptor α subunits at the postsynaptic membranes. Since the developmental changes in $GABA_A$ receptor subunit expression were especially prominent amongst the α subunits, we have focused here on α subunit expression.

First, we assessed the relative contribution of α1, α3 and α4 subunits to synaptic responses before (pn6) and at the end (pn21) of neonatal development, using subunit-specific pharmacology. Then we analysed the kinetic contribution of each α subunit in its native setting. We confirmed the results obtained with the pharmacological experiments for the α1 subunit by two independent genetic methods: α1 knockout mice and α1 antisense oligonucleotide deletion. Our data are in line with a general concept that in particular α subunit-expression is causally related to the decay kinetics of sIPSCs (Vicini *et al.* 2001).

METHODS

Animals and preparations

Non-anaesthetized Wistar rats were decapitated, and their brains quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF, mM: 125 NaCl, 25 $NaHCO_3$, 3 KCl, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 10 D(+)-glucose (carboxygenated with 5 % CO_2–95 % O_2, 304 mosmol l^{-1}, pH 7.4)). Coronal sections (400 μm thick) of the visual cortex were cut using a Leica VT1000S vibratome slicer. Slices were stored for up to 8 h in continuously carboxygenated ACSF at room temperature.

In some experiments, tissue cultures were used. These were made from the visual cortex of pn6/7 Wistar rat pups as previously described (Baker & Van Pelt, 1997). Two pieces of cortex were incubated, with their ventral sites touching, for 2 weeks in a chemically defined medium. During the second week of culturing, antisense oligonucleotides directed against the $GABA_A$ receptor α1 subunit were present in some cultures. The oligonucleotide sequence used as antisense probe was 5′-GCT GGT TGC TGT AGG-3′. As a control, we used a random sequence (5′-GTC GGG GTC TCT CTG-3′). The oligonucleotides were present at a concentration of 10 μM in the culture medium and refreshed every 48 h (see also Brussaard *et al.* 1997).

Mice lacking the α1 subunit of the $GABA_A$ receptor were of a mixed 50 % C57BL6–50 % 129SvEv background, as previously described (Sur *et al.* 2001) and measured during adulthood (> 3 months). Both wild-type and knockout mice were sedated by ketamine (I.P., ~0.1 mg (g body weight)$^{-1}$) 20 min prior to decapitation, as required during transport from the stable to the lab, by the Animal Welfare Committee of the Vrije Universiteit Amsterdam, in accordance with the Dutch law. All experimental methods were approved by the Animal Welfare Committee of our university.

Cellular recordings

In situ whole-cell voltage-clamp recordings were made of randomly selected neurons of layer II–III of the visual cortex using an Axopatch 200A amplifier (Axon Instruments, Union City, CA, USA) and borosilicate glass (Harvard Apparatus Ltd, UK) electrodes with tip resistances of 2–5 MΩ. All experiments were performed at 33 °C using ACSF with 20 μM DNQX and 20 μM APV (both from Sigma) to block the ionotropic glutamate receptors. The pipettes were filled with (mM): 135 CsCl, 1 $CaCl_2$, 10 EGTA, 10 Hepes, 2 MgATP, 296 mosmol l^{-1}, pH 7.2 (with CsOH). All spontaneous IPSCs (sIPSCs) could be blocked by the specific $GABA_A$ receptor antagonist bicuculline methiodide (10 μM, Sigma). For zolpidem (Tocris, UK), bretazenil (a kind gift from Roche Nederland, NL) and flunitrazepam (Bufa B.V., NL) we used stock solutions in DMSO. The final DMSO concentration was maximally 0.1 %. Furosemide (Sigma) was freshly dissolved each day using equimolar NaOH. There was no tetrodotoxin (TTX) added to the extracellular solution, implying that the sIPSCs measured were a mixture of action potential-evoked and miniature IPSCs.

Data analysis

Experimental data were stored on digital tapes and analysed later using the Computer Disk Recorder v1.3 and the Whole Cell Program v2.3 (both kindly provided by Dr J. Dempster, Strathclyde University, UK). Sampling rate was 5 kHz, with 1 kHz low-pass filtering. Events were detected using current and time thresholds. All automatically detected events were individually checked. Only single events with a sharp rising phase starting from a stable base line were accepted. Previous work by others on the rise times of sIPSCs in melanotropes of *Xenopus laevis* (Borst *et al.* 1994) showed that rise times of more than 1 ms may occur, even in the absence of dendritic filtering. However, variation in rise time of sIPSCs may also arise from dendritic filtering. To exclude dendritically filtered sIPSCs from further analysis, within each experiment, a correlation diagram was made of the 10–90 % rise time *versus* peak current of individual sIPSCs. The maximal 10–90 % rise time, at which no correlation between rise time and peak current was observed, was determined per experiment. At pn6, 94 % of all sIPSCs were accepted, having a maximal rise time of 3.16 ± 0.46 ms. At pn21, 96 % of all sIPSCs were accepted, having a maximal rise time of 2.78 ± 0.68 ms. Moreover, in our recordings there was no difference in the compensated RC-time constants between pn6 (RC τ = 223 ± 138 μs) and pn21 (210 ± 139 μs; $P > 0.4$; Mann-Whitney test). The resistance × capacitance (RC) time constants were calculated by multiplication of the compensated series resistance (pn6: 9.4 ± 5.4 MΩ; pn21: 9.6 ± 4.3 MΩ) and the membrane capicitance (pn6: 24.4 ± 8.8 pF; pn21: 25.3 ± 17.5 pF). Both parameters were read off the amplifier. The average input resistance was 752 ± 244 MΩ (pn6) and 543 ± 278 MΩ (pn21) (all values are means ± S.D.).

Therefore, it is unlikely that the variations in recording conditions and/or subsequent analysis introduced bias towards one of the two ages under investigation. One might argue that setting the rise time criterion for analysis per individual experiment, may have biased selection of sIPSCs depending on the developemental stage being recorded from. Instead we could have used one objective strict rise time criterion (e.g. < 0.6 ms, see Nusser *et al.* 2001). Application of this criterion to our data would have rejected most of the events and would have introduced a bias towards neurons having a high frequency of sIPSCs.

Inter-event times (sIPSC interval times) were measured between all accepted sIPSCs, irrespective of their rise times. From all sIPSCs that were not dendritically filtered, we measured the peak current and the synaptic current decay time constant (τ_{decay}). The τ_{decay} was calculated from mono-exponential fits to the decay phase of each individual sIPSC. We established that the experimental decay data were best described by mono-exponential fits (Aikake's Information Criterion, see also Bozdogan (1987)). Possibly, the relatively small amplitudes of the sIPSCs, and therefore the less optimal signal to noise ratio of our recordings,

made discrimination between fast desensitization and deactivation of $GABA_A$ receptors impossible in this experimental setup.

For each neuron, histograms were made of the peak currents, τ_{decay} values and interval times of all the accepted sIPSCs. Neurons which had less than 50 accepted control sIPSCs were rejected from further analysis. Typically, we analysed approximately 400 sIPSCs per condition per neuron. Peak current and τ_{decay} histograms were not normally distributed (Kolmogorov–Smirnov (K–S) test). Instead, they were skewed towards larger values. These distributions could be well fitted with lognormal curves. In contrast, the interval time histograms were best fitted with mono-exponential functions, as described previously (Brussaard *et al.* 1996).

