Molecular Pharmacology Fast Forward. Published on January 5, 2005 as doi:10.1124/mol.104.006437

Developmental Changes of GABA Synaptic Transient in Cerebellar Granule Cells

Andrea Barberis, Congyi Lu, Stefano Vicini and Jerzy W. Mozrzymas

AB,CL,SV, & JWM: Department of Physiology and Biophysics, Georgetown University School of Medicine, Washington, DC 20007

JWM::Laboratory of Neuroscience, Department of Biophysics, Wrocław Medical University, Chałubińskiego 10, 50-368 Wrocław, Poland

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: GABA Synaptic Transient during development

Number of text pages: 28

Number of words in abstract: 237

Number of words in Introduction: 756

Number of words in Discussion: 1391

Number of Tables: 0

Number of Figures: 6

Number of references: 39

List of non-standard abbreviations:

mIPSC - miniature Inhibitory Postsynaptic Current

TPMPA - 1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid

SR-95103 - 2-(carboxy-3'-propyl)-3-amino-4-methyl-6-phenylpyridazinium chloride

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

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. While several lines of evidence indicate that developmental changes of GABAergic synaptic currents 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 mIPSCs to a quickly dissociating competitive antagonist, TPMPA, in neurons cultured for 6-8 days in vitro 6-8, "young" and for 12-14 days in vitro, "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 due to a stronger GABA release in young neurons. The change of mIPSCs sensitivity to TPMPA during development was not affected by the deletion of $\alpha 1$ subunit supporting its presynaptic origin. The effects of a second quickly dissociating antagonist, SR-95103, on young, old, and $\alpha 1$ -/- 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 days in vitro 6-8 and in days in vitro 12-14 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.

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

The shape of the post synaptic currents is crucial for signal integration in the CNS. 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 (e.g. Okada et al., 2000; Renger et al., 2001; Choi et al., 2003; Calcagnotto et al., 2002). Despite intense investigations, the mechanisms of such modifications are not fully elucidated. Several studies demonstrated that changes in the shape of post-synaptic 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 non-equilibrium conditions of post-synaptic receptor activation, resulting from an extremely rapid time course of the synaptic neurotransmitter transient (Clements 1992; Mozrzymas et al., 1999; Barberis et al., 2000; Mozrzymas et al., 2003b; Mozrzymas 2004), make the post-synaptic responses extremely sensitive to variations in synaptic transmitter release. It should be emphasized that the above mentioned non-equilibrium results from the rate of synaptic GABA clearance being comparable to the upper limit of $GABA_A$ receptor activation rate (Barberis et al., 2000; Mozrzymas 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 2003b, Barberis 2004; Mozrzymas 2004). Hence, it is convenient to define the strength of the synaptic pulse as the integral of the GABA

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

concentration synaptic time course (assuming an exponential time course with peak A_t and decay time constant τ_t, integral ~ $A_t \cdot \tau_t$). The importance of synaptic agonist transient time course has been recently emphasized, for instance, in processes such as plasticity (Choi et al., 2003) and variability of the quantal size (Barberis et al., 2004; Liu et al., 1999). Moreover, Renger et al., (2001) have 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 1992; Overstreet et al., 2002; Liu et al. 1999; Barberis et al. 2004). The differences in the GABA synaptic transient in young (days in vitro 6-8) and old (days in vitro 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 mIPSCs block strongly relies on the strength of the pre-synaptic GABA release, as 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 to the time duration of the GABA synaptic transient. As TPMPA and SR-95103 show dissociation time constants ~ 0.46 and ~ 2.4 ms respectively (Jones et al., 2001), these competitive antagonists appear to be a suitable tool to unmask differences in the synaptic GABA transient (Barberis et al.,

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

2004, Overstreet et al., 2002). However, due to its faster unbinding rate, TPMPA is more efficient than SR-95103. We found that mIPSCs recorded in CGCs from old (days in vitro 12-14) cultures were blocked by competitive antagonists to a larger extent with respect to the ones recorded from young (days in vitro 6-8) cultures, indicating a stronger agonist release in young neurons. Importantly, in days in vitro 12-14 cultures prepared from α1 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 days in vitro 6-8 and days in vitro 12-14 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, taken together, demonstrate that the uniquantal GABA release is stronger in young cultures than in the old ones.

