Early postnatal switch in GABA$_A$ receptor $\alpha$-subunits in the reticular thalamic nucleus

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Pangratz-Fuehrer S, Sieghart W, Rudolph U, Parada I, Huguenard JR. Early postnatal switch in GABA$_A$ receptor $\alpha$-subunits in the reticular thalamic nucleus. J Neurophysiol 115: 1183–1195, 2016. First published December 2, 2015; doi:10.1152/jn.00905.2015.—The GABAergic neurons of the thalamic reticular nucleus (nRt) provide the primary source of inhibition within the thalamus. Using physiology, pharmacology, and immunohistochemistry in mice, we characterized postsynaptic developmental changes in these inhibitory projection neurons. First, at postnatal days 3–5 (P3-5), inhibitory postsynaptic currents (IPSCs) decayed very slowly, followed by a biphasic injection neurons. First, at postnatal $\alpha_1$-subunit (early) subunit expression in nRt. Zolpidem, selective for $\alpha_1$- and $\alpha_3$-containing GABA$_A$R, augmented only mature IPSCs, whereas clonazepam enhanced IPSCs at all stages. This effect was blocked by the $\alpha_5$-specific inverse agonist L-655,708, but only in immature neurons. In $\alpha_3$(H126R) mice, in which $\alpha_3$-subunits were mutated to become benzodiazepine insensitive, IPSCs were enhanced compared with those in wild-type animals in early development. Third, tonic GABA$_A$R activation in nRt is age dependent and more prominent in immature neurons, which correlates with early expression of $\alpha_3$-containing GABA$_A$R. Thus neonatal nRt neurons show relatively high expression of $\alpha_3$-subunits, which contributes to both slow synaptic and tonic extrasynaptic inhibition. The postnatal switch in GABA$_A$R subunits from $\alpha_3$ to $\alpha_1$ could facilitate spontaneous network activity in nRt that occurs at this developmental time point and which is proposed to play a role in early circuit development.

GABA$_A$R $\alpha_3$; subunit turnover; IPSCs; tonic current; mouse

THE $\gamma$-AMINOBUTYRIC ACID (GABA)-ergic signaling system undergoes significant changes during development. Altered gating kinetics and allosteric modulation lead to functional and pharmacological differences in these receptors (Cherubini and Conti 2001; Macdonald and Olsen 1994; McKernan and Whitting 1996; Mody and Pearce 2004). The developmental switch in the $\alpha$-subunit expression has been associated with a shift in kinetics of GABA-mediated inhibitory postsynaptic potentials, which become faster in several brain regions within the first 2 postnatal weeks (Hollrigel and Soltesz 1997; Okada et al. 2000; Vicini et al. 2001). This phenomenon of slow GABAergic transmission during early development may be required to ensure synaptic activation in times of sparse synaptic connectivity (Dunning et al. 1999). On the other hand, studies in the developing hippocampus have shown that GABA-mediated excitation could drive epileptiform activity (Dzhala and Staley 2003; Khalilov et al. 2003). Studies in humans and animal models suggest that mutations or alterations in GABA$_A$ receptor (GABA$_A$R) subunits might be linked to epilepsy in children (Brooks-Kayal 2005; Brooks-Kayal et al. 1998; Harvey et al. 1997; Swann 2004; Zhang et al. 2004). For example, a hyperthermia model of perinatal seizures is associated with persistent low expression of $\alpha_1$-subunit and a smaller amplitude of GABAergic currents (Cossette et al. 2002). A failure in the developmental subunit switch might lead to persistent changes in circuitry that could be pathological, such as in generalized childhood absence epilepsy (AE), which has a strong dependence on proper GABA$_A$R function (Huguenard and McCormick 2007). Notably, several mutations of GABA$_A$Rs have been associated with AE (Kang and Macdonald 2004; Maljevic et al. 2006; Marini et al. 2003; Tan et al. 2007; Wallace et al. 2001). Absence seizures appear to derive from the intrathalamic network responsible for generation of sleep spindle oscillations (Huguenard and Prince 1994; von Krosigk et al. 1993), although other network substrates are proposed (Leresche et al. 2012; Pinault and O’Brien 2005). In particular, the GABAergic thalamic reticular nucleus (nRt) plays a central role in regulating experimental AE by controlling this form of spindle activity (Avanzini et al. 1993; Huntsman et al. 1999; Sohal and Huguenard 2003). It has been shown that mutant mice lacking functional inhibition within the nRt display hypersynchronous thalamic epileptiform activity and absence-related seizures (DeLorey et al. 1998; Huntsman et al. 1999). In our previous work, we found indications for nRt-specific location of endogenous benzodiazepine (BZ)-binding site ligands (endozepines) (Christian et al. 2013). Synaptic inhibitory responses in nRt are functionally mature at postnatal day 12 (P12) (Huntsman and Huguenard 2000), a stage when thalamic spindle generation is already robust (Jacobsen et al. 2001). However, in neonatal mice younger than P12, a unique pattern of spontaneous giant inhibitory network responses has been observed (Pangratz-Fuehrer et al. 2007). These early network responses, proposed to play a role in circuit formation, depend in part on depolarizing GABA responses but also may rely on the particular GABA$_A$R composition. Thus GABA$_A$R within nRt may impact absence seizure susceptibility in the developing brain. Although the specific developmental sequence of
GABA<sub>R</sub> expression in humans is not well understood, such changes may explain in part the age-dependent incidence of AE in children (Caraballo and Dalía 2013). In the present study, we used immunohistochemistry, voltage-clamp recordings, and α-subunit-specific pharmacology in wild-type (WT) and mutant mice carrying a BZ-insensitive GABA<sub>R</sub> α<sub隼</sub>-subunit to assess the functional properties of inhibition in the nRT in the period before the spindle-generating circuitry is mature.

