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Developmental Changes of GABA Synaptic Transient in Cerebellar Granule Cells

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ABSTRACT

The time course of synaptic currents is largely determined by the microscopic gating of the postsynaptic receptors and the temporal profile of the synaptic neurotransmitter concentration. Although several lines of evidence indicate that developmental changes of GABAergic synaptic current time course are clearly correlated with a switch in postsynaptic receptors, much less is known about the modification of GABA release during development. To address this issue, we studied the sensitivity of miniature inhibitory postsynaptic currents (mIPSCs) to a quickly dissociating competitive antagonist, 1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid (TPMPA), in neurons cultured for 6 to 8 days in vitro (DIV) ("young") and for 12 to 14 DIV ("old"). mIPSCs recorded in young neurons were significantly more resistant to the block by TPMPA. This observation was interpreted as a consequence of a more efficient displacement of TPMPA from $GABA_A$ receptors caused by a stronger GABA release in young neurons. The change in mIPSC sensitivity to TPMPA during development was not affected by the deletion of α_1 subunit, supporting its presynaptic origin. The effects of a second quickly dissociating antagonist, SR-95103 [2-(carboxy-3′-propyl)-3-amino-4-methyl-6-phenylpyridazinium chloride], on young, old, and α_1 −/− neurons were qualitatively the same as those obtained with TPMPA. Moreover, the analysis of current responses to ultrafast GABA applications showed that the unbinding rates of TPMPA in DIV 6 to 8 and in DIV 12 to 14 neurons are not significantly different, ruling out the postsynaptic mechanism of differential TPMPA action. Thus, we provide evidence that presynaptic GABA uniquantal release is developmentally regulated.

The shape of the postsynaptic currents is crucial for signal integration in the central nervous system. The amplitude and time course of these currents are known to undergo considerable changes during development, pathological conditions, and in a variety of modulatory processes (Okada et al., 2000; Renger et al., 2001; Calcagnotto et al., 2002; Choi et al., 2003). Despite intense investigations, the mechanisms of such modifications are not fully elucidated. Several studies demonstrated that changes in the shape of postsynaptic currents are attributable to variation in the number and gating properties of the postsynaptic receptors (Nusser et al., 1997; Okada et al., 2000). However, the lack of saturation at both inhibitory and excitatory synapses (Frerking et al., 1995; Auger and Marty, 1997; Mellor and Randall, 1997; Liu et al., 1999; Perrais and Ropert, 1999; McAllister and Stevens, 2000; Mozrzymas et al., 2003b; Barberis et al., 2004) raises the possibility that modulation of neurotransmitter release could also shape postsynaptic responses. In addition, the strong nonequilibrium conditions of postsynaptic receptor activation, resulting from an extremely rapid time course of the synaptic neurotransmitter transient (Clements et al., 1992; Mozrzymas et al., 1999, 2003b; Barberis et al., 2000; Mozrzymas 2004), make the postsynaptic responses extremely sensitive to variations in synaptic transmitter release. It should be emphasized that the above-mentioned nonequilibrium results from the rate of synaptic GABA clearance being comparable with the upper limit of $GABA_A$ receptor activation rate (Barberis et al., 2000; Mozrzymas et al., 2003a). In these conditions, the extent of postsynaptic receptor activation depends not only on the peak concentration but also on the time duration of the synaptic agonist pulse (Mozrzymas et al., 2003b; Barberis et al., 2004; Mozrzymas 2004). Hence, it is convenient to define the strength of the synaptic pulse as the integral of the GABA concentration synaptic time course (assuming an exponential time course with peak A_t and decay time constant τ_t, integral $\sim A_t \times \tau_t$). The impor-

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ABBREVIATIONS: CGC, cerebellar granule cell; mIPSC, miniature inhibitory postsynaptic current; DIV, day(s) in vitro; TPMPA, 1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid; SR-95103, 2-(carboxy-3′-propyl)-3-amino-4-methyl-6-phenylpyridazinium chloride; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

tance of synaptic agonist transient time course has been recently emphasized, for example, in processes such as plasticity (Choi et al., 2003) and variability of the quantal size (Liu et al., 1999; Barberis et al., 2004). Moreover, Renger et al. (2001) found that in glutamatergic synapses, the agonist release undergoes a developmental regulation. In contrast, in GABAergic synapses, a developmental modulation of transmitter release remains an open question. In the present work, we investigated the impact of the GABA synaptic transient on miniature GABAergic currents during development in the cerebellar granule cells (CGCs) in culture. The kinetics of the synaptic transient can be inferred by using quickly dissociating competitive antagonists (Clements et al., 1992; Liu et al., 1999; Overstreet et al., 2002; Barberis et al., 2004). The differences in the GABA synaptic transient in young (DIV 6–8) and old (DIV 12–14) cultures were investigated by studying the sensitivity of mIPSCs to the quickly dissociating $GABA_A$ receptor competitive antagonists TPMPA (Ragozzino et al., 1996: Jones et al., 2001) and SR-95103 (Overstreet et al., 2002). In the presence of competitive antagonist, the amount of mIPSC block strongly relies on the strength of the presynaptic GABA release, because antagonist and GABA compete for the same binding site. In particular, a prerequisite for an efficient displacement of competitive antagonist by synaptic agonist is that the dissociation time constant of the antagonist is comparable with the time duration of the GABA synaptic transient. Because TPMPA and SR-95103 show dissociation time constants of ~0.46 and ~2.4 ms, respectively (Jones et al., 2001), these competitive antagonists seem to be a suitable tool to unmask differences in the synaptic GABA transient (Overstreet et al., 2002; Barberis et al., 2004). However, because of its faster unbinding rate, TPMPA is more efficient than SR-95103. We found that mIPSCs recorded in CGCs from old cultures were blocked by competitive antagonists to a larger extent with respect to the ones recorded from young cultures, indicating a stronger agonist release in young neurons. It is noteworthy that in DIV 12 to 14 cultures prepared from α_1 knockout mice, both TPMPA and SR-95103 exerted the same effect as in wild-type neurons at the same culture period, further indicating the presynaptic origin of the their differential sensitivity in DIV 6 to 8 and DIV 12 to 14 cultures. In addition, the analysis of current responses to ultrafast GABA applications provided evidence that the TPMPA unbinding rates in the two groups of cultured neurons were not significantly different. These data together demonstrate that the uniquantal GABA release is stronger in young cultures than in old ones.