Materials and Methods

Mutant mouse production and CGC cell culture

Heterozygous α_1 subunit deficient mice were previously described (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 3rd postnatal day from tail snips, and identification of the knock-out allele achieved with PCR as described in Ortinski et al. (2004). Primary cultures of mouse cerebellar granule neurons were prepared as recently described in details by Ortinski et al. (2004). Briefly, mouse

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

pups (postnatal day 7, P7) were sacrificed by decapitation (procedure in agreement with the guidelines of the Georgetown University Animal Care and Use Committee), the cerebella were dissociated with trypsin (0.25 mg/ml, Sigma, St. Louis, MO) and plated in 35 mm Nunc dishes at a density of $1.1x10^6$ cells/ml on glass coverslips (Fisher Scientific, Pittsburgh, PA) coated with poly-L-lysine (10 μg/ml; Sigma). The cells were cultured in basal Eagle's medium supplemented with 10% bovine calf serum, 2 mM glutamine, and 100 μg/ml gentamycin (all from Invitrogen Corporation Carlsbad, CA), and incubated at 37^oC in 5% CO_2. The final concentration of KCl in the culture medium was adjusted to 25 mM (high K^+). At days in vitro 5 the medium was replaced with low (5 mM) K^+ medium (MEM supplemented with 5 mg/ml glucose, 0.1 mg/ml transferrin, 0.025 mg/ml insulin, 2 mM glutamine, 20 μg/ml gentamicin, Invitrogen and cytosine arabinofuranoside 10 μM, Sigma). Granule cells were distinguished from the interneurons according to their different shape and size. CGCs appeared smaller than interneurons, displayed a characteristic round shape and had lower whole-call 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, Union City, CA) at a holding potential (V_h) of -60 mV. The intrapipette solution contained (in mM) KCl 145, $MgCl_2$ 5, 1,2-bis(2-aminophenoxy)ethane-N,N,N'-tetraacetic acid (BAPTA) 10, ATP 2, HEPES 10 (pH 7.4

with KOH). The composition of the standard external solution was (in mM) NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, HEPES 10 (pH 7.4 with NaOH). Stock solution of TPMPA (Sigma, St Louis MO) and SR95103 (a gift from Sanofi Research) were prepared in water. GABA was applied to excised patches using the ultrafast perfusion system based on a piezoelectric-driven theta-glass application pipette (Jonas et al., 1995). The piezoelectric translator was from Physik Instrumente (preloaded HVPZT translator 40 μm, Waldbronn, Germany) and theta-glass tubing from Hilgenberg (Malsfeld, Germany). The open tip recordings of the liquid junction potentials revealed that a complete exchange of solution occurred within 80-120 μs. A minimum duration of drug application was ~ 1 ms (when applying shorter pulses, often oscillations appeared). The characteristics of the time course (rise time, 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 5-20 minutes. Since current responses were recorded every 0.5-2 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). 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. As Glutamatergic AMPA receptors mediated EPSCs show

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

decay kinetics faster by at least one 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 4-8 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 50-100 kHz using the analog-to-digital converter Digidata 1322A (Axon Instruments) and stored on the computer hard disk. For the acquisition and analysis, PClamp 9.0 (Axon Instruments) software was used.

Analysis

The decaying phase of the currents was fitted with a function in the 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 mIPSCs averaged amplitude in control conditions and in presence of $GABA_A$ antagonists have been compared after count matching to the largest event (Stell & Mody, 2002). This procedure allows avoiding the antagonist-induced distortions due to the loss of events falling below the detection threshold.

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

The kinetic modelling 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 6B,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 modelled as the sum of occupancies of the open states in the model (Fig. 6A).

Data are expressed as mean ± SEM. 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 the Student's unpaired *t*-test was used.

Results

TPMPA differentially inhibits GABAergic mIPSCs in CGCs from young (days in vitro 6-8) and old (days in vitro 12-14) cultures. In order 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