**MATERIALS AND METHODS**

*Animals.* Wild-type and mutant mice (α<sub>隼</sub>H126R) pups of either sex were used at P3-20. Mutants were mice homozygous for a histidine-to-arginine point mutation at position 126 of the GABA<sub>R</sub> α<sub隼</sub>-subunit (5–6 backcrosses to the 129/SvJ background) that were generated as described previously (Low et al. 2000). Some confirmatory experiments utilized P5-10 Sprague-Dawley rat pups (Simonsen Laboratories, Gilroy, CA). Experiments were performed in accordance with approved procedures (Protocol 12321/0) established by the Administrative Panel on Laboratory Animal Care at Stanford University. Mice were deeply anesthetized by intraperitoneal injection with pentobarbital (50 mg/kg) until unresponsive and were then decapitated. Brains were blocked, removed, and placed in ice-cold (4°C) oxygen-equilibrated (95% O<sub>2</sub>-5% CO<sub>2</sub>) “cutting” solution containing 126 NaCl, 26 NaHCO<sub>3</sub>, 10 glucose, 2.5 KCl, 2 MgCl<sub>2</sub>·6H<sub>2</sub>O, 2 CaCl<sub>2</sub>·2H<sub>2</sub>O, and 1.25 NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O for 1 h before recording.

*Electrophysiology.* Whole cell patch-clamp recordings were obtained from nRT neurons in brain slices maintained in a chamber with a constant flow of aCSF perfusion (2 ml/min) equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Experiments were conducted at room temperature. Glass electrodes (tip resistance 2.5–3.3 MΩ; KG-33, borosilicate glass; Garner Glass, Claremont, CA) were pulled in multiple stages using a Flaming-Brown micropipette puller (model P-87; Sutter Instruments, Novato, CA) and filled with high-chloride solution, which contained (in mM) 135 CsCl, 5 lidocaine -ethyl bromide (QX-314), 2 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>·6H<sub>2</sub>O, and 1.25 NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O for 1 h before recording.

*Data collection and analysis.* Data were filtered at 2 kHz, collected, and sorted with locally written software [Metatope, WDetecta, and WinScanSelect (J. R. Huguenard)] and then analyzed using pCLAMP 9 (Axon Instruments, Union City, CA) and Origin (MicroCal Software, Northampton, MA). The following properties of averaged IPSCs (n > 50 events/cell) were determined: rise times (the time required to rise from 10 to 90% of peak amplitude), 90% widths (the width at 10% maximum amplitude), half widths, peak current amplitude, frequency, and the weighted decay time constant (τ<sub>slow</sub>), which was calculated using the following equation: τ<sub>slow</sub> = ([τ<sub>fast</sub>·τ<sub>slow</sub>]/[τ<sub>fast</sub> + τ<sub>slow</sub>]).

The analysis for tonic current was performed as reported previously (Sipila et al. 2007). Briefly, we calculated an all-points histogram of the recorded voltage-clamp current (>30 s) for each experimental condition either immediately before drug application (control) or at the end of the period of application at which time drug equilibrium was achieved. Gaussian fits were obtained for the histograms, and these were skewed, with IPSCs contributing to a “negative tail” with currents more inward than those occurring between sIPSCs. To minimize the contribution of IPSCs to the fitted Gaussian curves, bins on the negative side of the peak with bins filled to less than half of the peak amplitude were excluded from the analysis (Sipila et al. 2007). In addition, in a separate analysis on a subset of cells, we confirmed that analysis of baseline noise, as measured during IPSC-free periods of recording (Porcello et al. 2003), provided qualitatively similar results, showing increases in baseline noise during GABA application and decreases in the presence of GBZ. All data are means ± SD. Student’s t-test was used to assess statistical significance for drug effects, and one-way ANOVA followed by Tukey’s post hoc test was used for comparisons between group means unless otherwise stated. Differences were regarded significant if P < 0.05. Higher levels of significance are indicated as P < 0.01 or P < 0.001.

**Immunocytohistochemistry.** Animals younger than P5 were deeply anesthetized by intraperitoneal injection with pentobarbital sodium (50 mg/kg) until unresponsive and were then decapitated, whereas older mice were anesthetized and then perfused through the ascending aorta with 0.1 M phosphate-buffered saline (PBS; pH 7.4). Brains were removed and placed in 4% paraformaldehyde in 0.1 M PBS for 1 h. Finally, the sections were treated with 0.4 mM 3,3-diaminobenzidine (DAB) and then mounted on Aclar. The sections were blocked in 10% normal goat serum and then incubated with the primary antibodies (1:1000 dilution) against the postsynaptic marker gephyrin (Sigma) and 0.01% H<sub>2</sub>O<sub>2</sub> for 1 h. The sections were rinsed in PBS and mounted on Aclar. The sections were blocked in 10% normal goat serum and then incubated with the primary antibodies (1:1000 dilution) against the postsynaptic marker gephyrin (Sigma) and 0.01% H<sub>2</sub>O<sub>2</sub> for 1 h. The sections were rinsed in PBS and mounted on Aclar.

**Immunofluorescence staining.** Sections were reacted with 0.5% H<sub>2</sub>O<sub>2</sub> for 30 min to inactivate endogenous peroxidase and then treated with 50% alcohol for 10 min. After being rinsed in PBS, free-floating sections were blocked in 10% normal goat serum and then incubated with primary antibodies diluted in PBS and 0.4% Triton X-100 at 4°C. On the following day, sections were rinsed in PBS and incubated in biotinylated secondary antibodies diluted in goat anti-rabbit serum (Vectorstain Elite ABC kit; catalog no. PK 6101; Vector Laboratories, Burlingame, CA) for 1 h. After being rinsed in PBS, the sections were incubated with AB reagents from the same ABC kit for 1 h. Finally, the sections were treated with 0.4% 3,3′-diaminobenzidine (Sigma) and 0.01% H<sub>2</sub>O<sub>2</sub> for 10 min and then mounted on slides and coveredslipped with DPX mounting medium (catalog no. 3176-1-6; Aldrich) for microscopy.