Materials and Methods

Mutant Mouse Production and CGC Cell Culture. Heterozygous α_1 subunit-deficient mice were described previously (mixed genetic background C57BL/6J, strain 129/Sv/SvJ, and FVB/N; Vicini et al., 2001) and were interbred to produce wild-type (+/+), heterozygous (+/−), and homozygous (−/−) knockout mice. Genotyping was performed from total genomic cDNA isolated at the third postnatal day from tail snips, and identification of the knockout allele achieved with polymerase chain reaction was as described in Ortinski et al. (2004). Primary cultures of mouse cerebellar granule neurons were prepared as recently described in detail by Ortinski et al. (2004). In brief, mouse pups (postnatal day 7) were sacrificed by decapitation (procedure in agreement with the guidelines of the Georgetown University Animal Care and Use Committee), and the cerebella were dissociated with trypsin (0.25 mg/ml; Sigma-Aldrich, St. Louis, MO) and plated in 35-mm Nunc (Nalge Nunc International, Naperville, IL) dishes at a density of 1.1×10^6 cells/ml on glass coverslips (Fisher Scientific Co., Pittsburgh, PA) coated with poly-L-lysine (10 μg/ml; Sigma-Aldrich). The cells were cultured in basal Eagle's medium supplemented with 10% bovine calf serum, 2 mM glutamine, and 100 μg/ml gentamicin (all from Invitrogen, Carlsbad, CA) and incubated at 37°C in 5% CO_2. The final concentration of KCl in the culture medium was adjusted to 25 mM (high K^+). At DIV 5, the medium was replaced with low (5 mM) K^+ medium [minimal essential medium supplemented with 5 mg/ml glucose, 0.1 mg/ml transferrin, 0.025 mg/ml insulin, 2 mM glutamine, and 20 μg/ml gentamicin (Invitrogen) and 10 μM cytosine arabinofuranoside (Sigma-Aldrich)]. Granule cells were distinguished from the interneurons according to their different shape and size. CGCs looked smaller than interneurons, displayed a characteristic round shape, and had lower whole-cell capacitance. Immunocytochemical studies, where GABAergic cells (interneurons) were stained with antibodies for markers of GABAergic neurons, confirmed the accuracy of the method (not shown).

Electrophysiological Experiments. The current responses were recorded in the outside-out mode of the patch-clamp technique using the Axopatch 1D amplifier (Axon Instruments Inc., Union City, CA) at a holding potential (V_h) of −60 mV. The intrapipette solution contained 145 mM KCl, 5 mM $MgCl_2$, 10 mM BAPTA, 2 mM ATP, and 10 mM HEPES (pH 7.4 with KOH). The composition of the standard external solution was 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). Stock solutions of TPMPA (Sigma-Aldrich) and SR-95103 (a gift from SANOFI Research Center, Montpellier, France) were prepared in water. GABA was applied to excised patches using the ultrafast perfusion system based on a piezoelectric-driven θ-glass application pipette (Jonas, 1995). The piezoelectric translator was from Physik Instrumente (Waldbronn, Germany) (preloaded HVPZT translator 40 μm), and θ-glass tubing was from Hilgenberg (Malsfeld, Germany). The open tip recordings of the liquid junction potentials revealed that a complete exchange of solution occurred within 80 to 120 μs. A minimum duration of drug application was ~1 ms (when applying shorter pulses, oscillations often occurred). The characteristics of the time course (rise time and time constants of deactivation) of current responses to rapid GABA applications showed little cell-to-cell variability, and the values of these parameters estimated from different cells were pooled. The analysis of current amplitudes required comparison of recordings made on the same patch. Stable recordings (less than 10% of rundown) of current responses to ultrafast GABA applications were available for approximately 5 to 20 min. Because current responses were recorded every 0.5 to 2 min, the impact of rundown was small. Controls and recordings in the presence of TPMPA were alternated.

All experiments were performed at room temperature (22–24°C). mIPSCs were recorded in the whole-cell configuration in the presence of tetrodotoxin (0.5 μM; Sigma-Aldrich). mIPSCs were captured by using the sliding template algorithm with pClamp9 software. Synaptic events with amplitude smaller than 4 times standard deviation of the baseline noise were excluded from the analysis. Because glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor-mediated excitatory postsynaptic currents show decay kinetics faster by at least 1 order of magnitude with respect to GABAergic mIPSCs, we distinguished between them by properly setting the parameters of the pClamp 9 software sliding template. In the whole-cell mode, the series resistance (R_s) was in the range 4 to 8 MΩ. Both mIPSCs and currents elicited by brief GABA pulses were recorded in symmetrical chloride at holding potential −60 mV.

The current signals were low-pass filtered at 10 kHz and sampled at 50 to 100 kHz using the analog-to-digital converter Digidata 1322A (Axon Instruments Inc.) and stored on the computer hard disk. For the acquisition and analysis, pClamp 9.0 (Axon Instruments Inc.) software was used.