Whether a specific neuron was sensitive for a certain ligand was tested by comparing the appropriate sIPSC parameter (interval time, peak current or τ_{decay}) of all sIPSCs before and after ligand application with a Kolmogorov–Smirnov test. Ratios of sensitive neurons at pn6 and pn21 were tested for significant difference using Fisher's exact test. In order to do this, a neuron was termed 'sensitive' if indicated as such by the K–S test. The amplitudes of the pharmacological effects were compared between pn6 and pn21 using Student's *t* test. Throughout this paper, the level of statistical significance used was 5 %, unless otherwise stated.

RESULTS

Postnatal changes in peak current and τ_{decay} of IPSCs

The $GABA_A$ receptor subunit expression pattern is highly plastic during neonatal development of the visual cortex, as has been demonstrated by mRNA expression studies (Laurie *et al.* 1992) and protein measurements using antibodies (Fritschy *et al.* 1994). The changes in subunit expression, which take place between pn6 and pn21, correlate well in time with alterations in the peak currents and decay time constants (τ_{decay}) of spontaneous IPSCs (sIPSCs) mediated by $GABA_A$ receptors (Laurie *et al.* 1992; Fritschy *et al.* 1994). However, we have only just begun to comprehend the causal relation between α subunit transcription and postsynaptic activity.

As an overall starting point towards this goal, we investigated whether $GABA_A$ receptors with different kinetics are specifically targeted to synapses with low or high receptor numbers. We plotted histograms of peak currents *versus* τ_{decay} values (Fig. 1) of all sIPSCs from all experiments carried out at different developmental stages. Immature

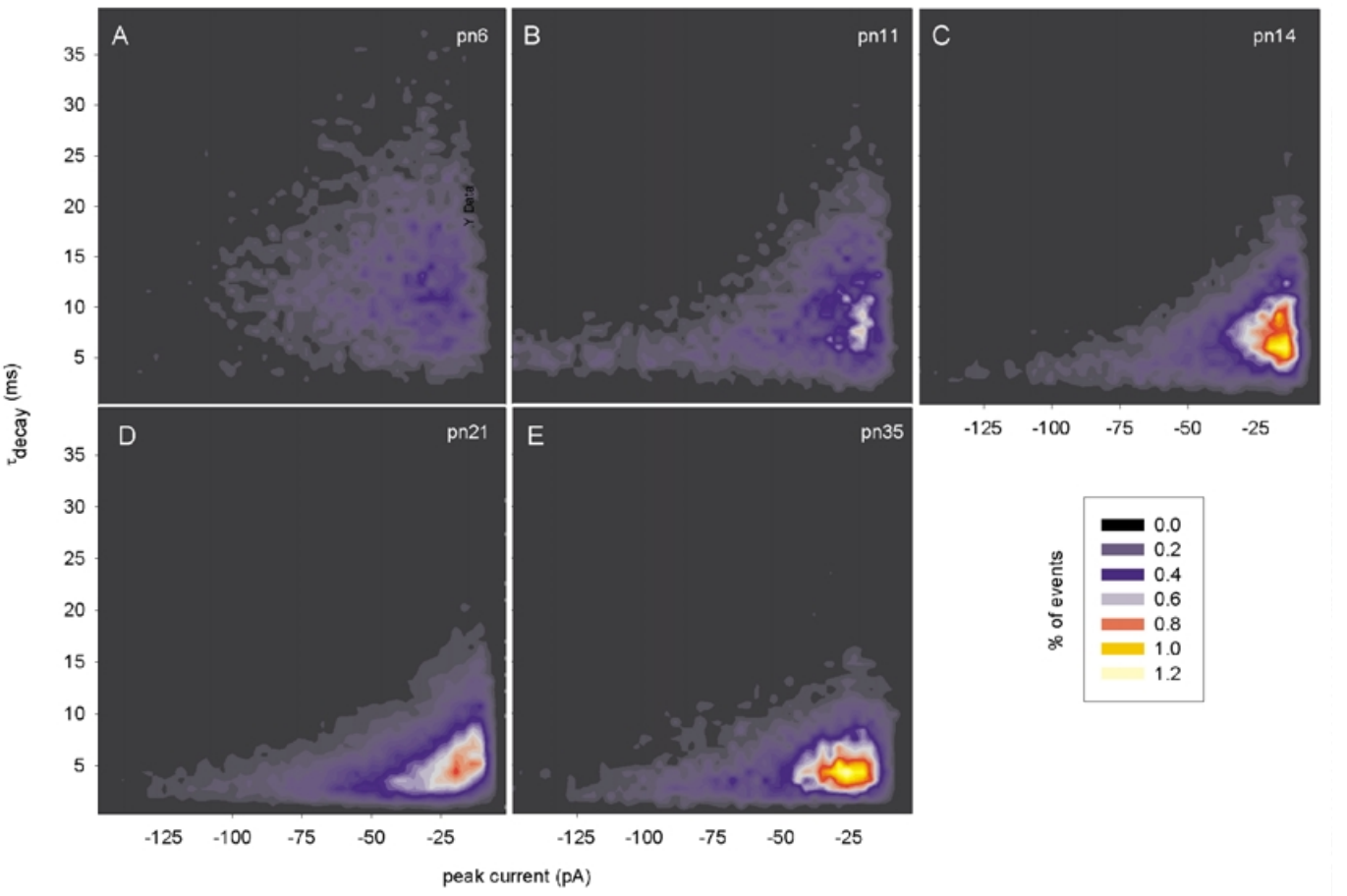

Figure 1. Development of postsynaptic GABAergic currents

Colour coded histograms of peak current *vs.* τ_{decay} of all sIPSCs at different stages of neocortical development. The percentage of sIPSCs with a particular peak current– τ_{decay} combination per bin is indicated by a colour coding (black, no sIPSCs; white, highest number of sIPSCs). Number of neurons used per group: pn6, $n = 60$; pn11, $n = 24$; pn14, $n = 33$; pn21, $n = 119$ and pn35, $n = 8$.

neurons (at pn6) displayed a heterogeneous distribution of all combinations of peak current and τ_{decay}: all combinations of peak currents and τ_{decay} values occurred equally often (Fig. 1*A*). Then during the neonatal development, large slow-decaying sIPSCs disappeared first, whereas simultaneously large fast-decaying sIPSCs appeared (pn11, Fig. 1*B*). Between pn14 and pn21, the large fast-decaying sIPSCs disappeared gradually (Fig. 1*C* and *D*) and eventually also the small slow-decaying sIPSCs vanished (pn35, Fig. 1*E*), leaving the small fast-decaying sIPSCs. Thus, the large heterogeneity at pn6 changed into a rather clustered population of sIPSCs at pn35.

The average 10–90 % rise time of the sIPSCs decreased during development (1.58 ± 0.83 ms (pn6; $n = 60$ experiments; average ± S.D. of all sIPSCs pooled) *vs.* 1.16 ± 0.83 ms (pn21; $n = 119$ experiments; $P < 0.05$; Mann-Whitney test). This may indicate that the passive conductance properties of the neurons changed during development, also affecting the τ_{decay} of sIPSCs. However, since the RC-time constants of individual recordings did not differ between the distinct developmental stages (see Methods) and since within each indivual experiment we selected sIPSCs that were not obviously under the influence of dendritic filtering, we conclude that the shift in decay of sIPSCs is largely due to developmental changes in $GABA_A$ receptor properties, including subunit switching. In order to analyse the specific contribution of subunit switching to the decay properties of $GABA_A$ receptors during development, we subsequently analysed the functional contribution of some of the α subunits, both by means of pharmacological and genetic manipulations.