**Immunofluorescence staining.** We assessed the distribution of GABA<sub>R</sub> α<sub隼</sub>-and α<sub隼</sub>-subunits and the postsynaptic marker gephyrin in mice from P4-30. For double immunofluorescence of GABA<sub>R</sub> subunit antibodies with single-cell morphology, biocytin (0.05%; Sigma) was included in the internal pipette solution to fill neurons during electrophysiological recordings. Horizontal slices were resectioned at 35 μm with a freezing microtome, mounted on slides, and processed using the water bath antigen-retrieval method (Jiao et al. 2004).
RESULTS

Slow IPSCs dominate early GABAergic transmission in nRt. We recorded isolated sIPSCs from nRt neurons in mouse thalamic slices at P13-16 in voltage-clamp mode at −60 mV and in the presence of APV (100 μM) and DNQX (20 μM) to block ionotropic glutamate receptors. Analysis of sIPSC properties revealed significant developmental changes. On the basis of distinct age-dependent sIPSC characteristics, we organized cells into the following four age groups: P3-5, P6-8, P9-11, and P12-15. At P3-5, we recorded spontaneous activity from 61 of 74 cells (82.4% of all neurons); the remaining 13 cells (17.6%) were silent. In four of these silent cells, brief trains of electrical stimulation within nRt triggered subsequent barrages of spontaneous IPSC activity, suggesting that absence of sIPSCs in some cells was related to low activity levels rather than lack of postsynaptic receptors. Example traces recorded at four different developmental stages show that at P5 (Fig. 1A), sIPSCs occurred at low frequencies with significantly slower decay times compared with all other age groups (mean τ_{dw} at P5: 156 ± 8 ms). In contrast, sIPSCs at P6-8 (Fig. 1B) decayed significantly faster (mean τ_{dw} at P6-8: 95.4 ± 5.5 ms, n = 13; P < 0.001). Surprisingly, at P9-11, sIPSC decay rates became slower again (Fig. 1C). This temporary slowing in τ_{dw} resulted in >30% increase of the weighted decay time constant compared with P6-8 (mean τ_{dw} at P9-11: 126.1 ± 4.8 ms, n = 25; P < 0.01; Fig. 1F). At P12-16 (Fig. 1D), decay times were significantly faster compared with P3-5 and P9-11 and similar to durations in P17 mice or older (mean τ_{dw} at P12-16: 83.4 ± 9.1 ms, n = 13, and at P17-20: 76.8 ± 4.1 ms, n = 8; P < 0.001; Fig. 1D). We next analyzed the distribution of τ_{dw} for each age group (Fig. 1E). Decay constants were calculated from a minimum of 110 well-resolved individual sIPSCs recorded from 5–6 cells per age group, and the histogram was fitted with the best single Gaussian distribution. Data are plotted on a log ordinate with square root abscissa to facilitate display of distributions with both brief and long-lasting events (Sigworth and Sine 1987). Figure 1E illustrates that although decay constants were generally overlapping in their distributions at all age groups, the relative contribution of slow events, especially those outside of the Gaussian distribution, was greatest at P3-5 and P9-11. The peaks in each distribution show a clear progressive leftward shift with each stage of development, with the exception of P9-11, where there appears to be a pause, or even a transient reversal of the trend. Note that at the three earliest developmental stages, the IPSC τ_{dw} distributions...
were poorly fit by single Gaussian distributions, with several bins in each case at the large end of the distribution (>100 ms) outside of the fitted Gaussian curve. Thus the peak for these curves is left-shifted (smaller) compared with the overall mean. The developmental pattern of change in sIPSC decay time follows a bimodal function with two distinct peaks at P3-5 and P9-11 (Fig. 1F). nRt neurons at these young ages also displayed developmentally dependent differences in peak current amplitude, rise time, and frequency (Fig. 1, G–I). Interestingly, the maturation pattern for these parameters followed a bireciprocal time course similar to that already shown for the decay time constant. For example, sIPSC peak current amplitudes were significantly larger between P6-8 (42.8 ± 0.4 pA; P < 0.01) and P9-11 (43.2 ± 0.3 pA; P < 0.001) compared with recordings from cells at P3-5 (40.8 ± 0.3 pA) and P12-16 (40.9 ± 0.3 pA). Furthermore, sIPSC rise times, calculated as the time required to rise from 10 to 90% of peak current amplitude, were significantly slower at P6-8 (2.12 ± 0.02 ms; P < 0.001) compared with P3-5, when they were fastest (1.52 ± 0.01 ms; P < 0.001), and with P9-11 (1.75 ± 0.01 ms) and P12-16 (1.72 ± 0.01 ms). Finally, although sIPSCs occurred at very low frequencies in neonatal neurons (P3-5: 0.89 ± 0.02 Hz; P < 0.001), there was a substantial increase (over 100%) in frequency within 2 days (P6-8: 2.23 ± 0.02 Hz). These rates were sustained until P9-11 (2.17 ± 0.02 Hz), when we observed a significant decrease (P12-16: 1.9 ± 0.02 Hz; P < 0.001) again (n = 15 cells per age group).

In summary, although sIPSC kinetic properties progressively became more adultlike during the developmental time period that we studied, there appears to be a short period at the beginning of the second postnatal week when inhibitory events were longer in duration, had larger amplitudes and slower rise times, and occurred at higher frequencies.

Developmental changes in activity dependence of sIPSCs in nRt. Several mechanisms may account for these developmental changes in sIPSC kinetic properties, including pre- and postsynaptic changes (see Discussion). One measure of presynaptic alterations is provided by TTX sensitivity, which can be used to probe the contribution of action potential-mediated synaptic responses to overall spontaneous events that also include action potential-independent synaptic response [miniature IPSCs (mIPSCs, or minis)]. Figure 2 shows the effect of TTX (1 μM) on sIPSCs at distinct developmental stages. An example of a recording from a P9 nRt cell (Fig. 2A) demonstrates that TTX blocked large-amplitude (>50 pA) sIPSCs (Fig. 2B). Thus the cumulative probability for sIPSCs was shifted to the left, especially for the upper quartile (Fig. 2C), suggesting that the majority of activity-dependent events were larger than ~50 pA. The sensitivity to TTX was correlated with the mean sIPSC peak amplitude. In Fig. 2D, the solid line represents the results of linear regression (R = 0.85, P < 0.001) between the TTX-induced reduction in sIPSC amplitude and control (i.e., pre-TTX) amplitude. Furthermore, TTX sensitivity is also associated with age, because only sIPSCs at P9-11 were found to be affected by TTX (mean reduction in amplitude = 32.5 ± 4.4% and in that frequency = 30.7 ± 5.6%; P < 0.05; Fig. 2E). It is important to note that sIPSCs at this developmental stage had significantly larger peak current amplitudes, as described in Fig. 1G. The lack of TTX efficacy in other age groups suggests that at these developmental stages, the vast majority of sIPSCs are minis, i.e., those independent on action potential generation and therefore generated through spontaneous vesicle release events.