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

reduced the mIPSCs peak amplitude by 30 ± 4 % at days in vitro 6-8, 45 ± 2 % at days in vitro 9, and by 54 ± 3% at days in vitro 12-14. ($p < 0.001$, $n = 8$, Fig. 1). The larger mIPSCs 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 (days in vitro12-14) cultures showed a considerably faster decaying kinetics (Fig.3A,B), τ_{mean} was 54.9 ± 2.7 and 21.3 ± 0.8 ms in young and old respectively, $p < 0.001$) as previously reported (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, $n = 7$, at days in vitro6-8 and 0.61 ± 0.03 ms, $n = 7$, at days in vitro12-14, $p < 0.05$). TPMPA slowed down the mIPSC onset in both young and old neurons (1.19 ± 0.15 ms, $n = 7$, at days in vitro6-8 and 0.75 ± 0.08 ms, $n = 7$, at days in vitro12-14, paired t-test $p < 0.05$ Fig 3C,D). In contrast, TPMPA did not significantly affect the mIPSC decaying phase (Fig. 3A,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 also the 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 of that obtained with TPMPA. In the presence of SR-95103 (3 μM) the mIPSCs peak amplitude was reduced by 42 ± 4 % and 57 ± 4 % in days in vitro 6-8 and days in vitro 12-14 cultures respectively ($p < 0.05$, $n = 6$, Fig. 2). As TPMPA show 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

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

presynaptic mechanism. However, since the acceleration of mIPSCs 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 knock out and wild type CGCs 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). Since the inhibition of mIPSCs induced by competitive antagonists critically depends on their binding and unbinding kinetics, it cannot be ruled out that the observed differential block of mIPSCs during development (Fig. 1) might reflect differences in these rate constants due to differential expression of postsynaptic $GABA_A$ receptor subtypes. In order to test this possibility, mIPSCs were recorded in CGCs culture from α_1 knock out 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 days in vitro12-14 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 previously reported (Ortinski et al., 2004). The rise time of mIPSCs in α_1 knockout neurons at days in vitro12-14 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 due to a low α_1 subunit expression, then these competitive blockers would be expected to exert a similar effect on mIPSCs recorded from days in vitro12-14 α_1 knock out cultures.

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

We found that the mIPSCs inhibition by TPMPA both at days in vitro 9 and days in vitro 12-14 in α_1 knock out cultures was similar to that observed in the wild type ones. In α_1 knockout cultures, in fact, TPMPA (200 μM) reduced the mIPSCs by 46 ± 1 % and 47 ± 1 % at days in vitro 9 and days in vitro 12-14, respectively (n = 6, Fig 1), values not significantly different from those obtained in the wild type cultures at the same age (45 ± 2 % and 54 ± 3 %, respectively) . These values, in contrast, were significantly different from that obtained in wild type cultures at days in vitro 6-8 ($p < 0.05$). As in wild type neurons, TPMPA (200 μM) slowed down the mIPSC onset (0.85 ± 0.06 ms, n = 6 at days in vitro 12-14, 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 days in vitro 12-14, application of SR-95103 (3 μM) reduced the amplitude by 57 ± 4 % and 54 ± 2 % in wild

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

type and α_1 knockout neurons, respectively, (Fig 2). Altogether, these data may suggest that the differential effect of TPMPA in young and old culture is not due to the different TPMPA-$GABA_A$ receptor binding and/or unbinding rate constants in different $GABA_A$ receptor subtypes expressed in young and old cultures.

TPMPA similarly affects GABA-evoked responses in days in vitro 6-8 and days in vitro 12-14 neurons. To further rule out that the differential action of TPMPA on synaptic currents recorded at days in vitro6-8 and days in vitro12-14 might involve different interaction of this drug with postsynaptic receptors in these two groups it is thus important to demonstrate that TPMPA action on $GABA_A$ receptors in these two groups is similar. In particular, as explained in details above, it is crucial to provide evidence that the unbinding rate of TPMPA from receptors in days in vitro6-8 and days in vitro12-14 is comparable. In order to address this issue, the current responses to rapidly applied GABA at saturating concentration (10 mM) were measured in the absence and presence of 200-400 μM of 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. Since the effective binding rate for TPMPA (k_{on}·[TPMPA], [TPMPA] = 200-400 μM) is at least two 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

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

larger current inhibition was observed (Fig. 1). However, it needs to be emphasized that application of saturating [GABA] for 1-3 ms differs substantially from synaptically applied agonist that is non-saturating and lasting for much shorter time (Mozrzymas et al., 1999, 2003b; Clements 1992; 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 days in vitro 6-8 and in days in vitro 12-14 neurons being 0.36 ± 0.02 and 0.23 ± 0.01 ms, respectively. Interestingly, the 10-90% rise time in days in vitro12-14 cells was significantly faster than that in days in vitro 6-8 ones ($p < 0.05$). Analogous recordings, performed in the presence of 200-400 μM TPMPA, revealed that the presence of this drug resulted in a strong slow down of the current onsets. Importantly, the extent of a decrease in current onset rate was very similar in days in vitro6-8 and days in vitro12-14 neurons (at 400 μM TPMPA, rise time ~ 0.73 ± 0.05 and 0.68 ± 0.13 respectively Fig. 4A, B and 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 days in vitro6-8 and days in vitro12-14, 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, 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

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

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 vs synaptic receptor substypes (Banks and Pearce, 2000; Mozrzymas et al., 1999). 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.