Pharmacological indications for subunit switching of $GABA_A$ receptors

For the subsequent pharmacological characterization of the synaptic $GABA_A$ receptors, we took pn6 as a starting point for neonatal development of the rat visual cortex. At that time, cortical layer formation has been completed and a period of massive synaptogenesis starts (Blue & Parnavelas, 1983). GABAergic synapses in pn21 rats were considered matured, since we did not find significant differences in either peak currents or τ_{decay} values of sIPSCs between pn21 and pn35 (K–S test). Since rats open their eyes around pn13, we measured 1 week before and 1 week after eye opening. The pharmacological screening was used to study the functional, postsynaptic contribution of the different α subunits present in the visual cortex. For α1, we used 100 nM zolpidem (Ruano *et al.* 1992; Renard *et al.* 1999; Vicini *et al.* 2001) and for α4, 100 μM furosemide (Korpi & Lüddens, 1997; Thompson *et al.* 1999). Ligands available for α3 are less specific. We used the best one available – 1 μM bretazenil (Puia *et al.* 1992). We realise that the α subunit selectivity of bretazenil is far from optimal. Therefore, the interpretation of the results obtained may have to be considered with caution.

First, we tested whether the sIPSCs at pn6 and pn21 were affected by the application of 100 nM zolpidem (Fig 2*A* and *C*). We compared the τ_{decay} of the sIPSCs before and after zolpidem application by means of a K–S test. Since 100 nM zolpidem preferentially elongates the τ_{decay} of sIPSCs mediated by the α1 subunit containing $GABA_A$ receptors (Ruano *et al.* 1992; Renard *et al.* 1999), we could assess the α1 contribution at pn6 and at pn21. However, aspecific reactions of zolpidem with non-α1 subunit-containing $GABA_A$ receptors at this concentration do occur and may lead to up to 25 % τ_{decay}-elongation (Vicini *et al.* 2001). Therefore, we introduced a specificity threshold for the zolpidem effect, which was set at a relative effect of 30 %. Accordingly, we present three categories of neurons in Fig. 2*D*: neurons in the first category showed no significant elongation of τ_{decay} upon zolpidem application (and thus probably have no or few synaptic α1 subunits). The second category comprises neurons that did show a significant zolpidem effect, but below the 30 % threshold. Hence in these neurons, the effects cannot with certainty be attributed to only α1 subunits. Finally, in the third category are the neurons that showed large (> 30 %), significant zolpidem effects, which we interpret as having a large portion of postsynaptic α1 receptors. When comparing pn6 and pn21, we observed not only an increase in the fraction of cells that were zolpidem sensitive (Fig. 2*D*), but also an increase in the relative effect of zolpidem per neuron (Fig. 2*E*). This implies that at pn21 there are not only more neurons expressing α1 at their synapses than at pn6, but also that the overall fraction of α1 subunits per synapse increases with neonatal development.

In a similar way, we analysed the sensitivity to the other two ligands. The second agent tested was bretazenil at 1 μM, which elongates the τ_{decay} of all $GABA_A$ receptors but has been reported to have the largest effect on α3-containing $GABA_A$ receptors (Puia *et al.* 1992). As shown in Fig. 3*A*–*C*, bretazenil clearly affected the τ_{decay} values of sIPSCs in immature tissue, but had hardly any effect in mature neurons. All neurons tested at pn6 showed clear potentiation by bretazenil, whereas only half the number of neurons at pn21 showed small effects (Fig. 3*D*). Furthermore, the effects in bretazenil-sensitive neurons decreased significantly with development (Fig. 3*E*). We conclude that the immature $GABA_A$ receptors are particularly affected by 1 μM bretazenil, in contrast to the mature receptor types at pn21. Since α3 is the only α subunit that is abundant at pn6, but is downregulated at pn21 (Laurie *et al.* 1992), whereas all other α subunits remain constant or are even upregulated, we hypothesize that the bretazenil action observed here can be largely attributed to specific binding of bretazenil to α3-containing $GABA_A$ receptors.

Finally, we used the $GABA_A$ receptor antagonist furosemide at a relatively low concentration of 100 μM to test for the

contribution of α4-containing $GABA_A$ receptors to the synaptic activity (Korpi & Lüddens, 1997; Thompson *et al.* 1999). Only one out of five immature neurons tested showed an effect of furosemide (both on sIPSC amplitude and sIPSC frequency). In contrast, most mature neurons displayed furosemide effects (Fig. 4*A–G*). Thus, the α4 subunit is almost absent in immature cells, but plays a significant role in a subset of mature neurons.

From the pharmacological screening, we conclude that $GABA_A$ receptors at pn6 are especially sensitive to bretazenil, in contrast to the $GABA_A$ receptors at pn21, which are particularly affected by zolpidem.

Pharmacological classification of sIPSC kinetics

In order to test whether one can predict the average τ_{decay} of a neuron on the basis of the $GABA_A$ receptor pharmacology of a particular neuron, we studied the putative correlation between the response to the different reagents used and the average τ_{decay} in individual neurons under control conditions (i.e. before application). Figure 5*Aa–c* show scatter plots of the average τ_{decay} and the average pharmacological effect of all neurons tested. To this end, we have superimposed the immature and the mature neurons and analysed putative correlations in the data sets thus obtained.

The zolpidem effect was negatively correlated with the average control τ_{decay}. This implies that neurons with, on average, faster sIPSCs show larger zolpidem effects than neurons with $GABA_A$ receptors that mediate, on average, slower sIPSCs (Fig. 5*Aa*). The opposite is true for bretazenil, indicating that bretazenil-sensitive $GABA_A$ receptors are preferentially located in neurons with, on average, slower sIPSCs (Fig. 5*Ab*). Finally, furosemide showed a (non-significant) trend towards a negative correlation, pointing at a possible, modest contribution of furosemide-sensitive $GABA_A$ receptors to the fast sIPSCs (Fig. 5*Ac*).

We next addressed the question of whether the correlations between $GABA_A$ receptor pharmacology and τ_{decay}, as described above on the cellular level, could also be found at the level of individual synapses. Therefore, we compared