Analysis. The decaying phase of the currents was fitted with a function in the following form:

$$y(t) = \sum_{i=1}^{n} A_i \exp(-t/\tau_i) \quad (1)$$

where, A_i are the fractions of respective components ($\Sigma A_i = 1$), and τ_i are the time constants. Deactivation time course was well fitted with a sum of two exponentials ($n = 2$). The averaged deactivation time constant τ_m was calculated using the formula $\tau_m = \Sigma A_i \tau_i$.

The averaged amplitude of the mIPSCs in control conditions and in the presence of $GABA_A$ antagonists have been compared after count matching to the largest event (Stell and Mody, 2002). This procedure allows avoiding the antagonist-induced distortions caused by the loss of events falling below the detection threshold.

The kinetic modeling was performed with the Channel Lab 2.0 software (developed by S. Traynelis for Synaptosoft, Decatur, GA), which converted the kinetic model (Fig. 6A) into a set of differential equations and solved them numerically assuming, as the initial condition, that at $t = 0$, no bound or open receptors were present. In model simulations aiming to model the effect of TPMPA on current responses (Fig. 6, B and C), the initial condition was selected as equilibrium binding of TPMPA to $GABA_A$ receptors in the absence of GABA. The current responses to GABA applications were modeled as the sum of occupancies of the open states in the model (Fig. 6A).

Data are expressed as mean ± S.E.M. The amplitudes of both synaptic currents and of current responses to rapid GABA applications were measured in the presence of TPMPA, and a comparison was made to the peaks of control currents measured from the came cell or excised patch. Thus, for analysis of amplitudes, Student's paired t test was used. For other parameters, such as rise time and deactivation kinetics, the data were pooled and Student's unpaired t test was used.

Results

TPMPA Differentially Inhibits GABAergic mIPSCs in CGCs from Young and Old Cultures. To investigate the strength of the GABA synaptic transient in young and old CGCs cultures, we studied the effect of the fast-off competitive antagonist TPMPA on GABAergic mIPSCs. We found that the inhibition exerted by TPMPA (200 μM) on mIPSCs was dependent on time in culture, being significantly weaker in the young cultures with respect to the old ones. TPMPA reduced the mIPSC peak amplitude by 30 ± 4% at DIV 6 to 8, by 45 ± 2% at DIV 9, and by 54 ± 3% at DIV 12 to 14 ($p < 0.001$; $n = 8$; Fig. 1). The larger mIPSC current inhibition by these competitive antagonists in old cultures with respect to the young ones suggests that in younger cultures the GABA synaptic transient might be characterized by a larger strength. Besides an increased sensitivity to TPMPA, the mIPSCs recorded in old cultures showed a considerably faster decaying kinetics (Fig. 3, A and B); τ_{mean} was 54.9 ± 2.7 and 21.3 ± 0.8 ms in young and old, respectively; $p < 0.001$) as reported previously (Ortinski et al., 2004). The rising phase of mIPSCs (measured as 10–90% rise time) was also found to accelerate with development (1.02 ± 0.19 ms at DIV 6–8, $n = 7$; and 0.61 ± 0.03 ms at DIV 12–14, $n = 7$; $p < 0.05$). TPMPA slowed down the mIPSC onset in both young and old neurons (1.19 ± 0.15 ms at DIV 6–8, $n = 7$; and 0.75 ± 0.08 ms at DIV 12–14, $n = 7$; paired t test, $p < 0.05$; Fig. 3, C and D). In contrast, TPMPA did not significantly affect the mIPSC decaying phase (Fig. 3, A and B).

The lack of TPMPA effect on the mIPSC decay is expected because the unbinding of this drug is much faster than the time constants describing the deactivation process (see model simulations, Fig. 6C). By using the fast-off competitive antagonist SR-95103, the differential inhibition of mIPSCs recorded in young and old neurons was qualitatively the same as that obtained with TPMPA. In the presence of 3 μM SR-95103, the mIPSC peak amplitude was reduced by 42 ± 4 and 57 ± 4% in DIV 6 to 8 and DIV 12 to 14 cultures, respectively ($n = 6$; $p < 0.05$; Fig. 2). Because TPMPA shows a faster off-rate than SR-95103, the fact that the differential inhibition of mIPSCs in young and old cultures was more pronounced with TPMPA with respect to SR-95103 further indicates a presynaptic mechanism. However, because the acceleration of mIPSC decay has been shown to be related to a change in the postsynaptic receptors (Ortinski et al., 2004), we needed to elucidate whether the competitive antagonists we used could differentially interact with the distinct postsynaptic receptor subunits found in young and old cultures.

TPMPA Similarly Inhibits GABAergic mIPSCs in α_1 Knockout and Wild-Type CGC Cultures. Different $GABA_A$ receptor subtypes are known to be differentially expressed during development (Laurie et al., 1992). In particular, in cerebellum, α_3 and α_2 $GABA_A$ subunits are abundantly expressed in newborn mice and are progressively replaced by α_1 in adults (Laurie et al., 1992). In cerebellar neurons, both in culture and brain slices, such developmental α subunit expression pattern is responsible for the changes in the GABAergic mIPSCs decay kinetics as demonstrated using α_1 −/− mice (Vicini et al., 2001; Ortinski et al., 2004). Because the inhibition of mIPSCs induced by competitive antagonists critically depends on their binding and unbind-