In order 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 (days in vitro6-8 and days in vitro12-14), 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), that matches very well our experimental observations (Fig. 3). This finding is consistent with a very fast unbinding of TPMPA (approximately 0.46 ms). Thus, after 1-3 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

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

agonist after 1-3 ms application) there is a nearly full occupancy of binding sites by 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 (e.g. 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 10-90% rise time at days in vitro12-14 was significantly shorter than that at days in vitro6-8 (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, see e.g. Mozrzymas et al., 2003a). Assuming thus 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).

Discussion

The different sensitivity of mIPSCs to TPMPA provides evidence that in young CGCs (days in vitro6-8), the synaptic GABA pulse is stronger than in the old ones (days in vitro12-14). 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 subtypes expression and a pronounced modification in the mIPSCs kinetics. As previously 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 (Gingrich et al., 1995; Verdoorn, 1994). Since the

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

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, since α_3-containing receptors are characterized by a binding rate constant (k_{on}) for GABA almost two 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.

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 (increased strength of synaptic agonist pulse) were the similar TPMPA and SR-95103 sensitivity of mIPSCs in days in vitro12-14 α_1- knockout and wild type neurons (Fig.1). Moreover, the lack of significant difference in the TPMPA unbinding rates (k_{off}) in days in vitro6-8 and days in vitro12-14 neurons as deduced from recordings of current responses to ultrarapid GABA applications (Fig. 4) also argue against a post-synaptic source of such differential inhibition by TPMPA. Although the protocol used in our experiments does not give a 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 days in vitro6-8 and days in vitro12-14 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 (Overstreet et al., 2002; Mozrzymas et al., 1999, 2003b) is expected to be several folds larger than that of TPMPA. Thus, when unbinding of TPMPA molecule

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

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 due to 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 as 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 a prolonged GABAergic mIPSCs, favoring thus 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 low affinity of the postsynaptic receptors, would favor the agonist spill-over from the synaptic cleft and an increased tonic GABA concentration in the vicinity of the synapse. Spill over 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. Taking additionally 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 the 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 due to an increase in the peak or to a prolongation of agonist presence (slower clearance). It needs thus 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 while 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 e.g. in (Barberis et al., 2004; Nusser et al., 2001) and in a recent review (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 works 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; Kruck 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), GABA switch from depolarizing to hyperpolarizing (Cherubini et al., 1990; Borodinsky et al., 2003), 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.

References

Auger C and Marty A (1997) Heterogeneity of functional synaptic parameters among single release sites. *Neuron* **19**:139-150.

Banks MI and Pearce RA (2000) Kinetic differences between synaptic and extrasynaptic $GABA_A$ receptors in CA1 pyramidal cells. *J Neurosci* **20**:937-48.

Barberis A, Cherubini E and Mozrzymas JW (2000) Zinc inhibits miniature GABAergic currents by allosteric modulation of $GABA_A$ receptor gating. *J Neurosci* **20**:8618-8627.

Barberis A, Petrini E and Cherubini E. (2004) Presynaptic source of quantal size variability at GABAergic synapses in rat hippocampal neurons in culture. *Eur J Neurosci* 20:1803-1810.

Barbour B (2001) An evaluation of synapse independence. *J Neurosci.* 2001 **21**:7969-84.

Borodinsky LN, O'Leary D, Neale JH, Vicini S, Coso OA and Fiszman ML (2003) GABA-induced neurite outgrowth of cerebellar granule cells is mediated by GABA(A) receptor activation, calcium influx and CaMKII and erk1/2 pathways. *J Neurochem* **84**:1411-20.

Cherubini E, Rovira C, Gaiarsa JL, Corradetti R and Ben Ari Y (1990) GABA mediated excitation in immature rat CA3 hippocampal neurons. *Int J Dev Neurosci* **8**:481-90.