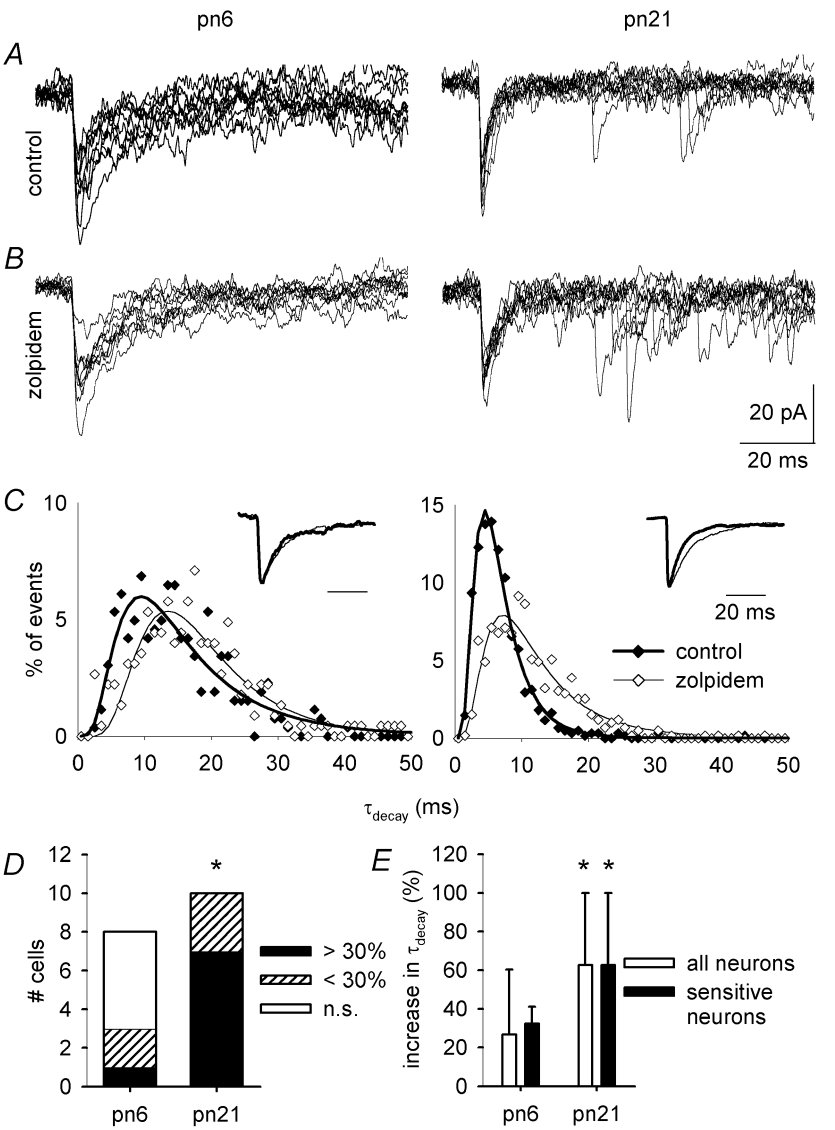

Figure 2. The contribution of the zolpidem-sensitive, postsynaptic $GABA_A$ receptors increases during neonatal development

A, example sIPSCs aligned to their rising phases. *B*, the same as *A*, but in the presence of 100 nM zolpidem. *C*, the effect of zolpidem increases with development. τ_{decay} histograms of equal numbers of sIPSCs pooled from all experiments before (◆) and after (◇) zolpidem application. Insets: average sIPSCs, control (thick line) and in the presence of zolpidem (thin line), normalized to peak current. *D*, the fraction of zolpidem-sensitive neurons increases between pn6 and pn21. Some neurons showed a significant but relatively small increase in τ_{decay} (hatched area). * $P < 0.05$ increase in the fraction of zolpidem-sensitive neurons (Fisher's exact test). *E*, average increase of τ_{decay} upon zolpidem application in all neurons (open bar) and in those neurons that showed a significant τ_{decay} increase (filled bar). * $P < 0.05$ as compared to pn6 (Student's *t* test).

the τ_{decay} values of equal numbers of sIPSCs coming from all experiments before and after ligand application (Fig. 5*Ba–Cc*).

Histograms were made of the τ_{decay} values of all these sIPSCs. The difference in distributions before and after application gives information about the typical decay kinetics of the sIPSCs mediated by $GABA_A$ receptors that were sensitive to the compound being tested. In the case of zolpidem, for instance, sIPSCs mediated by α1-containing $GABA_A$ receptors had an increased τ_{decay} after application. This means that the fast sIPSCs that were present before, but not after, zolpidem application were affected by zolpidem and thus may be mediated by α1-containing $GABA_A$ receptors. The same reasoning holds true for bretazenil, which works in a similar way to zolpidem. For the antagonist furosemide, we tested for the sIPSCs that had disappeared as a consequence of furosemide application.

Analysis of α1 subunit deletion

The above pharmacological analysis indicates that shortening of sIPSC kinetics with development is caused by changes in α subunit expression of $GABA_A$ receptors. To further substantiate the causal relation between α subunit expression and sIPSC decay kinetics, we chose to corroborate our pharmacological analysis for the α1 subunit by means of genetic manipulation, where the subunit contribution was altered, while putative development of the passive cable properties of the dendrites could still take place. To this end, we made use of two independent genetic manipulation methods. We measured acute slices from adult $GABA_A$ receptor α1 knockout mice (Sur *et al.* 2001) and organotypical slice cultures from rats (Baker & Van Pelt, 1997) treated with antisense oligonucleotides against the α1 subunit. In both preparations we investigated the effects of α1 deletion on the decay kinetics of the sIPSCs.

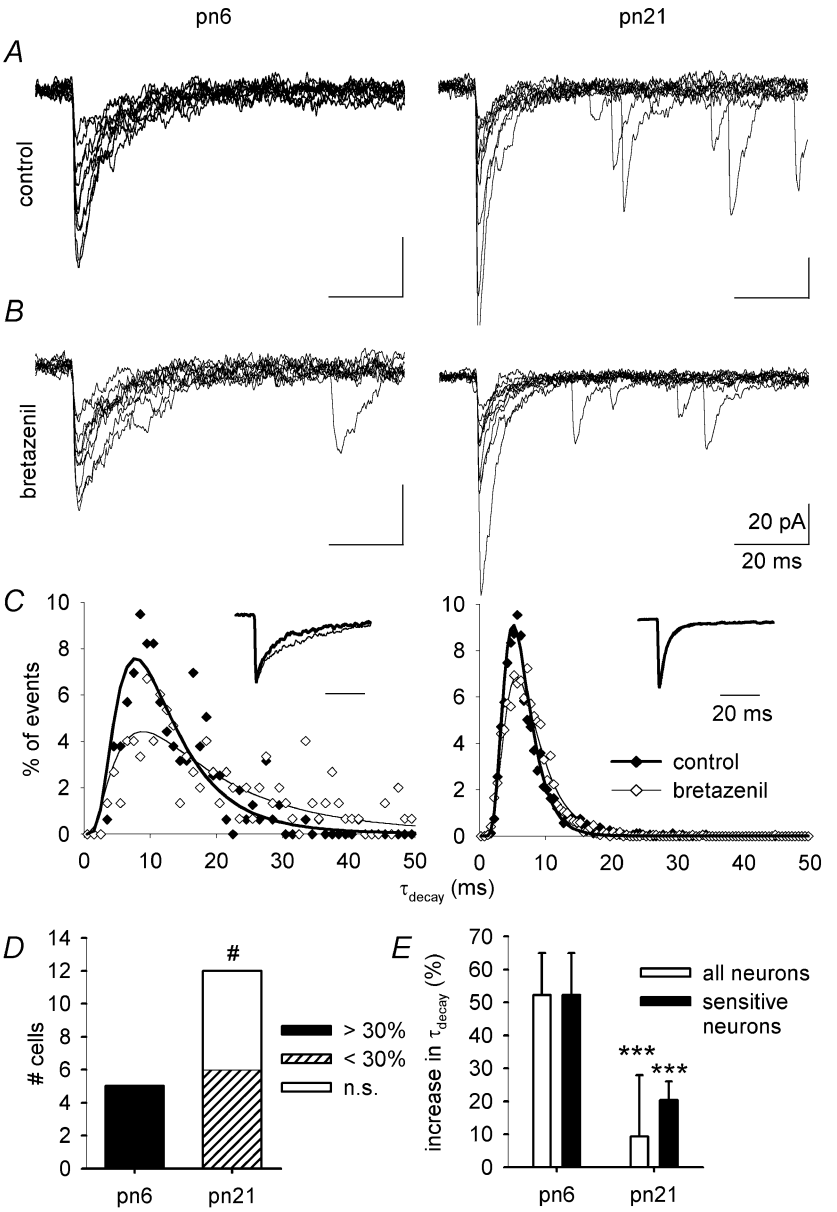

Figure 3. The contribution of the bretazenil-sensitive, postsynaptic $GABA_A$ receptors decreases during neonatal development

A, example sIPSCs aligned to their rising phases. *B*, the same as *A*, but in the presence of 1 μM bretazenil. *C*, the effect of bretazenil increases with development. Histograms of equal numbers of sIPSCs pooled from all experiments before (◆) and after (◇) bretazenil application. Insets: average sIPSCs, control (thick line) and in the presence of bretazenil (thin line), normalized to peak current. Note that at pn21 both lines overlap. *D*, the fraction of bretazenil-sensitive neurons decreases during neonatal development. Some pn21 neurons showed a significant but relatively small increase of τ_{decay} (hatched area). # $P < 0.1$ for the decrease in fraction of bretazenil-sensitive neurons (Fisher's exact test). *E*, average increase of τ_{decay} upon bretazenil application in all neurons (white bar) and in those neurons that showed a significant τ_{decay} increase (black bar). *** $P < 0.001$ as compared to pn6 (Student's *t* test).