Fig. 2. Large sIPSCs at P9-11 are tetrodotoxin (TTX) sensitive. A: sample trace obtained at P9 shows reduction in amplitude and frequency of sIPSCs in presence of TTX (1 μM); B: scatter plot of the same neuron illustrates sensitivity of large events to TTX blockade. Each point is the average amplitude of all sIPSCs within a given 3-s epoch. In the same cell, in C, the cumulative distribution of IPSC amplitudes demonstrates a reduction in the large-amplitude events after exposure to TTX, without changes in contribution of smaller (<20 pA) events. Pooled data in D demonstrate that the degree of TTX blockade is dependent on mean sIPSC amplitude. The solid line indicates the results of linear regression (R = 0.85, P < 0.001) between TTX effect and peak amplitude, suggesting that mainly currents >50 pA are blocked. Note that the largest TTX-dependent reduction in sIPSC amplitude occurred in two P9-11 neurons (filled circles) in which control (CTR) sIPSC amplitude was >100 pA. Red arrow marks the cell displayed in A, B, and C. E: age dependence of TTX sensitivity with significant reductions in amplitude (32.5 ± 4.4%; P < 0.05) and frequency (30.7 ± 5.6%; P < 0.05) only at P9-11. Values are means ± SE; for P3-5: n = 7; P6-8: n = 9; P9-11: n = 7; P12-15: n = 5. *P < 0.05; paired Student’s t-test.
Developmental postsynaptic changes in IPSCs in nRt cells. As described earlier, the kinetic properties of IPSCs are strongly influenced by the α-subunit isoform in the underlying GABA_A Rs. Therefore, we hypothesized that if these changes in IPSC morphology were caused by a developmental change of the α-subunit, this process should be reflected by altered sensitivity to α-subunit-specific benzodiazepine (BZ) modulators. In our first set of experiments, we tested for the contribution of the α_3-subunit in immature nRt cells by using mice with an α_3(H126R) point mutation (Low et al. 2000) that selectively renders GABA_A Rs containing the α_3-subunit insensitive to BZs but with otherwise normal function.

Clonazepam strongly enhances immature sIPSCs. In these experiments, we recorded sIPSCs from immature nRt neurons in α_3(H126R) mice and their WT controls under control conditions and in the presence of CZP (100 nM). If BZ site-sensitive α-subunits other than α_3 contribute to synaptic GABA_A family function, the application of CZP would be expected to enhance sIPSCs in nRt cells from α_3 mutant mice. However, if the response to CZP in the WT is mediated mainly by α_3-containing GABA_A Rs, a reduced enhancement of sIPSCs in the α_3 mutant mice compared with WT controls would be anticipated. Based on a previous study that demonstrated transient perinatal expression of α_3 nRt (Studer et al. 2006) and our observation of slower sIPSC kinetics in neonatal nRt cells, we used an α_3-specific site ligand to test for the presence of GABA_A Rs containing the α_3-subunit (Pearce 1993; Zarnowska et al. 2009).

We performed sequential recordings, first in control conditions, then in the presence of CZP, and finally during coapplication of CZP with the α_3-subunit-selective inverse agonist L-655,708 (L-655; 15 nM), which antagonizes BZ effects. Figure 3 shows examples of the CZP-induced slowing of sIPSCs and the partial reversibility of this effect during L-655 application in immature nRt neurons, but not in cells older than P10. At P5, CZP prolonged sIPSCs not only in WT but also in α_3(H126R) neurons (Fig. 3, A and B). In contrast, recordings at P15 (Fig. 3, C and D) showed reduced (WT) or abolished [α_3(H126R)] sensitivity to CZP. The CZP-induced effect on decay time could be partially reversed by coapplication of L-655 in both genotypes at P5, but not at P15 (Fig. 3, A–D). These drug-induced changes in τ_d and amplitude are presented in multiple comparison plots in Fig. 3, E1 and F1, respectively. They demonstrate the effect on individual nRt cells in WT and α_3 mutant mice for two developmental stages (each set of 3 symbols connected by 2 lines represents an individual cell). Figure 3, E2 and F2, shows the normalized population data for both age groups and genotypes: the effect of CZP on τ_d (Fig. 3E2) was significantly stronger in P4-8 neurons of WT compared with α_3 mutant mice [mean increase in WT τ_d: 84.5 ± 21.8%, n = 6; P < 0.01; and in α_3(H126R): 38.4 ± 6.2%, n = 14; P < 0.01]. For P11-16, CZP elicited a significant increase in τ_d in WT but not in α_3 mutant mice [WT: 35.3 ± 4.5%, n = 11; P < 0.01; and α_3(H126R): 6.8 ± 2.2%, n = 10]. Coapplication of L-655 with CZP reversed the increase in decay time in P4-8 animals; however, this reversal was only statistically significant in α_3 mutant mice [mean effect on τ_d in presence of CZP and L-655 compared with control values in WT: 49.3 ± 14.6%, n = 4; and in α_3(H126R): 21.5 ± 5.9%, n = 6; P < 0.05]. At P11-16, coapplication of L-655 with CZP was without any effect in either genotype. We also observed significant effects of CZP on IPSC amplitudes (Fig. 3F2). In general, there were more pronounced increases in the amplitude of sIPSCs at P4-8 [mean increase in WT: 30.7 ± 8.1%, n = 6; P < 0.01; and in α_3(H126R): 18.5 ± 4.0%, n = 14; P < 0.01] than at P11-16 [mean increase in WT 12.8 ± 3.6%, n = 11; P < 0.05; and in α_3(H126R): 4.3 ± 3.5%, n = 10]. Figure 3F2 also shows that L-655 had a significantly greater effect on the amplitude of α_3 mutant sIPSCs compared with WT [mean effect on amplitude in presence of CZP and L-655 compared with control values in WT: 19.6 ± 4.3%, n = 4; P < 0.05; and in α_3(H126R): −11.8 ± 3.1%, n = 6; P < 0.05]. At P4-8, coapplication of L-655 produced a larger reduction in sIPSC amplitude compared with the effect on decay time, but this effect was not observed in older mice. In conclusion, we found that CZP potentiates α_3 mutant sIPSCs at P4-8 but not at P11-16, indicating the presence of an α-subunit distinct from α_3. In addition, the CZP-induced increase was significantly stronger in WT compared with α_3(H126R) mice, which suggests that α_3-subunits are already expressed, i.e., the expression of both GABA_A R α-subtypes overlaps. Finally, L-655 had a noticeable effect only on immature IPSCs. This suggests that GABA_A Rs contain the α_3-subunit only in developing nRt neurons.