Calcagnotto ME, Barbarosie M, Avoli M (2002) Heterotopic neurons with altered inhibitory synaptic function in an animal model of malformation-associated epilepsy. *J Neurosci* **22**:7596-605.

Choi S, Klingauf J and Tsien RW (2003) Fusion pore modulation as a presynaptic mechanism contributing to expression of long-term potentiation. *Philos Trans R Soc Lond B Biol Sci* **358**:695-705.

Clements JD, Lester RA, Tong G, Jahr CE and Westbrook GL (1992) The time course of glutamate in the synaptic cleft. *Science* **258**:1498-1501.

Frerking M, Borges S and Wilson M (1995) Variation in GABA mini amplitude is the consequence of variation in transmitter concentration. *Neuron* **15**:885-895.

Gingrich KJ, Roberts WA and Kass RS (1995) Dependence of the GABAA receptor gating kinetics on the alpha-subunit isoform: implications for structure-function relations and synaptic transmission. *J Physiol* **489**:529-43.

Jonas P (1995) Fast application of agonists to isolated membrane patches, in *Single-channel recording* (Sakmann B, Neher E eds) pp.231–243. Plenum, New York.

Jones MV and Westbrook GL (1995) Desensitized states prolong $GABA_A$ channel responses to brief agonist pulses. *Neuron* **15**:181-191.

Jones MV, Jonas P, Sahara Y and Westbrook GL (2001) Microscopic kinetics and energetics distinguish $GABA_A$ receptor agonists from antagonists. *Biophys J* **81**:2660-2670.

Kleinle J, Vogt K, Luscher HR, Muller L, Senn W, Wyler K, Streit J. (1996) Transmitter concentration profiles in the synaptic cleft: an analytical model of release and diffusion. *Biophys J*. **71(5)**:2413-26.

Kruk PJ, Korn H, Faber DS. (1997) The effects of geometrical parameters on synaptic transmission: a Monte Carlo simulation study. *Biophys J.* **73(6)**:2874-90.

Laurie DJ, Wisden W and Seeburg PH (1992) The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* **12**:4151-4172.

Lavoie AM, Tingey JJ, Harrison NL, Pritchett DB and Twyman RE (1997) Activation and deactivation rates of recombinant $GABA_A$ receptor channels are dependent on alpha-subunit isoform. *Biophys J* **73**:2518-2526.

Liu G, Choi S and Tsien RW (1999) Variability of neurotransmitter concentration and nonsaturation of postsynaptic AMPA receptors at synapses in hippocampal cultures and slices. *Neuron* **22**:395-409.

Mellor JR and Randall AD (1997) Frequency-dependent actions of benzodiazepines on GABAA receptors in cultured murine cerebellar granule cells. *J Physiol* **503**:353-369.

McAllister AK and Stevens CF (2000) Nonsaturation of AMPA and NMDA receptors at hippocampal synapses. *Proc Natl Acad Sci U S A* **23**:6173-6178.

Mozrzymas JW, Barberis A, Michalak K and Cherubini E (1999) Chlorpromazine Inhibits Miniature GABAergic Currents by Reducing the Binding and by Increasing the Unbinding Rate of $GABA_A$ Receptors. *J Neurosci* **19**:2474-2488.

Mozrzymas JW, Barberis A, Mercik K and Zarnowska ED (2003a) Binding sites cooperativity, singly bound states and conformation coupling shape GABA-evoked currents. *J Neurophysiol* **89**:871-883.

Mozrzymas JW, Zarnowska ED, Pytel M and Mercik K (2003b) Modulation of GABA(A) receptors by hydrogen ions reveals synaptic GABA transient and a crucial role of the desensitization process. *J Neurosci* **23**:7981-7992.

Mozrzymas JW (2004) Dynamism of $GABA_A$ receptor activation shapes the 'personality' of inhibitory synapses. *Neuropharmacol* (in press).

Nusser Z, Cull-Candy S and Farrant M (1997) Differences in synaptic $GABA_A$ receptor number underlie variation in GABA mini amplitude. *Neuron* **19**:697-709.

Nusser Z, Naylor D and Mody I (2001) Synapse-specific contribution of the variation of transmitter concentration to the decay of inhibitory postsynaptic currents. *Biophys J* **80**:1251-1261.

Okada M, Onodera K, Van Renterghem, Sieghart W and Takahashi T (2000) Functional correlation of GABA(A) receptor alpha subunits expression with the properties of IPSCs in the developing thalamus. *J Neurosci* **20**:2202-22028.