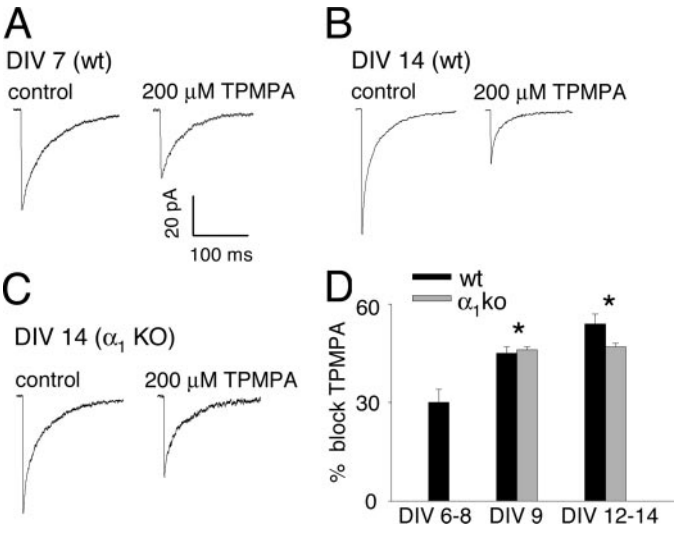

Fig. 1. mIPSC amplitude is differentially affected by TPMPA in DIV 6 to 8 and DIV 12 to 14 neurons. A, examples of averaged GABAergic mIPSCs recorded from a DIV 7 (DIV7) neuron in control conditions (left) and in the presence of TPMPA (200 μM). B, examples of averaged mIPSCs recorded from a day in vitro 14 (DIV14) neuron in control conditions (left) and in the presence of 200 μM TPMPA (right). C, averaged mIPSCs recorded from a DIV 14 α_1 knockout neuron in control conditions (left) and in the presence of 200 μM TPMPA (right). D, statistics of the mIPSCs inhibition by TPMPA at different DIV (indicated below the columns) in wild-type (black columns) and α_1 knockout (gray columns) neurons. Columns represent the percentage of the mIPSC peak amplitude block observed in the presence of 200 μM TPMPA. Asterisks above the columns indicate significant differences with respect to the control conditions.

ing kinetics, it cannot be ruled out that the observed differential block of mIPSCs during development (Fig. 1) might reflect differences in these rate constants because of differential expression of postsynaptic $GABA_A$ receptor subtypes. To test this possibility, mIPSCs were recorded in CGCs culture from α_1 knockout mice and compared with the wild-type ones at the same age in vitro. Because of the lack of α_1 subunit in these cultures, the switch α_3/α_2 to α_1 cannot occur and thus old cultures show the α_3/α_2 phenotype (Ortinski et al., 2004). This trend is reflected by the fact that in DIV 12 to 14 cultures of α_1 knockout neurons, the deactivation kinetics was much slower than in corresponding culture of wild-type neurons (Fig. 3, τ_{mean} was 21.3 ± 0.8 and 43.3 ± 2.7 in wild-type and α_1 knockout, respectively; $p < 0.001$), as reported previously (Ortinski et al., 2004). The rise time of mIPSCs in α_1 knockout neurons at DIV 12 to 14 was 0.68 ± 0.02 ($n = 6$; Fig. 3D). This value is not significantly different from that observed in wild-type neurons at the same culture age. If a weak competitive antagonist inhibition of mIPSCs in young wild-type cultures was caused by a low α_1 subunit expression, then these competitive blockers would be expected to exert a similar effect on mIPSCs recorded from DIV 12 to 14 α_1 knockout cultures.

To test this possibility, the sensitivity of mIPSCs to TPMPA was studied and compared in α_1 knockout and wild-type cultures at DIV 9 and DIV 12 to 14. In this time window, in wild-type cultures, α_1 subunit starts to be significantly expressed (at approximately DIV 9) and becomes predominant with respect to α_3/α_2 (at approximately DIV 12 to 14; Ortinski et al., 2004).

We found that the mIPSCs inhibition by TPMPA both at DIV 9 and DIV 12 to 14 in α_1 knockout cultures was similar to that observed in the wild-type ones. In α_1 knockout cultures, in fact, 200 μM TPMPA reduced the mIPSCs by 46 ± 1 and 47 ± 1% at DIV 9 and DIV 12 to 14, respectively ($n = 6$; Fig. 1), values not significantly different from those obtained in the wild-type cultures at the same ages (45 ± 2 and 54 ± 3%, respectively). These values, in contrast, were significantly different from that obtained in wild-type cultures at DIV 6 to 8 ($p < 0.05$). As in wild-type neurons, 200 μM TPMPA slowed down the mIPSC onset (0.85 ± 0.06 ms at DIV 12 to 14, $n = 6$; Fig. 3D). Again, the block by SR-95103 of mIPSCs in old wild-type and α_1 knockout cultures was similar to that observed with TPMPA. At DIV 12 to 14, application of 3 μM SR-95103 reduced the amplitude by 57 ± 4 and 54 ± 2% in wild-type and α_1 knockout neurons, respectively (Fig. 2). Together, these data may suggest that the differential effect of TPMPA in young and old culture is not caused by the different TPMPA-$GABA_A$ receptor binding and/or unbinding rate constants in different $GABA_A$ receptor subtypes expressed in young and old cultures.

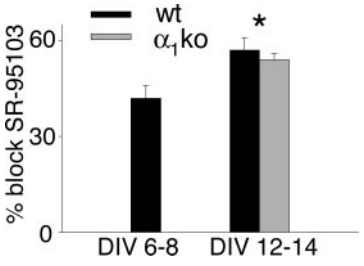

Fig. 2. mIPSC amplitude is differentially affected by 3 μM SR-95103 in DIV 6 to 8 (DIV6–8) and DIV 12 to 14 (DIV12–14) neurons. Black columns show the statistics of the mIPSC peak amplitude block observed in the presence of 3 μM SR-95103 in DIV 6 to 8 (DIV6–8) and DIV 12 to 14 (DIV12–14) wild-type neurons. Gray column shows the statistics of the mIPSC peak amplitude block observed in the presence of 3 μM SR-95103 in DIV 12 to 14 α_1 knockout neurons. Asterisks above the columns indicate significant differences with respect to the control conditions.