As shown in Fig. 6*A–D*, sIPSCs from the α1 knockout mice had longer τ_{decay} values than the sIPSCs from the wild-type mice. Apparently, the postsynaptic $GABA_A$ receptor pool is different after deletion of the α1 subunit (in line with the findings of Vicini *et al.* (2001)). Since these measurements were made in constitutive knockouts, compensation by, for instance, other α subunits is quite possible. Therefore, we also used another genetic manipulation. In organotypical slice cultures, made from pn6 rats, we applied antisense oligonucleotides against the α1 subunit (Fig. 6*E–H*). We argue that the elegance of this method is that α1 deletion now only occurs during the week before measurement, leaving less opportunity for compensatory mechanisms. Also in these experiments we found an elongation of the average τ_{decay} (Fig. 6*G*). With respect to possible compensatory mechanisms, it should be noted that the peak currents of the sIPSCs in the antisense-treated cultures were severely reduced (Fig. 6*E*), whereas

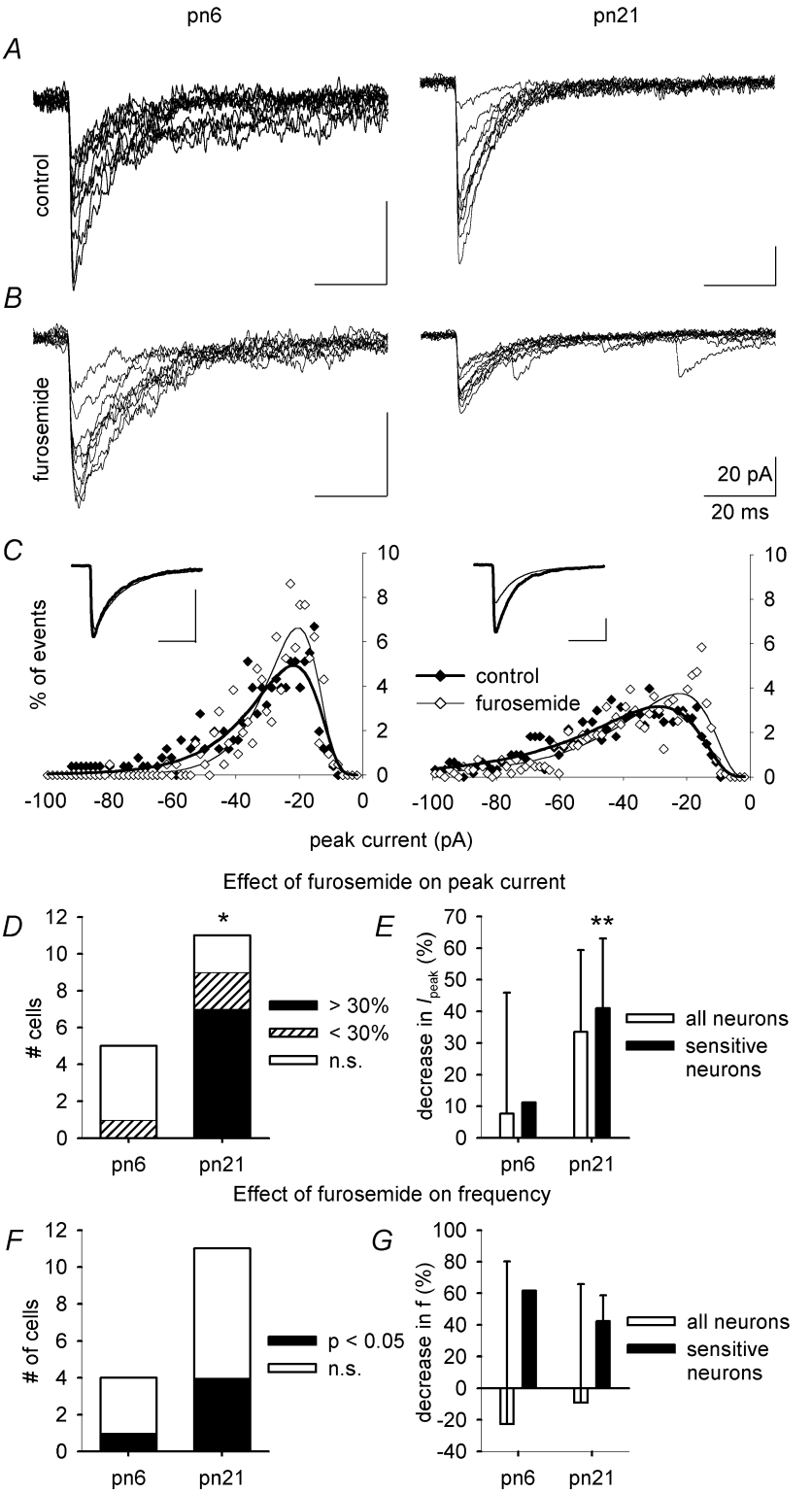

Figure 4. The contribution of furosemide-sensitive, postsynaptic $GABA_A$ receptor increases during neonatal development

A, example sIPSCs aligned to their rising phases. *B*, the same as *A*, but in the presence of 100 μM furosemide. *C*, the effect of furosemide increases with development. Peak current histograms of equal numbers of sIPSCs pooled from all experiments before (◆) and after (◇) furosemide application. Insets : average sIPSCs, control (thick line) and in the presence of furosemide (thin line). *D*, the fraction of neurons displaying a decrease of peak current upon furosemide application increases between pn6 and pn21. Some neurons show a significant but relatively small decrease of peak current (hatched area). * $P < 0.05$ for increase in fraction of furosemide-sensitive neurons (Fisher's exact test). *E*, average decrease of peak current upon furosemide application in all neurons (open bar) and in those neurons that showed a significant peak current decrease (filled bar). ** $P < 0.01$ as compared to pn6 (Student's *t* test). *F*, there is no difference in the fraction of neurons that show a decrease of sIPSC frequency upon furosemide application (Fisher's exact test). In order to calculate the sIPSC frequency, dendritically filtered sIPSCs were also used. *G*, average decrease in sIPSC frequency upon furosemide application in all neurons (open bar) and in those neurons that showed a significant decrease in sIPSCfrequency (filled bar). The small increases in frequency upon furosemide application are not statistically different from no effect (Student's *t* test). There were no significant changes between pn6 and pn21 (Student's *t* test).

those in the knockouts did not differ from the wild-types (Fig. 6*A*). This could point towards compensatory upregulation of other subunits in the knockouts, but not in the antisense-treated cultures.