α_3(H126R) GABA_A Rs are insensitive to zolpidem. Previous studies demonstrated that CZP potentiates not only GABA_A Rs containing α_3- and α_5-subunits but also those containing α_1- or α_2-subunits (Dunning et al. 1999; Macdonald and Olsen 1994; Rudolph and Mohler 2004; Pritchett and Seeburg 1990). Because CZP dramatically enhanced GABA_A Rs in immature nRt cells, we wanted to determine whether immature GABA_A Rs also express the α_1-isofrom. The imidazopyridine zolpidem (ZLP) is a BZ ligand with high selectivity for receptors containing the α_1-subunit and moderate affinity to α_2- or α_5-subunit, but with no sensitivity to α_3-containing GABA_A Rs (Maric et al. 1999). The rationale behind this approach is that if ZLP significantly enhanced sIPSCs at P4-8, the underlying GABA_A combination should contain the α_1-subunit, or potentially the α_2- or α_5-subunit. However, if sIPSC properties remained unchanged under ZLP application, then the presence of GABA_A Rs containing α-subunits that are insensitive to ZLP, such as the α_3-subunit, would be indicated. Figure 4 shows recordings of two age groups of nRt cells from WT and α_3(H126R) slices in control conditions and in the presence of ZLP. For recordings from P5 neurons, ZLP showed only a minor, nonsignificant effect on sIPSC from WT (Fig. 4A) and had no noticeable effect in α_3 mutant mice (Fig. 4B). In contrast, ZLP increased averaged sIPSCs at P15 in neurons of WT (Fig. 4C) but not α_3(H126R) mice (Fig. 4D). The effects of ZLP on sIPSC τ_d and amplitude from individual recordings in WT and α_3 mutant mice are illustrated in Fig. 4, E1 and F1, respectively. Finally, Fig. 4, E2 and F2, summarizes how ZLP sensitivity changed during development. We found that at P4-8, sIPSC τ_d was not significantly affected in WT (6.5 ± 4.2%, n = 4). ZLP sensitivity increased during development in WT GABA_A Rs such that at P11-16, ZLP increased sIPSC τ_d by 35.3 ± 3.2% (n = 6; P < 0.01). In contrast, there was no developmental change in ZLP sensitivity in mice carrying the BZ-insensitive GABA_A R α_5-subunit; at all ages the sIPSCs remained ZLP insensitive. These results further support the conclusion that GABA_A Rs in immature (P4-8) neurons express α_3-subunits.

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Early expression of \( \alpha_5 \) GABA\(_A\)R subtype. On the basis of our previous observations, we suggest that differences in the kinetic properties of inhibitory synaptic currents result from changes in the underlying molecular structure of the GABA\(_A\)R. We used immunohistochemistry to confirm that \( \alpha_5 \)-subunits are transiently expressed in immature nRt cells and to determine whether there might be a developmental period in which they are coexpressed with the \( \alpha_3 \)-subunit. Figure 5 shows the distribution of GABA\(_A\)R subtypes in horizontal sections of thalamic slices at P5 (left) and at P20 (right). These slices have been processed with subunit-specific antibodies for GABA\(_A\)R subunits \( \alpha_1 \) (Fig. 5A), \( \alpha_3 \) (Fig. 5B), and \( \alpha_5 \) (Fig. 5C). For \( \alpha_1 \)-subunit-containing GABA\(_A\)Rs, we did not observe antibody staining in nRt cells at P5 (Fig. 5A1) or P20 (Fig. 5A2). However, intense immunoreactivity (IR) could be detected in the globus pallidus and slightly less in caudate putamen at both ages. As also shown in Fig. 5A1, only weak labeling for \( \alpha_1 \)-subunit IR could be seen in the ventrobasal complex (VB) in slices at P5, in contrast to a robust expression at P20 (Fig. 5A2). Note that this delayed expression of \( \alpha_1 \) (preceded by \( \alpha_2 \)) IR in VB has been described in earlier studies (Peden et al. 2008). The \( \alpha_3 \)-subunit showed strong nRt staining at P5 and persistence at a somewhat lower level at P20 (Fig. 5B). We found prominent staining for \( \alpha_3 \) only during early development (Fig. 5C1). Staining for \( \alpha_3 \) IR was no longer present in nRt at P20 (Fig. 5C2). The above results highlight developmental changes in nRt GABA\(_A\)R subunit immunoreactivity that correspond to the functional changes in synaptic inhibition. Although it is possible that knockin of BZ insensitivity to the \( \alpha_3 \) GABA\(_A\)R subunit might result in a different developmental profile, the initial characterization of these mice indicated that overall GABA\(_A\) receptor expression was not disrupted (Low et al. 2000).