Ortinski PI, Lu C, Takagaki K, Fu Z and Vicini S (2004) Expression of Distinct {alpha} Subunits of GABAA Receptor Regulates Inhibitory Synaptic Strength. *J Neurophysiol* Apr 21 [Epub]

Overstreet LS, Westbrook GL and Jones MV (2002) Measuring and modeling the spatiotemporal profile of GABA at the synapse, in *Transmembrane Transporters* (Quick M. ed) pp 259–275, Wiley, New York

Overstreet LS and Westbrook GL (2003) Synapse density regulates independence at unitary inhibitory synapses. *J Neurosci* **23**:2618-2626.

Pawlu C, DiAntonio A and Heckmann M (2004) Postfusional control of quantal current shape. *Neuron* **27**:607-618.

Perrais D and Ropert N (1999) Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic GABAA receptors in central synapses. *J Neurosci* **19**:578-588.

Ragozzino D, Woodward RM, Murata Y, Eusebi F, Overman LE, Miledi R. (1996) Design and in vitro pharmacology of a selective gamma-aminobutyric acidC receptor antagonist. *Mol Pharmacol*. **50(4)**:1024-30.

Renger JJ, Egles C and Liu GA (2001) Developmental switch in neurotransmitter flux enhances synaptic efficacy by affecting AMPA receptor activation. *Neuron* **29**:469-84.

Stell BM and Mody I (2002) Receptors with different affinities mediate phasic and tonic GABA(A) conductances in hippocampal neurons. *J Neurosci* **15:**RC223

Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL and Homanics GE (2001) GABA(A) receptor alpha1 subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. *J Neurosci* **21**:3009-3016.

Verdoorn (1994) Formation of heteromeric gamma-aminobutyric acid type A receptors containing two different alpha subunits. *Mol Pharmacol*. **45**:475-80.

Footnotes

Supported by NIMH grant MH64797

Corresponding Author: Andrea Barberis, Department of Physiology and Biophysics, Georgetown University School of Medicine, 225 Basic Research Bldg. 3900 Reservoir Rd, NW Washington, DC 20007, Tel. 202 6878096, Fax 202 6877407, Email: barberis@sissa.it

AB,CL,SV, & JWM: Department of Physiology and Biophysics, Georgetown University School of Medicine, Washington, DC 20007

JWM::Laboratory of Neuroscience, Department of Biophysics, Wrocław Medical University, Chałubińskiego 10, 50-368 Wrocław, Poland

Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Fig 1. mIPSC amplitude is differentially affected by TPMPA in days in vitro6-8 and days in vitro12-14 neurons. **A.** Examples of averaged GABAergic mIPSCs recorded from a days in vitro7 (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 days in vitro14 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 days in vitro (indicated below the bars) in wild type (*black bars*) and α_1 knockout (*grey bars*) neurons. Bars represent the percentage of the mIPSC peak amplitude block observed in the presence of 200 μM TPMPA. Asterisks above the bars indicate significant differences with respect to the control conditions.

Fig 2. mIPSC amplitude is differentially affected by SR-95103 (3 μM) in days in vitro6-8 (DIV6-8) and days in vitro12-14 (DIV12-14) neurons. Black bars show the statistics of the mIPSC peak amplitude block observed in the presence of SR-95103 (3 μM) in wild type in days in vitro 6-8 (DIV6-8)and days in vitro 12-14 (DIV12-14)wild type neurons. Grey bar shows the statistics of the mIPSC peak amplitude block observed in the presence of SR-95103 (3 μM) in days in vitro 12-14 α_1 knockout neurons. Asterisks above the bars indicate significant differences with respect to the control conditions.