The fact that deletion of the α1 subunit, either in knockout animals or in antisense-treated slice cultures, led to a lengthening of the decay of the sIPSCs showed that subunit switching can be held responsible for the developmental shift in decay of the sIPSCs. Thus the role of putative changes in passive electrical properties occurring with development can be largely excluded. The 10–90 % rise time of accepted sIPSCs in wild-type (WT) animals was 0.96 ± 0.42 ms, whereas in α1 −/− animals it was 1.09 ± 0.52 ms (obtained from ~7000 sIPSCs pooled from

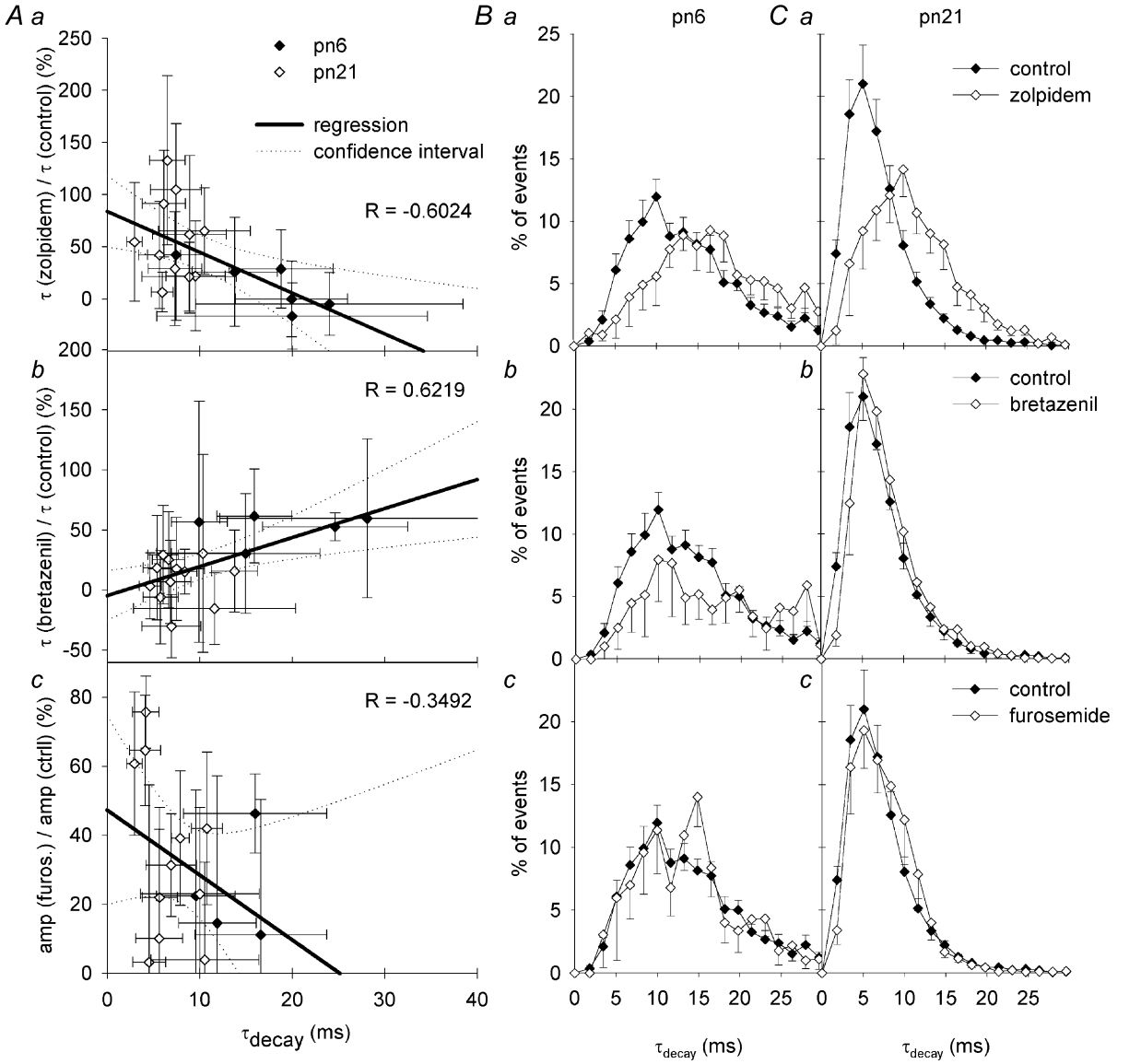

Figure 5. The contribution of the different, pharmacologically classified $GABA_A$ receptor types to the overall sIPSC kinetics of neurons

Aa–Ac, the average (± S.D.) τ_{decay} of all neurons tested (filled symbols: pn6; open symbols: pn21) before ligand application *versus* the pharmacogical effect (as a percentage of control; average ± S.D.). The parameters used for the pharmacological effects were: τ_{decay} (for zolpidem to assess the role of α1 and bretazenil for α3) and peak current (for furosemide (α4)). A regression line was fitted (continuous line) with its 95 % confidence interval (dotted lines). This resulted in a negative correlation for zolpidem ($P < 0.05$, Pearson test) and a positive correlation for bretazenil ($P < 0.01$, Pearson test). The negative correlation found between τ_{decay} and furosemide-induced peak current decrease was not significant ($P = 0.2$, Pearson test). The correlation coefficients (R) are indicated. *Ba–Cc,* averaged τ_{decay} histogram constructed from all histograms from individual neurons before (filled symbols) and after (open symbols) ligand application. Histograms were from pn6 (*Ba–Bc*) and pn21 (*Ca–Cc*). It can be seen that all α subunits contribute to faster τ_{decay} values at pn21 than at pn6.

$n = 17$ for WT and $n = 21$ for $\alpha 1$ −/− experiments, respectively; $P < 0.05$; Student's t test), whereas their RC τ values were not significantly different (i.e. 131 ± 62 μs *versus* 124.4 ± 50 μs, respectively; $P > 0.7$; Student's t test). In $\alpha 1$ −/− mice the morphological development of visual cortex neurons is unaltered in Golgi staining (data not shown). Moreover, the fact that RC times of individual recordings were not altered indicates that the alterations in passive properties cannot be held responsible for the small but significant shift in rise time of sIPSCs coming from WT *versus* $\alpha 1$ −/− mice. Alternatively, we cannot exclude that some of the variation in rise times of sIPSCs may also depend on the subunit composition of the postsynaptic $GABA_A$ receptors being active (see also Haas & Macdonald (1999)). The finding that average rise time in $\alpha 1$ −/− mice was longer than in $\alpha 1$ +/+ mice corroborates this view.

DISCUSSION

We have studied the functional contribution of distinct pharmacological subtypes of $GABA_A$ receptor to the postsynaptic receptor activity in the rat visual cortex before and after neonatal development. We found that the fraction of postsynaptic $GABA_A$ receptors that was sensitive to bretazenil decreased between pn6 and pn21, whereas the effects of zolpidem and furosemide increased. Thus, in neonatal neurons there is probably a predominant functional synaptic contribution of the $\alpha 3$ subunit, although the contribution of $\alpha 1$ cannot be excluded. In matured neurons, the $\alpha 1$ subunit is probably the most prevalent, followed by α 4 and to a lesser extent also $\alpha 3$ (Fig. 7). These conclusions based on the analysis the experiments using subunit-specific pharmacology are in