**Fig. 3.** Immature synaptic GABA\(_A\) receptors (GABA\(_A\)Rs) are highly benzodiazepine (BZ) sensitive and not strictly dependent on \( \alpha_3 \)-subunits. Properties of sIPSCs at different age groups in CTR, 100 nM clonazepam (CZP) and 100 nM CZP + 20 nM L-655,708 (L-655). A and B: averaged sIPSCs from P5 wild-type (WT) and \( \alpha_3 \)(H126R) mutant (\( \alpha_3 \)) mice, respectively, show strong CZP-dependent increase together with significant L-655-dependent reduction in sIPSC amplitude and decay time. C and D: CZP efficacy at P15 in WT but not \( \alpha_3 \)(H126R); L-655 was ineffective in both genotypes at this age. E and F: CZP-induced increase and partial L-655-dependent reduction of sIPSC \( \tau_{\text{wa}} \) (C1) and amplitude (F1) in individual WT and \( \alpha_3 \)(H126R) cells at P4-8 (filled circles) but not at P11-16 (open circles), when CZP efficacy was weaker in WT and absent in \( \alpha_3 \) neurons. E2 shows normalized responses indicating a decrease in CZP efficacy for WT and \( \alpha_3 \)(H126R) sIPSC \( \tau_{\text{wa}} \), from P4-8 to P11-16. CZP efficacy increased at both age groups in WT but only at P4-8 in \( \alpha_3 \)(H126R). F2 shows an average increase of 30.7% (n = 6) in WT amplitude at P4-8 and significantly less increase of 18.5% (n = 10) in \( \alpha_3 \)(H126R). At P4-8, WT sIPSC amplitude was decreased to less than control by L-655. There was a 12.8% CZP-dependent increase in WT amplitude at P11-16 but no effect in \( \alpha_3 \)(H126R) cells. No effect of L-655 on amplitude was found at this developmental stage. Statistical significance: *P < 0.05; **P < 0.01, CZP compared with control, +P < 0.05, ++P < 0.01, L-655 compared with CZP. \( \alpha_3 \) compared with WT at P4-8; Student’s t-test.
The α5-subunit-containing GABA<sub>A</sub>R has been identified at extrasynaptic locations in hippocampus (Caraiscos et al. 2004; Crestani et al. 2002). To differentiate between synaptic and extrasynaptic location of immature GABA<sub>A</sub>Rs, we used confocal microscopy for either α<sub>5</sub>- or α<sub>5</sub>-subunit IR double-stained with the scaffold protein gephyrin, which is implicated in the synaptic localization of GABA<sub>A</sub>Rs (Luschek and Keller 2004; Yu et al. 2007). α<sub>5</sub> IR and gephyrin costaining was quite rare at P5, whereas at P30 multiple clusters could be observed near presumed cell bodies (Fig. 6, A1 and A2, arrows). This finding indicates that the extent of synaptic α<sub>5</sub>-containing GABA<sub>A</sub>Rs increases during development. In contrast, colocalization of α<sub>5</sub> with gephyrin was sparse but present at P5, but largely absent at P30 (Fig. 6, B1 and B2). The small number of gephyrin clusters colocalized with either α<sub>5</sub>- or α<sub>5</sub>-subunit IR during the first postnatal week suggests that many GABA<sub>A</sub>Rs are not associated with synaptic locations at this stage.

### Tonic currents mediated by immature GABA<sub>A</sub>Rs have distinct pharmacology

Because varying combinations of GABA<sub>A</sub>Rs containing α<sub>5</sub> subtypes have been shown to mediate tonic currents (Mohler 2006; Semyanov et al. 2004), we tested whether tonic GABA<sub>A</sub>R activation mediated by α<sub>5</sub>-subunits plays a role in the nRt. Tonic GABA<sub>A</sub>R activation could be developmentally regulated similar to other brain regions (LoTurco et al. 1995; Nuñez and Mody 2002; Stell and Mody 2002), such as in immature hippocampal pyramidal neurons, where a significant tonic current can only be detected during early development (Demarque et al. 2002). Therefore, we tested the response of bath-applied GABA (5–10 μM) and the GABA<sub>A</sub>R antagonist gabazine (GBZ; 0.2 and 10 μM) on currents recorded at two stages of development, perinatal (P5) and mature (P24-30; Fig. 7). Figure 7A shows that 5 μM GABA induced an inward shift in the baseline holding current (I<sub>hold</sub>) at both ages, but with a much larger inward shift at P5 (top) compared with P24 (bottom). This GABA-induced shift in I<sub>hold</sub> could be reversed by subsequent GBZ application, and post-GBZ levels were more positive than baseline, especially in P5 cells, indicating that some tonic GABA<sub>A</sub> conductance was active even in the absence of exogenous GABA. The shifts in I<sub>hold</sub> were accompanied by changes in background noise (I<sub>SD</sub>), as illustrated in Fig. 7C. These differences in background noise are correlated with magnitude of active tonic GABA conductance and correspond in part to the modulation of stochastic channel openings resulting from the activation and inhibition of tonically active GABA<sub>A</sub>Rs by GABA and GBZ, respectively (Brickley et al. 1996; Kaneda et al. 1995; Wall and Usowicz 1997). Analysis of the cells from Fig. 7A revealed that bath-applied GABA caused an inward shift in I<sub>hold</sub> as well as an increase in background noise of currents at P5 (Fig. 7B, top), whereas it had less effect at P24 (Fig. 7B, bottom).

Coapplication of low-dose GBZ (0.2 μM) decreased the GABA-induced background noise at P5, but not at P20-26. We estimated I<sub>SD</sub> from Gaussian fits of all-point-histograms based on the current amplitude [see MATERIALS AND METHODS (Sipila et al. 2007)]. The all-points histograms for the two representative neurons from Fig. 7A are shown in Fig. 7B. For Fig. 7C, we calculated the mean I<sub>SD</sub> from Gaussian fits from all-points histograms under control, GABA, and low- and high-dose GBZ conditions. Results obtained by using I<sub>SD</sub> as an imperfect estimate of tonic GABA<sub>A</sub> receptor activation (it will be contaminated by other biological and instrument noise) suggest higher amplitudes of intrinsic GABA<sub>A</sub>R tonic conductances for the older compared with younger age groups (mean I<sub>SD</sub> in control at P5: 5.3 ± 0.6 pA and at P24-31: 6.5 ± 0.4 pA).

In addition, we found higher GABA sensitivities in immature neurons such that at P5, GABA increased I<sub>SD</sub> to 15.7 ± 1.7 pA.