TPMPA Similarly Affects GABA-Evoked Responses in DIV 6 to 8 and DIV 12 to 14 Neurons. To further rule out that the differential action of TPMPA on synaptic currents recorded at DIV 6 to 8 and DIV 12 to 14 might involve different interaction of this drug with postsynaptic receptors in these two groups, it is important to demonstrate that TPMPA action on $GABA_A$ receptors in these two groups is similar. In particular, as explained in detail above, it is crucial to provide evidence that the unbinding rates of TPMPA from receptors in DIV 6 to 8 and DIV 12 to 14 are comparable. To address this issue, the current responses to rapidly applied GABA at saturating concentration (10 mM) were measured in the absence and presence of 200 to 400 μM TPMPA. After a sufficiently long pretreatment at this TPMPA concentration, it is expected that this drug would reach a steady-state occupancy of the agonist binding sites at $GABA_A$ receptors. In these conditions, a response (activation) of receptors occupied by TPMPA to the application of rapid and saturating GABA concentrations would be delayed by the time needed for TPMPA to unbind. Because the effective binding rate for TPMPA (k_{on}·[TPMPA] = 200–400 μM) is at least 2 orders of magnitude smaller than that for GABA (at 10 mM), the amplitude of the current response is expected be only slightly affected by the presence of TPMPA. This prediction qualitatively differs from TPMPA effect observed on the synaptic currents, where a much larger current inhibition was observed (Fig. 1). However, it needs to be emphasized that application of saturating [GABA] for 1 to 3 ms differs substantially from synaptically applied agonist that is nonsaturating and lasting for much shorter time (Clements et al., 1992; Mozrzymas et al., 1999, 2003b; Overstreet et al., 2002).

Taking into account these assumptions, current responses to ultrafast saturating GABA applications were recorded in control conditions and in the presence of TPMPA. In the absence of TPMPA, the rising phases (measured as 10–90% rise times) were very fast both in DIV 6 to 8 and in DIV 12 to 14 neurons, being 0.36 ± 0.02 and 0.23 ± 0.01 ms, respectively. Interestingly, the 10 to 90% rise time in DIV 12 to 14 cells was significantly faster than that in DIV 6 to 8 ones ($p < 0.05$). Analogous recordings, performed in the presence of 200 to 400 μM TPMPA, revealed that the presence of this drug resulted in a strong slow down of the current onsets. It is noteworthy that the extent of a decrease in current onset rate was very similar in DIV 6 to 8 and DIV 12 to 14 neurons (at 400 μM TPMPA, rise time ~0.73 ± 0.05 and 0.68 ± 0.13, respectively; Fig. 4, A–C). The amplitudes of currents as well as deactivation kinetics were only slightly affected (Fig. 4D). The effect of 200 μM TPMPA on the rising phase and amplitude of current responses was very similar to that observed at 400 μM TPMPA (not shown). The deactivation kinetics of control current responses showed a trend to accelerate with time of culture (τ_{mean} ~39.7 ± 2.9 and 27.8 ± 1.7 ms for DIV 6–8 and DIV 12–14, respectively; $p < 0.05$). Thus, the deactivation kinetics of current responses and synaptic currents showed a similar pattern of changes during the considered

period of culture (Figs. 3 and 5). The acceleration of the decaying phases of the current responses (Fig. 5) seems also to qualitatively reproduce the trend observed in mIPSCs. It has to be pointed out that both the onset and decaying kinetics of current responses and mIPSCs show quantitative differences. The main source of this discrepancy could be attributable to different extrasynaptic versus synaptic receptor substypes (Mozrzymas et al., 1999; Banks and Pearce, 2000). In addition, in the case of mIPSCs, the onset kinetics could be additionally affected by electrotonic filtering. Moreover, it is likely that mIPSCs rising phase shows a larger sensitivity to agonist concentration profile than the decay kinetics.

To further verify the predictions of the approach applied to compare the unbinding rates of TPMPA from the $GABA_A$ receptors in the two considered groups (DIV 6–8 and DIV 12–14), model simulations of current responses to saturating [GABA] (10 mM) in control conditions and in the presence of TPMPA were performed. For the model simulations presented in Fig. 6A, the gating scheme of Jones and Westbrook (1995) was used with the rate constants taken from Barberis et al. (2000). The binding and unbinding rates for TPMPA were taken from Jones et al. (2001). As shown in Fig. 6B, the model simulations predicted a decrease in the current onset rate by a value comparable with the unbinding rate of TPMPA. In addition, in the presence of 400 μM TPMPA, only a small decrease in amplitude is obtained (Fig. 6B). These predictions of the model simulations are in good agreement with our experimental data (Fig. 3). Moreover, the effect of TPMPA on the current deactivation (especially the later phase) is predicted to be negligible (Fig. 6C), which matches very well with our experimental observations (Fig. 3). This finding is consistent with a very fast unbinding of TPMPA (approximately 0.46 ms). Thus, after 1- to 3-ms application of saturating [GABA], the majority of receptors unbind TPMPA, and the binding site becomes rapidly occupied by GABA. This implies that at the starting point for the deactivation process (removal of agonist after 1- to 3-ms application), there is a nearly full occupancy of binding sites by