Fig 3. Developmental changes in decay and onset kinetics of mIPSCs and their modulation by TPMPA. **A.** Averaged normalized and superimposed GABAergic mIPSCs from days in vitro 6 (DIV6), days in vitro 14 (DIV14) wild type and from days in vitro 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 bars). Averaged traces were fitted by a sum of exponential functions and the mean decay time constants were calculated as describe in Methods. In none of the considered groups, the values of mean decay time constants were significantly affected by TPMPA. Asterisks above the bars 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 a young days in vitro 7 (DIV 7) and old, days in vitro 14 (DIV 14) neurons. **D.** Statistics for the 10-90% rise time of mIPSCs in control (*black bars*) and in presence of 200 μM TPMPA (*gray bars*) in days in vitro 6-8 wild type, days in vitro 12-14 wild type and days in vitro 12-14 α1 knockout cultures. Asterisks above the bars 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 days in vitro6-8 and days in vitro12-14 neurons. **A, B.** Normalized current responses evoked by ultrafast brief (3 ms) pulse of saturating (10 mM) GABA recorded in patches excised from days in vitro 6 (A) and days in vitro 14 neurons (B) in the presence (*thin line*) and absence (*thick line*) of 400 μM TPMPA. **C.** Statistics of the TPMPA effect on the 10-90% rise times of current responses to 10 mM GABA in control

conditions (*black bars*) and in the presence of 400 μM TPMPA (*gray bars*). The culture period is indicated below the bars. Asterisks above the bars indicate significant differences with respect to the control conditions. **D.** Statistics of the TPMPA effect on current amplitude. Bars represent the current amplitudes in the presence of 400 μM TPMPA relative to the controls obtained from the same cells.

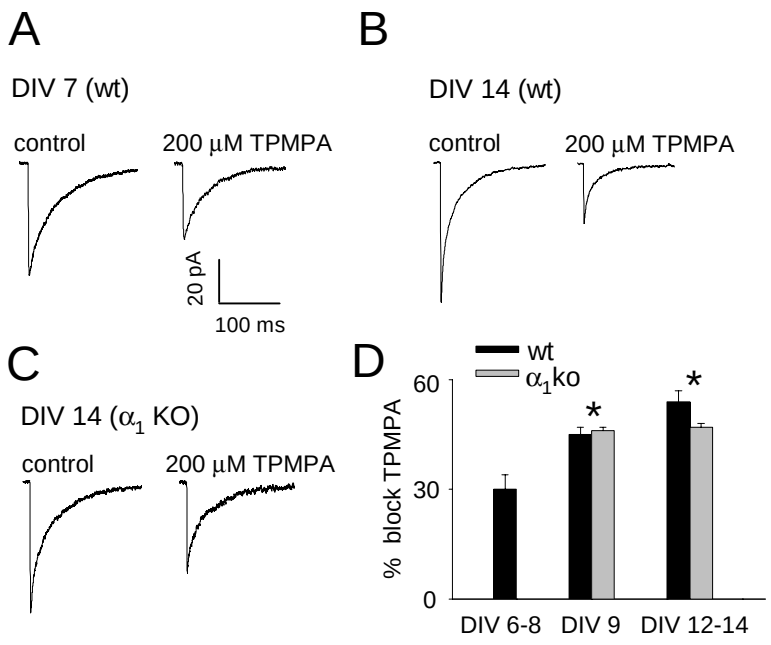
Fig 5. The decaying phase of current responses to rapid applications of brief and saturating GABA concentrations accelerates with development in vitro. A. Typical normalized current responses to saturating GABA (10 mM, 3 ms) recorded from a patch excised from days in vitro 6 and days in vitro14 neurons. Note that the decay of current recorded from the days in vitro14 neuron is considerably faster. B. Statistics of the mean decaying time constants in days in vitro 6-8 and days in vitro 12-14 neurons in control conditions (*black bars*) and in the presence of TPMPA (*gray bars*). TPMPA had no significant effect in either of the two groups of neurons. Asterisk above the bars indicates significant difference with respect to the value of τ_{mean} obtained in days in vitro6-8 neurons.

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.

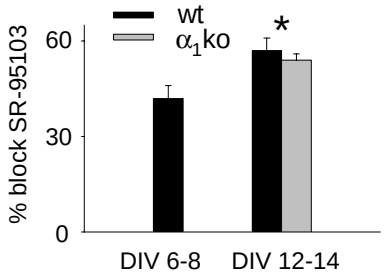
Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