### Fig. 4. Immature GABA<sub>A</sub>Rs are relatively insensitive to zolpidem (ZLP). Averaged sIPSCs of a representative P5 cell illustrate mild increase of decay time by ZLP (100 nM) in WT (A) but not α3(H126R) neurons (B). At P15, ZLP strongly slowed sIPSC decay in WT (C) but not in α3(H126R) (D). E: ZLP effects on sIPSC duration. In E1, individual cells from WT mice illustrate greater increases in τ<sub>rise</sub> at P9-15 (open circles) compared with P4-8 (filled circles). In contrast, ZLP did not have any effect in α3(H126R) mice at any age. In E2, at P4-8, ZLP failed to increase sIPSC τ<sub>rise</sub> in either WT (6.5 ± 4.2%, n = 4; not significant, NS) or α3(H126R) (1.3 ± 0.9%, n = 4, NS). In contrast, at P11-15, the mean increase in τ<sub>rise</sub> was 35.3 ± 3.2% in WT (n = 6; P < 0.01), whereas there was no effect in the α3 mutant (n = 5). The effect of ZLP on sIPSC τ<sub>rise</sub> was significantly different between WT and α3(H126R) at P11-15 (P < 0.01), but not in younger mice. F: ZLP effects on sIPSC amplitude. In F1, there was insignificant increase in sIPSC amplitude at all ages in WT, but not for mutant cells. In F2, there was no significant effect on sIPSC amplitude in any age group or genotype. Statistical significance: *P < 0.05; **P < 0.01, ZLP compared with control. †P < 0.05; ‡P < 0.01, WT compared with α3(H126R); Student’s t-test.
In contrast, there was significantly less enhancement in the older group (11 ± 0.8 pA). Interestingly, during coapplication with GBZ at low doses of 0.2 μM, the GABA-enhanced \( I_{SD} \) was significantly reversed at P5 (51 ± 1.8%) but had only a weak effect at P24 (26 ± 3.2%; Fig. 7D; mean \( I_{SD} \) after 0.2 μM GBZ at P5: 7.8 ± 1.5 pA; \( P < 0.05 \); and at P24-30: 8.2 ± 0.9 pA; \( P < 0.05 \)). Furthermore, high-dose GBZ (10 μM) application decreased \( I_{SD} \) to less than in control conditions and, as such, had a stronger effect in immature neurons (86 ± 1.4%) compared with the older age group (74 ± 3.6%; mean \( I_{SD} \) after 0.2 μM GBZ at P5: 2.1 ± 0.2 pA; \( P < 0.05 \); and at P24-30: 2.7 ± 0.2 pA, \( n = 6 \) per age group).

We also calculated the shift in \( I_{hold} \) for each condition (GABA, low- and high-dose GBZ; not shown). There was a significantly larger shift of the GABA-enhanced \( I_{hold} \) at P5, increasing \( I_{hold} \) from 60.2 ± 7.3 to 167.4 ± 20.0 pA in cells of the younger age group compared with an increase in more mature neurons from 13.9 ± 6.6 to 47.9 ± 12.0 pA (\( P < 0.001 \)). However, low concentrations of GBZ (0.2 μM) reduced the GABA-enhanced \( I_{hold} \) to 66.6 ± 17.0 pA at P5, which is close to the level seen under control conditions, and at P24-30, we measured 25.5 ± 6.4 pA (\( n = 6; P < 0.05 \), independent Student’s \( t \)-test). Our results suggest that tonic GABA \(_A\)R-mediated signaling occurs more robustly in immature and juvenile nRt neurons and that the degree of tonic GABA \(_A\)R activation (i.e., magnitude of tonic current) is age dependent, presumably because of differences in the underlying \( \alpha \)-subunit composition.

**DISCUSSION**

Previously, we described the presence of thalamic giant depolarizing potentials (tGDPS) in neonatal nRt neurons. These GABAergic events appear to play a role in circuit formation (Pangratz-Fuehrer et al. 2007). The present study examined how altered GABA \(_A\)R subunit compositions affect intrathalamic inhibition during early development. Briefly, we showed that 1) the GABA \(_A\)R \( \alpha_3 \)-subunit is transiently expressed in nRt cells during the first two postnatal weeks, 2) the decline of \( \alpha_5 \) expression is paralleled by a decrease of IPSC decay, 3) key electrophysiological properties of sIPSCs undergo a biphasic change; and 4) tonic GABA \(_A\)R-mediated conductance is stronger during the first postnatal week.

*Does bidirectional change in IPSC kinetics reflect transient \( \alpha_3/\alpha_5 \) coexpression?* One of the main findings of this study is the bimodal time course for IPSC decay time constants in nRt neurons between P3 and P18. Slowest IPSC decay times were observed at P3, becoming faster at P6-8. Surprisingly, at P9-11, we found a temporal slowing, followed by acceleration in cells older than P12. Multiple reasons could account for this bimodal time course. Given that the GABA \(_A\)Rs \( \alpha_5 \)-subunit is transiently expressed in neonatal nRt neurons, as shown here and previously (Poulter et al. 1992; Studer et al. 2006), differ-
ences in decay time would be expected as a result of decreased expression of α_5 and replacement with other α-subunits. We tested this hypothesis with double staining for α_3 and α_5 at several developmental stages. Indeed, at P5 we found strong expression of α_5 with less prominent expression of α_3, whereas this ratio had reversed at P11. Although there is precedent for progressive shortening of IPSC decay time during development due to subunit turnover (Dunning et al. 1999; Hollrigel and Soltesz 1997; Hutcheon et al. 2004; Okada et al. 2000; Vicini et al. 2001), this would not account for the transient slowing of sIPSCs at P9-11, which suggests another mechanism independent of GABAAR subunit changes. Recently, it has been shown that editing of RNA transcripts encoding the α_3-subunit undergoes developmental regulation (Rula et al. 2008). Editing activity peaked at P7; thus non-edited cells dominate during the first postnatal week. GABAARs containing non-edited α_3-subunits exhibit faster activation and slower deactivation times compared with edited receptors. The fact that we recorded sIPSCs with fastest rise and slowest decay times at P3-5 argues for a coexpression of non-edited α_3-subtype together with GABAAR containing α_5. Faster sIPSC kinetics at P6-8 might correspond to increased numbers of edited α_3 GABAAR accompanied by declining α_5-subunits. ZLP, which is selective for α_1 and α_3, but not α_5, had no effect on sIPSC duration of neonatal nRt neurons (Fig. 4). CZP had significantly stronger effects in immature WT compared with mutant mice, indicating that α_3 already contributes to functional inhibition at P3-5. The lack of significant ZLP effect at this stage, however, suggests that the contribution of α_3 is minor.