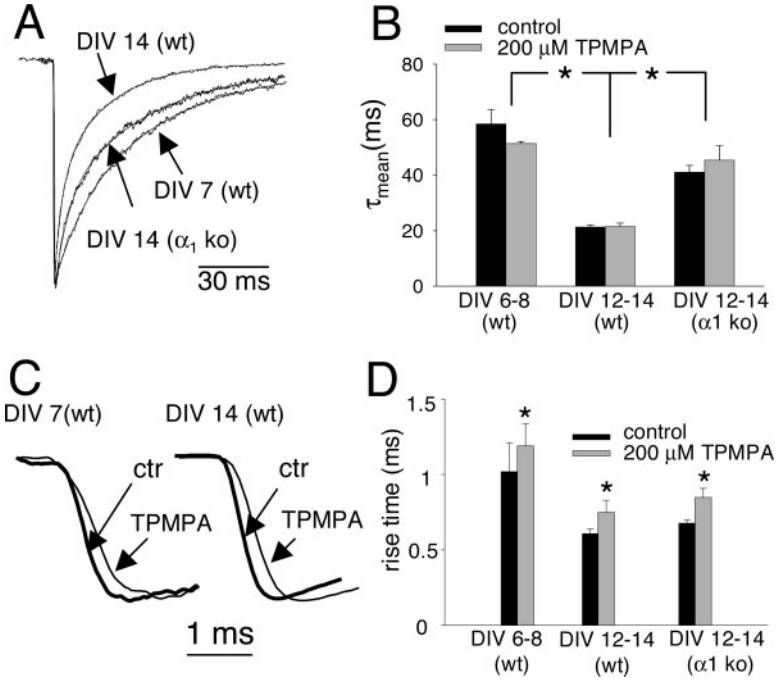

Fig. 3. Developmental changes in decay and onset kinetics of mIPSCs and their modulation by TPMPA. A, averaged normalized and superimposed GABAergic mIPSCs from DIV 6 (DIV6), DIV 14 (DIV14) wild-type, and from DIV 14 (DIV14) α_1 knockout neurons. B, statistics of the mIPSCs decay kinetics in wild-type and α_1 knockout neurons at different developmental stages (indicated below the columns). Averaged traces were fitted by a sum of exponential functions, and the mean decay time constants were calculated as describe under *Materials and Methods*. In none of the considered groups were the values of mean decay time constants significantly affected by TPMPA. Asterisks above the columns indicate significant differences between the considered groups (unpaired t test). C, averaged and superimposed mIPSCs (shown in expanded time scale) in control condition (thick line) and in the presence of 200 μM TPMPA (thin line) in both young DIV 7 (DIV 7) and old DIV 14 (DIV 14) neurons. D, statistics for the 10 to 90% rise time of mIPSCs in control (black columns) and in presence of 200 μM TPMPA (gray columns) in DIV 6 to 8 wild-type, DIV 12 to 14 wild-type, and DIV 12 to 14 α_1 knockout cultures. Asterisks above the columns represent significant differences with respect to the control conditions (paired t test; $p < 0.05$).

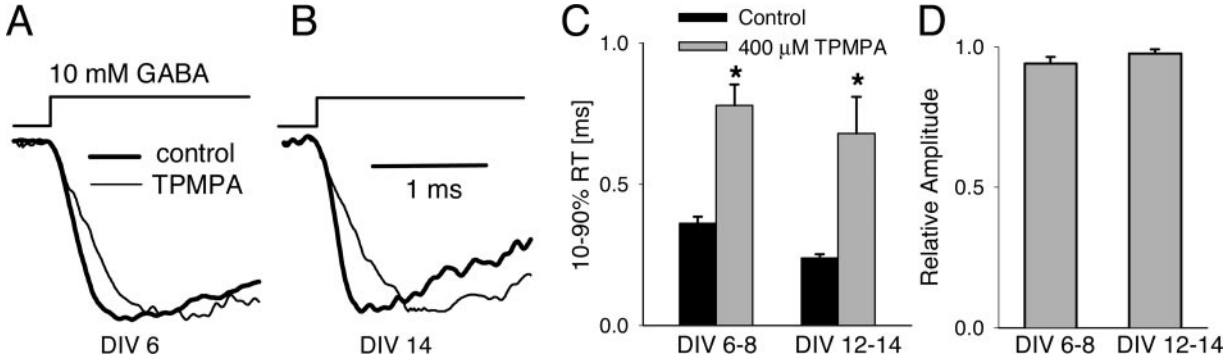

Fig. 4. TPMPA induces a similar reduction of the onset rate of current responses to saturating GABA in DIV 6 to 8 and DIV 12 to 14 neurons. A and B, normalized current responses evoked by ultrafast brief (3-ms) pulse of saturating (10 mM) GABA recorded in patches excised from DIV 6 (A) and DIV 14 neurons (B) in the presence (thin line) and absence (thick line) of 400 μM TPMPA. C, statistics of the TPMPA effect on the 10 to 90% rise times of current responses to 10 mM GABA in control conditions (black columns) and in the presence of 400 μM TPMPA (gray columns). The culture period is indicated below the columns. Asterisks above the columns indicate significant differences with respect to the control conditions. D, statistics of the TPMPA effect on current amplitude. Columns represent the current amplitudes in the presence of 400 μM TPMPA relative to the controls obtained from the same cells.

GABA. The deactivation process (especially in the case of responses to saturating [GABA]) is believed to be predominantly shaped by transitions between the fully bound states (coupling between open, desensitized, and closed states terminated by agonist unbinding; Jones and Westbrook, 1995). Thus, until the receptor is fully bound by GABA, TPMPA has no effect on deactivation. Agonist unbinding that occurs after GABA removal turns the receptor into the closed state with no possibility of further openings. Thus, once the receptor unbinds GABA, its contribution to shape the deactivation current is terminated independently of whether or not TPMPA binds to the vacant binding sites. The model simulations performed using the rate constants for $GABA_A$ receptor gating from other reports (Jones and Westbrook 1995; Mozrzymas et al., 2003a) gave exactly the same predictions for TPMPA effects (slower rise time, small TPMPA effect on amplitude, and negligible effect on deactivation), indicating that the above-described impact of TPMPA on current responses to saturating [GABA] is largely model-independent within the considered gating frame.