Fig 1 Barberis et al.2004 MOL6437



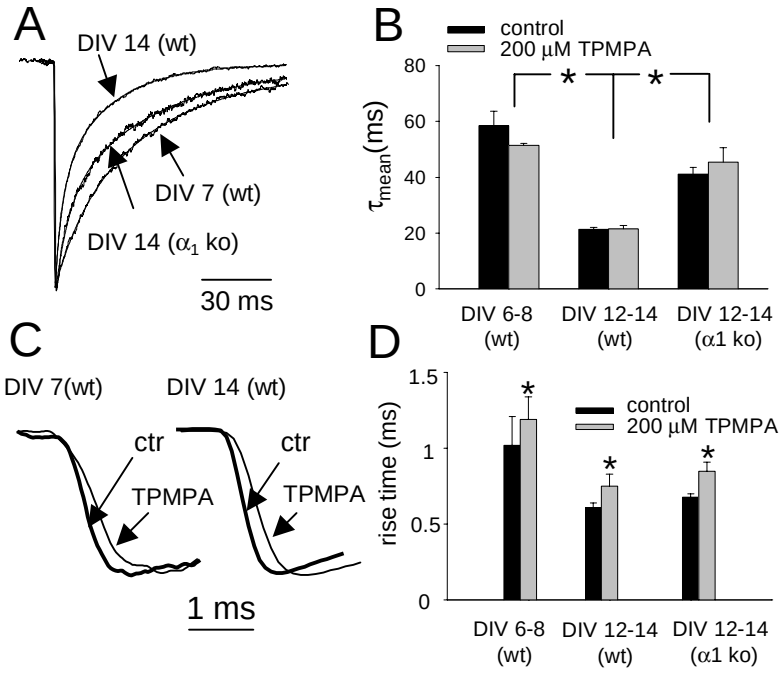
Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

Fig 2 Barberis et al.2004 MOL6437



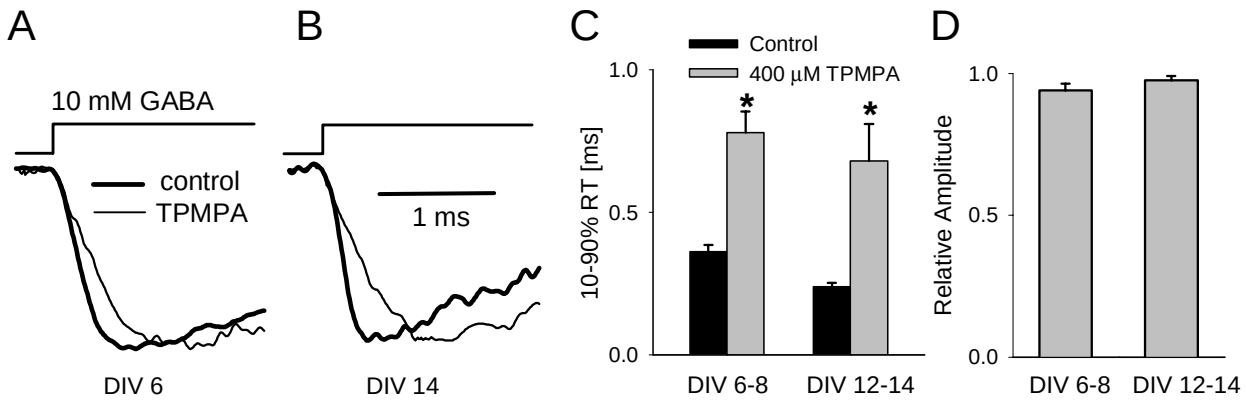
Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

Fig 3 Barberis et al.2004 MOL6437



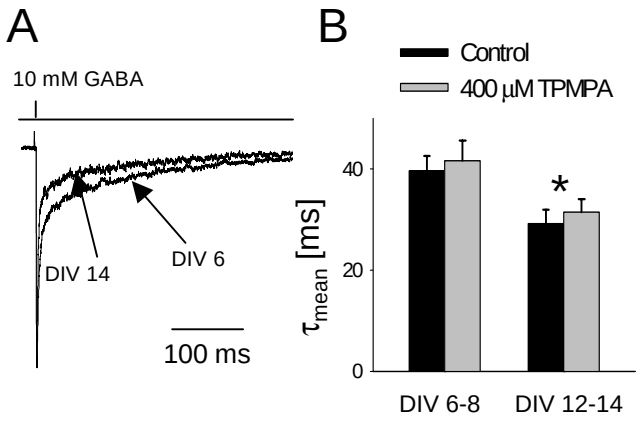
Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

Fig 4 Barberis et al.2004 MOL6437



Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

Fig 5 Barberis et al.2004 MOL6437



Molecular Pharmacology Fast Forward. Published on January 5, 2005 as DOI: 10.1124/mol.104.006437
This article has not been copyedited and formatted. The final version may differ from this version.

Fig 6 Barberis et al.2004 MOL6437