**Effect of TTX is age dependent.** A second key finding of this study was the apparent increase in activity-dependent inhibition at P6-8, which may contribute to the transient slow sIPSC kinetics and could explain why TTX blocked large sIPSCs exclusively at P9-11. Activity-dependent events may have different kinetic properties, as evidenced by the multimodal distribution of decay times at this age (Fig. 1E). This suggests that the activity-dependent release of GABA is capable of activating even extrasynaptic GABAARs (Herd et al. 2013; Rovo et al. 2014). A related developmental progression in nRt cell intrinsic excitability has been demonstrated (Warren and Jones 1997). The ability of nRt cells to produce low-threshold spikes (LTS) evoking multiple action potentials is age dependent. Bursting was only observed after P10-11 and could then induce network activation via triggering of rebound spikes in relay neurons. This suggests an activity-dependent maturation of nRt neurons, particularly during the first postnatal week, when a “critical period” was already determined for structural plasticity of developing thalamocortical synapses (Crair and Malenka 1995).

**Tonic inhibition is more robust in immature nRt neurons.** A tonic GABAAR-mediated conductance was demonstrated in both immature and mature nRt neurons (Fig. 7). Tonic inhibition has been discovered in a variety of neuronal types (see review, Glykys and Mody 2007), including other thalamic...
nuclei such as VB and lateral geniculate nucleus (Belelli et al. 2005; Bright et al. 2007; Cope et al. 2005; Peden et al. 2008; Wafford et al. 2009). However, these studies did not detect GABA-mediated tonic currents in nRt.

Various factors may explain this difference. First, much of the work examining tonic inhibition in the thalamus used adult animals. The fact that we observed the largest tonic current in immature nRt neurons does not diminish their relevance, because synaptic GABAergic activity is already present at this age. Nonetheless, we cannot exclude the possibility that inefficient GABA uptake might at least partially explain the prominent tonic current in early postnatal nRt cells. However, another study found that even in immature tissue, blocking GABA transporters enhanced the endogenous tonic current twofold in hippocampal pyramidal neurons (Sipila et al. 2007), suggesting that transporter function is already functional at this stage and that GABA transporters might play a significant role in extrasynaptic signaling.

Similarly, a significant reduction in tonic current has been described in developing cortical neurons. For newborn mice, the charge contributed by tonic current accounted to nearly 100% of the total GABA charge but decreased to 50% by the second postnatal week (Sebe et al. 2010). A second factor that could contribute to differences between our observations and previous studies is the higher concentration of GABA (10 μM) used in our experiments, due to the fairly weak effect of 1 μM GABA. Taken together, these findings suggest functional differences in the underlying GABAAR in P5 compared with mature neurons. This selective sensitivity to low GBZ concentrations at P5 is inconsistent with decreased GABA update at that developmental stage in that decreased uptake would increase GABA concentrations leading to less, not more, blockade by the competitive antagonist GBZ. Given that mature nRt neurons express GABAARs and GBZ is subsequently less effective. A: an all-points histogram based on the steady holding current (I_{hold}) of the same neurons as in A illustrates high sensitivity of tonic current to GABA and GBZ at P5, but not at P24. In the latter, only little current was induced by GABA, and that which was produced was insensitive to low-dose GBZ. C: pooled data of Gaussian fits from all-points histograms of voltage-clamp I_{hold} show significant differences in background noise (I_{noise}) between age groups, which demonstrates that the degree of tonic GABAAR activation (i.e., magnitude of tonic current) is age dependent. Furthermore, data in D indicate that immature nRt neurons showed higher sensitivity to GBZ, suggesting that only high-dose GBZ can efficiently block tonic currents in mature nRt cells. Values are means ± SE; n = 6 per age group. *P < 0.05; **P < 0.01; ***P < 0.001; independent Student’s t-test.

Given that early in postnatal life, GABAergic transmission in nRt is excitatory (Pangratz-Fuehrer et al. 2007), we hypothesize that activation of the tonic current will depolarize the resting membrane potential of immature nRt neurons. The depolarizing influence on the intra-nRt network could act as a major drive contributing to tGDP generation, thus creating a recurrent excitatory network within nRt, which would likely be amplified by the reciprocal connectivity with thalamocortical (TC) neurons. In the adult, a significant GABAergic tonic current would hyperpolarize nRt neurons and suppress the intra-nRt network via shunting inhibition. The reduced burst activity of nRt neurons and the resultant attenuation of the inhibitory output received by TC neurons could explain the previously reported suppression of thalamic oscillations during pathological spike-wave discharges (Huguenard and Prince 1994; Huntsman et al. 1999; Sohal et al. 2000; von Krosigk et al. 1993). In the developing hippocampus, tonic inhibition can
block the generation of synaptic GABAergic inhibition (Grantyn et al. 2011), whereas a complete lack results in spontaneous gamma oscillations in vitro (Glykys et al. 2008).

Dysfunctional extrasynaptic inhibition can produce an epileptic phenotype (Zhu et al. 2011). In the thalamus, blockade of intra-nRT inhibition facilitates epileptiform discharge (Sohl and Huguenard 2003). Interestingly, the complete loss of the α3-subunit protein in the nRT (α3 knockout mouse) did not result in an overtly different behavioral phenotype but displayed retention of nRT GABA_A receptor signaling, suggesting that other α-subunits may compensate for the loss of α3 (Schofield and Huguenard 2007).

Conclusion. Together, these results suggest that the early expression of α3-subunits in nRT cells prolongs the duration of synaptic inhibitory responses. This slow mode of inhibition could be required for early circuit development. Specifically, because GABA is considered a promoter for maturation and synaptogenesis, slower transmission could enhance this function. Furthermore, α3-containing GABA_A Rs could mediate the early postnatal tonic current. Biphasic changes in GABAergic transmission could reflect the induction of network activation, with the developmental shortening of IPSCs promoting faster rhythmic oscillations at a time when fast signal transduction and higher levels of consciousness are required after the enhanced awareness of the external environment, i.e., the time of eye opening.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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