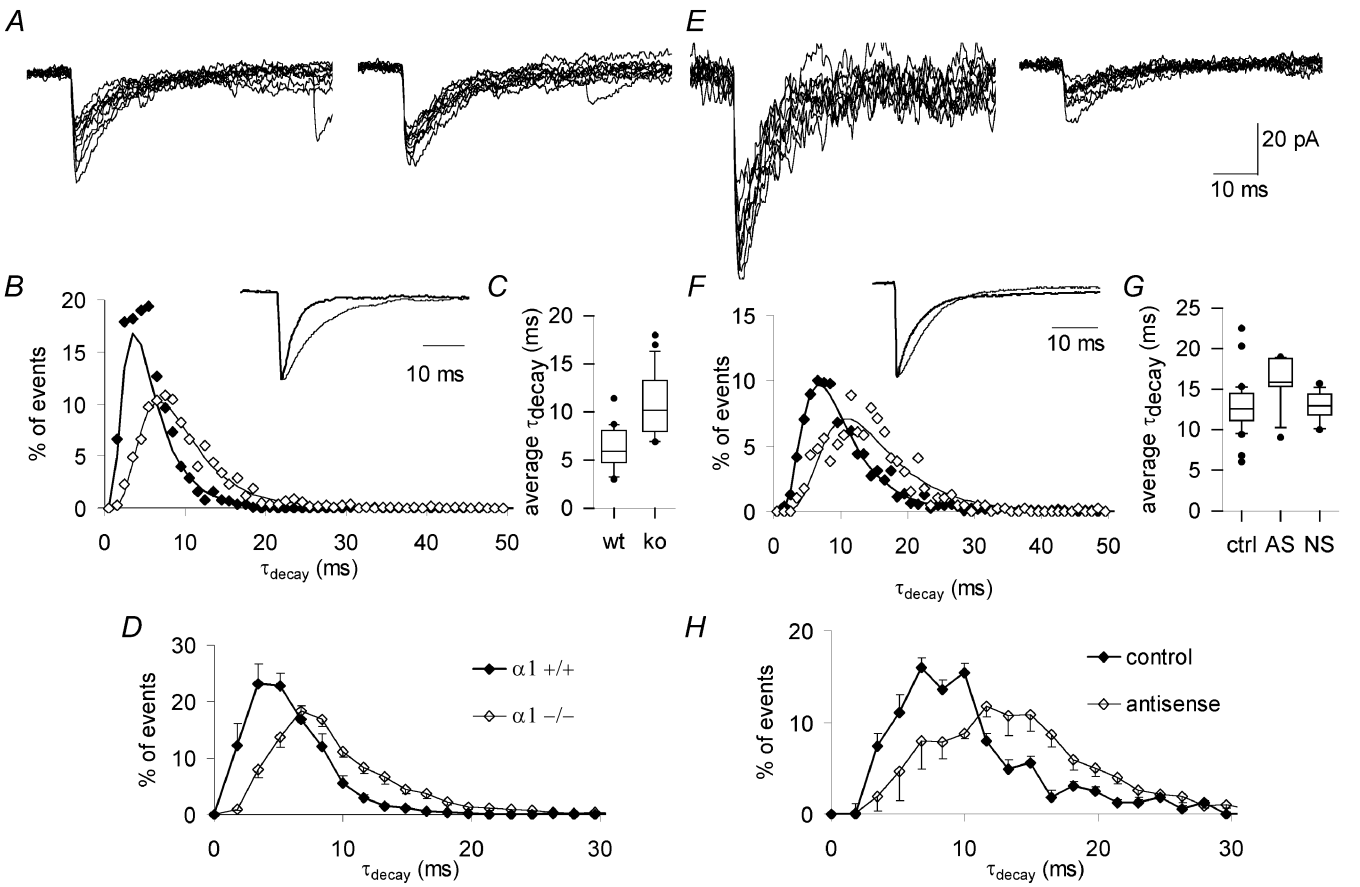

Figure 6. Deletion of the GABAA receptor $\alpha 1$ subunit results in sIPSCs with a longer τ_{decay}

A, example sIPSCs aligned to their rising phases from adult wild-type mice (left) and adult α 1 −/− mice (right). *E*, example sIPSCs aligned to their rising phases from untreated organotypical slice cultures from rat visual cortex (left) and from cultures that were grown in the presence of $\alpha 1$ subunit antisense (AS) oligonucleotide for one week (right). Both groups were measured after 15 days *in vitro*. *B* and *F*, τ_{decay} histograms made from equal number of sIPSCs from control (filled symbols) and $\alpha 1$ knockout (*B*) and $\alpha 1$ AS-treated cultures (*F*) (open symbols). Insets: averaged sIPSCs from control (thick line) and knockout (*B*)/antisense-treated cultures (*F*) (thin line), normalized to peak current. *C* and *G*, box plots of average τ_{decay} values. NS: non-sense sequence oligonucleotide, as mentioned in Methods. *D* and *H*, averaged histograms, calculated from the τ_{decay} histograms of all individual neurons. Number of neurons used per group: wild-type (WT), $n = 17$; knockout (KO), $n = 21$; control cultures, $n = 26$; AS-treated cultures, $n = 7$; NS-treated cultures, $n = 10$.

line with the quantitative mRNA transcript measurements performed previously (Laurie *et al.* 1992).

Furthermore, we demonstrate that the presence of synaptic $GABA_A$ receptor α subunits is very important for determining the decay kinetics of GABAergic sIPSCs. Both our pharmacological experiments and the genetic manipulations show that the α1 subunit is responsible for fast decay. The upregulation of α4-containing $GABA_A$ receptors is characterized by even faster sIPSCs, whereas α3-containing receptors in both juvenile and mature neurons appear to mediate slow decaying sIPSCs.

The α2, α5 and α6 subunits were not investigated. There is no good pharamacological tool available for the α2 subunit. However, in earlier experiments performed in our lab, it has been shown that α2 antisense deletion in visual cortex tissue cultures accelerates the decay kinetics, indicating that α2 contributes to $GABA_A$ receptors involved in slow decaying IPSCs (Brussaard *et al.* 1997). In acutely dissected tissue, the α5 subunit is present at low levels at pn6 (13 % of all α subunit expression), but virtually absent from mature neurons (data not shown). In cultured cortical neurons, α5 plays a larger role than in acutely sliced tissue (Dunning *et al.* 1999; Hutcheon *et al.* 2000). Unfortunately, we had no good ligand to test for α5 contribution. Since its modest role, however, this does not seem to be a major problem. The α6 subunit is not expressed in the visual cortex (Wisden *et al.* 1992).

Specificity of the pharmacology used

The specificity of the $GABA_A$ receptor α subunit-selective ligands currently available is under dispute. In particular, the specificity of bretazenil for α3 has been doubted (Ebert *et al.* 1997; Smith *et al.* 2001). While bretazenil has been reported to elicit the greatest enhancement at α3-containing receptors (Puia *et al.* 1992), this study did not take into account the lower GABA affinity of the $\alpha 3\beta\gamma 2$ receptor subtype relative to the other $GABA_A$ receptor subtypes used in these experiments. Consequently it would not be surprising to observe greater enhancement at the same concentration of GABA. Other studies on heterologous expression systems, for instance Smith *et al.* (2001), demonstrated a lack of subtype selectivity for bretazenil. However, here we show that bretazenil affected sIPSCs at pn6, but not at pn21. Since α3 is the only α subunit that is highly expressed at pn6 but hardly at pn21 (Laurie *et al.* 1992), we propose that, at least in the postsynaptic receptors of the visual cortex, bretazenil is exhibiting significant selectivity for α3-containing $GABA_A$ receptors.