As mentioned above, the 10 to 90% rise time at DIV 12 to 14 was significantly shorter than that at DIV 6 to 8 (Fig. 3). It is thus important to clarify to what extent this difference in the onset kinetics could obscure the differences in the unbinding of TPMPA in these two groups of neurons. At saturating [GABA], the onset rate is known to be largely determined by the transitions between fully bound states (mainly opening β_2 and desensitization d_2; Mozrzymas et al., 2003a). Assuming that the observed change in rise times reflects a modification of these transition rates, a series of simulations were performed in which β_2 and d_2 were modified over a wide range. We found that, although modifications of both rate constants strongly influenced the current onset kinetics, the simulated difference in rise times in the absence and presence of TPMPA was only slightly affected (not shown).

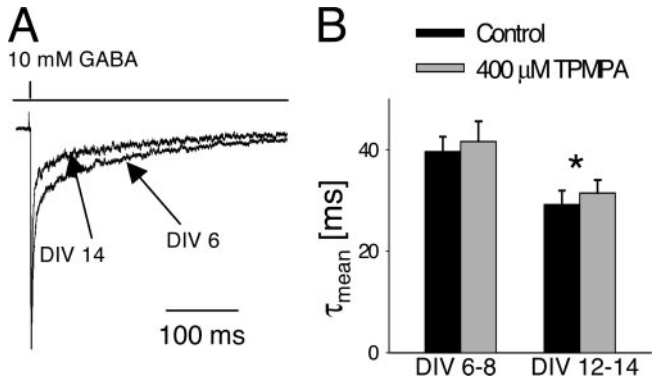

Fig. 5. Decaying phase of current responses to rapid applications of brief and saturating GABA concentrations accelerates with development in vitro. A, typical normalized current responses to saturating GABA (10 mM, 3 ms) recorded from a patch excised from DIV 6 and DIV 14 neurons. Note that the decay of current recorded from the DIV 14 neuron is considerably faster. B, statistics of the mean decaying time constants in DIV 6 to 8 and DIV 12 to 14 neurons in control conditions (black columns) and in the presence of TPMPA (gray columns). TPMPA had no significant effect in either of the two groups of neurons. Asterisk above the columns indicates significant difference with respect to the value of τ_{mean} obtained in DIV 6 to 8 neurons.

Discussion

The different sensitivity of mIPSCs to TPMPA provides evidence that in young CGCs (DIV 6–8), the synaptic GABA pulse is stronger than in the old ones (DIV 12–14). This observation suggests a developmental change in presynaptic mechanisms at GABAergic synapses. Interestingly, it has been recently reported that maturation of the glutamatergic synapses is related to a pronounced modulation of release mechanism (Renger et al., 2001). However, in this report, it has been proposed that the strength of glutamate release increases during development. Modulation of glutamate release has been also recently implicated as an important presynaptic mechanism contributing to expression of the long-term potentiation (Choi et al., 2003). It seems thus that an increasing body of evidence underscores a crucial role of presynaptic mechanisms, including agonist transient, in processes related to development and synaptic plasticity both in glutamatergic and GABAergic synapses. Interestingly, the decrease in the strength of GABA transient described here seems to be correlated with the developmental change of the $GABA_A$ receptor subtype expression and a pronounced modification in the mIPSC kinetics. As mentioned, the decrease in α_3 and the increase in α_1 subunit expression during development result in faster mIPSC decay. Moreover, α_3- and α_1-containing receptors have been reported to mediate currents showing slow and fast deactivation kinetics, respectively (Verdoorn, 1994; Gingrich et al., 1995). Because the amplitude and the duration of the GABA transient may influence the current amplitude and decay kinetics (Barberis et al., 2004), the reported changes in the synaptic pulse strength could contribute to changes of IPSCs with development (Ortinski et al., 2004). Moreover, because α_3-containing receptors are characterized by a binding rate constant (k_{on}) for GABA almost 2 orders of magnitude lower than the α_1-containing ones (Gingrich et al., 1995), it may be speculated that a large strength of the agonist pulse in young cultures is required to efficiently activate the α_3-containing receptors.

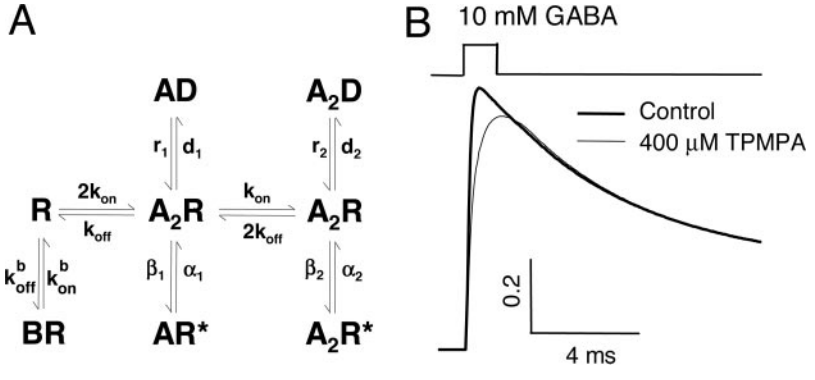

Fig. 6. Model simulation predicts that TPMPA slows down the onset but has little effect on amplitude and deactivation kinetics of currents elicited by rapid application of saturating GABA concentration. A, model of $GABA_A$ receptor gating (Jones and Westbrook, 1995) with a transition to the closed state with a binding site occupied by a competitive antagonist molecule (BR). The rate constants for the $GABA_A$ receptor gating were taken from Barberis et al. (2000) and the binding/unbinding rates for the competitive antagonist (TPMPA) were adopted from Jones et al. (2001). B, simulated current responses to 10 mM GABA applied for 2 ms in control conditions (thick line) and in the presence of 400 μM TPMPA (thin line). Note that besides the rising phase, the currents overlap predicting the lack of TPMPA effect on the current decaying phase.

The changes in postsynaptic receptor subtypes during development raise an important question to what extent the observed difference in the mIPSCs sensitivity to TPMPA and SR-95103 has a postsynaptic origin. The major arguments supporting the presynaptic mechanism (change of strength of synaptic agonist pulse) were the similar TPMPA and SR-95103 sensitivity of mIPSCs in DIV 12 to 14 α_1 knockout and wild-type neurons (Fig. 1). Moreover, the lack of significant difference in the TPMPA unbinding rates (k_{off}) in DIV 6 to 8 and DIV 12 to 14 neurons, as deduced from recordings of current responses to ultrarapid GABA applications (Fig. 4), also argues against a postsynaptic source of such differential inhibition by TPMPA. Although the protocol used in our experiments does not give direct insight into the binding rate of TPMPA, it is expected that the impact of difference in TPMPA binding to $GABA_A$ receptors in the DIV 6 to 8 and DIV 12 to 14 neurons is minor because the previous estimations of the binding rate of TPMPA yielded the value that is considerably lower than those typically obtained for GABA (Jones et al., 2001). Moreover, the peak of synaptic GABA concentration (Mozrzymas et al., 1999, 2003b; Overstreet et al., 2002) is expected to be several folds larger than that of TPMPA. Thus, when unbinding of TPMPA molecule coincides with synaptic GABA transient, it is more likely that a vacant binding site would be occupied by GABA rather than by TPMPA. In addition, the fact that the unbinding rates for TPMPA from young and old receptors are undistinguishable, could suggest that the binding rates are following the same trend.

A lower TPMPA and SR-95103 sensitivity of mIPSCs in young cultures could suggest a stronger displacement of this competitive antagonist by GABA as a result of a rapid binding of GABA. However, as mentioned above, α_3-containing receptors are characterized by a binding rate much slower than that in the case of receptors including α_1 subunit, arguing against a stronger TPMPA displacement by GABA in young cultures. A similar argument can be used for the α_2-containing receptor because their binding rate for GABA is comparable with that of the α_1-containing ones (Lavoie et al., 1997).

The observed difference in the strength of agonist transient during development could be of physiological significance. In neurons at early developmental stages (at which GABA is depolarizing; Cherubini et al., 1990; Borodinsky et al., 2003), a robust GABA release is required to exert a trophic effect by sustaining a calcium-mediated synaptogenesis. It is worth emphasizing that in young neurons such enhanced GABA release is correlated with prolonged GABAergic mIPSCs, thus favoring a long membrane depolarization that, in turn, would enhance the influx of calcium through the voltage-operated calcium channels. An additional possibility is that an increased strength of synaptic agonist release, in combination with a high affinity of the postsynaptic receptors, would favor the agonist spillover from the synaptic cleft and an increased tonic GABA concentration in the vicinity of the synapse. Spillover and resulting tonic GABA were recently reported to play an important role not only in mediating the shunting inhibition but also in controlling the degree of synapse independence (Overstreet and Westbrook, 2003). It is thus possible that the impact of GABA spilling over from the synapses changes during development but this issue would require a separate study. In contrast to what observed in young neurons, a weaker synaptic agonist pulse in adult CGCs where higher affinity α_1-containing receptors are predominant would be expected to yield a signal more localized to the synapse itself. In addition, taking into account that mIPSCs in adult neurons are short lasting, it may be proposed that the developmental decrease in the strength of the synaptic agonist release might contribute to an enhanced spatial and temporal resolution of GABAergic synaptic currents in adulthood.

In general, our major conclusion related to change in the agonist transient is expressed in terms of the strength of the synaptic pulse. The convenience of the use of this parameter is related to the fact that, within a relatively broad range of transient parameters (peak amplitude and time constant of clearance), it is difficult to strictly determine whether a stronger agonist release was caused by an increase in the peak or to a prolongation of agonist presence (slower clearance). It thus needs to be borne in mind that any modulation of the synaptic agonist transient has at least these two degrees of freedom. Clearly, an enhancement of a postsynaptic current may take place when the agonist clearance slows down, whereas the peak agonist amplitude remains unchanged. Recently, it has been reported that regulation of the release kinetics in the glutamatergic synapse strongly affected the time course of synaptic glutamate, giving rise to protraction of synaptic currents (Pawlu et al., 2004). Dependence of synaptic currents on agonist transient duration has been also discussed in Nusser et al. (2001) and Barberis et al. (2004) and in a recent review by Mozrzymas (2004).

It has to be pointed out that the clearance of the neurotransmitter in the cleft (in particular in vivo, where the synapses are tightly packed) is also thought to be strongly influenced by the geometry of the synapses, diffusion coefficient of the transmitter, and the number and affinity of GABA binding sites (including $GABA_A$ receptor, $GABA_B$ receptor and GABA transporters). Differences in these parameters in young and old neurons could be potentially responsible for the observed differential age-dependent inhibition by fast-off antagonists. However, although several studies have shown (at both excitatory and inhibitory synapses) that the neurotransmitter concentration peak and temporal profile critically depend on these parameters (Kleinle et al., 1996; Kruk et al., 1997; Barbour, 2001), much less is known about their developmental changes. When interpreting the data obtained from a simple model of cultured neurons, it is important to consider to what extent the developmental paradigm observed in vitro could reproduce that observed in vivo. Although neuronal cell cultures are in many respects different from the in vivo conditions, it has to be pointed out that several fundamental processes occurring in development such as changes in the IPSCs kinetics and frequency (Vicini et al., 2001; Ortinski et al., 2004), the GABA switch from depolarizing to hyperpolarizing (Cherubini et al., 1990; Borodinsky et al., 2003), and the replacement of α_3/α_2 subunit by α_1 subunit (Ortinski et al., 2004) seem to be reasonably reproduced in the cell culture. Thus, it seems plausible to propose that the described here change in the synaptic agonist strength occurs also during development in vivo.

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