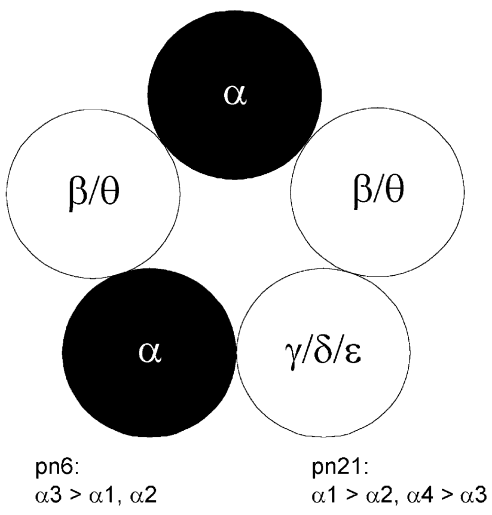

Figure 7. Putative subunit composition of $GABA_A$ receptors before and after neonatal development of the visual cortex

In immature synapses, the most abundant α subunit is α3, although α1 also occurs. In mature synapses, α1 is the dominant α subunit, but α4 also occurs. In addition, low levels of α3 may also be present.

Zolpidem does not potentiate δ subunit-containing $GABA_A$ receptors (Hevers & Lüddens, 1998). Mature, but not immature, neurons of the visual cortex have relatively high levels of δ subunit expression (Laurie *et al.* 1992; Pirker *et al.* 2000). This could make some $GABA_A$ receptors zolpidem insensitive, even when they have an α1 subunit. The described developmental upregulation of α1 may therefore be underestimated, although in the cerebellum, the δ subunit is exclusively located outside the synapse (Nusser *et al.* 1998).

$GABA_A$ receptor plasticity during neonatal development

Earlier studies have shown that the expression patterns of $GABA_A$ receptor subunits are spatially (Wisden *et al.* 1992; Pirker *et al.* 2000) and developmentally (Laurie *et al.* 1992; Fritschy *et al.* 1994) restricted. Most of these studies used mRNA experiments. In the present study we validate, at least for layers II–III of the visual cortex, these expression studies by showing that the described changes in transcription are reflected in plasticity of the functional, postsynaptic receptor proteins. Our results are further in agreement with those of Dunning *et al.* (1999) and Hutcheon *et al.* (2000), who showed that α1 and α4 are upregulated during *in vitro* development of dissociated embryonic cortical cells, whereas α3 is downregulated.

We have used randomly selected neurons in order to get an overall impression of the α subunit contribution of all neurons in layers II–III of the rat visual cortex, instead of only in a subset of neurons. Approximately 66 % of the neurons in layers II–IV are pyramidal cells (Winfield *et al.* 1980). The sIPSCs recorded in these neurons reflect synaptic input from all types of interneurons. In addition, interneuron–interneuron connections have been included in this study.

Kinetic contributions of the $GABA_A$ receptor α subunits

Our data support the general concept that, amongst other factors, α subunit expression is causally related to decay kinetics of sIPSCs (Hevers & Lüddens, 1998). This is in line with data obtained either in recombinant expression studies (in *Xenopus* oocytes or HEK cells) or by single-cell PCR of one or few α subunits (Dunning *et al.* 1999; Okada *et al.* 2000). It has been demonstrated that recombinant $GABA_A$ receptors containing the α2 (Lavoie *et al.* 1997) or α3 (Verdoorn, 1994) subunits decay more slowly than α1-containing $GABA_A$ receptors (see also Hevers & Lüddens, 1998). This has been confirmed by studies on an α1 knockout mouse line, generated independently from the one we used, for the cerebellum (Vicini *et al.* 2001) and by α2 antisense deletion in organotypical slice cultures of the visual cortex (Brussaard *et al.* 1997). Furthermore, it is in line with kinetic changes in neurons of the hypothalamus that display an endogenous α1 to α2 switch (Brussaard *et al.* 1997). The advantage of our present study on neonatal development is that we were able to study the kinetic properties of three abundant α subunits in their native settings, without having to consider the compensatory regulation of *in vitro* culture conditions or gene knockout mice. We have shown that the different α subunits are indeed associated with different kinetics, but that there is quite a large, heterogeneous range of action, especially for α3.

Finally, we have shown that deletion of the α1 subunit, either by generating a knockout mouse or by applying antisense oligonucleotides in an organotypical rat slice culture, yields very similar results when compared to our pharmacological analysis using 100 nM zolpidem in normal rat tissue. The differences between the decay properties of α1 knockout mice and antisense-treated cultures can be explained in two ways. At first, there are also differences in the controls (Fig. 6*D* and *H*). These can be caused by differences in expression of other α subunits, but also by other factors, like phosphorylation (Jones & Westbrook, 1997). Secondly, long term compensatory mechanisms in the α1 −/− mice may occur. In the antisense-treated cultures, a marked reduction of the peak currents was observed (Fig. 6*E*), whereas there were no differences in peak currents between α1 +/+ and α1 −/− mice (Fig. 6*A*). This may indicate that in the knockout mice, but not (or less) in the antisense-treated cultures, compensatory upregulation of other α subunits occurred.

Coexistence of different α subunits in specific neurons

All immature neurons, at pn6, show clear bretazenil-sensitivity. This implies that all these neurons probably express substantial amounts of postsynaptic α3 subunits. Therefore, the other α subunits present at that age must co-occur in α3-expressing neurons. The same holds true for zolpidem sensitivity in pn21 neurons. Since zolpidem affected virtually all pn21 neurons, we postulate that α1 may occur together with α2 and α4 in these neurons. This implies, in our view, that different α subunits are co-expressed in individual neurons, although one cannot exclude that a minor fraction of the neurons express only one α subunit isomer.

Differences in kinetic properties of sIPSCs mediated by the same α subunit between different synapses can be explained in four, not mutually exclusive, ways. The first explanation is that there is more than one type of α subunit per synapse, either in the same $GABA_A$ receptor (Verdoorn, 1994; Araujo *et al.* 1996) or in different receptors. This could imply that synapses with only α3 mediate slow-decaying sIPSCs, whereas synapses combining α3 and α1 mediate relatively fast-decaying synaptic currents.

Also the other, β and γ/δ, subunits can influence the kinetic properties of $GABA_A$ receptors. It has been described that δ subunit-containing $GABA_A$ receptors have faster kinetics than γ2-containing ones (Haas & Macdonald, 1999). Developmental upregulation of δ mRNA in the visual cortex (Laurie *et al.* 1992) may therefore also contribute to faster sIPSCs after neonatal development, although the possibility of synaptic localization of the δ subunit has been rejected in other brain areas (Nusser *et al.* 1998). Furthermore, post-translational modification, for instance by (de)phosphorylation, is known to contribute to alterations in the kinetics in some cell types (see for instance Jones & Westbrook, 1997; McDonald *et al.* 1998). However, since the α subunits display much larger changes during development than the other subunits, we argue that the changes in non-α subunits have less impact than changes in the expression of α subunits during development. Finally, also variations in the kinetics of GABA in the synaptic cleft may affect $GABA_A$ receptor kinetics (Draguhn & Heinemann, 1996; Nusser *et al.* 2001).

Conclusions

Developmental changes in α subunit expression occur in order to mediate faster decaying GABAergic IPSCs. Both at juvenile and mature stages, a predominant contribution of particular α subunits can be observed, with the α3 subunit being the most abundant juvenile α subunit and the α1 the dominant mature α subunit. The presence of other α subunits, mainly α1 at immature synapses and α4 at mature synapses, was confirmed. In addition, co-existence of more than one type of α subunit within single neurons is evident. Each individual α subunit generates its own kinetic receptor profile. However, combination with other α subunits may lead to intermediate receptor kinetics, thereby fine tuning the effect of transcriptional subunit switching during neonatal development.

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