RAPID REPORT

Functional segregation of synaptic $GABA_A$ and $GABA_C$ receptors in goldfish bipolar cell terminals

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The transmission of light responses to retinal ganglion cells is regulated by inhibitory input from amacrine cells to bipolar cell (BC) synaptic terminals. $GABA_A$ and $GABA_C$ receptors in BC terminals mediate currents with different kinetics and are likely to have distinct functions in limiting BC output; however, the synaptic properties and localization of the receptors are currently poorly understood. By recording endogenous GABA receptor currents directly from BC terminals in goldfish retinal slices, I show that spontaneous GABA release activates rapid $GABA_A$ receptor miniature inhibitory postsynaptic currents (mIPSCs) (predominant decay time constant (τ_{decay}), 1.0 ms) in addition to a tonic $GABA_C$ receptor current. The $GABA_C$ receptor antagonist (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) has no effect on the amplitude or kinetics of the rapid $GABA_A$ mIPSCs. In addition, inhibition of the GAT-1 GABA transporter, which strongly regulates $GABA_C$ receptor currents in BC terminals, fails to reveal a $GABA_C$ component in the mIPSCs. These data suggest that $GABA_A$ and $GABA_C$ receptors are highly unlikely to be synaptically colocalized. Using non-stationary noise analysis of the mIPSCs, I estimate that $GABA_A$ receptors in BC terminals have a single-channel conductance (γ) of 17 pS and that an average of just seven receptors mediates a quantal event. From noise analysis of the tonic current, $GABA_C$ receptor γ is estimated to be 4 pS. Identified $GABA_C$ receptor mIPSCs exhibit a slow decay (τ_{decay}, 54 ms) and are mediated by approximately 42 receptors. The distinct properties and localization of synaptic $GABA_A$ and $GABA_C$ receptors in BC terminals are likely to facilitate their specific roles in regulating the transmission of light responses in the retina.

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Inhibition in the retina is predominantly mediated by two subtypes of ionotropic GABA receptor, $GABA_A$ and $GABA_C$ receptors, and by glycine receptors. $GABA_A$ receptors are present in most retinal cell types whereas $GABA_C$ receptors are predominantly localized to bipolar cell (BC) synaptic terminals (Enz *et al.* 1996; Koulen *et al.* 1997; Wassle *et al.* 1998). Here they function to limit BC output (Lukasiewicz & Werblin, 1994; Zhang & Slaughter, 1995; Shen & Slaughter, 2001), resulting in reduced activation of postsynaptic NMDA receptors (Matsui *et al.* 2001; Sagdullaev *et al.* 2006) and more transient ganglion cell light responses (Zhang *et al.* 1997; Dong & Werblin, 1998).

BC terminals receive GABAergic input from amacrine cells, which form both reciprocal and conventional synapses at the terminal (Dowling & Boycott, 1966; Dowling & Werblin, 1969). Activation of amacrine cell synapses evokes a response in BC terminals that comprises both a fast $GABA_A$ receptor component and a slow $GABA_C$ receptor component (Hartveit, 1999; Vigh & von Gersdorff, 2005; Eggers & Lukasiewicz, 2006). The differing time courses are likely to arise from intrinsic differences in receptor kinetics, as $GABA_A$ receptor currents evoked by exogenous GABA are much more transient than $GABA_C$ receptor currents (Qian & Dowling, 1995; Lukasiewicz & Shields, 1998; Shields *et al.* 2000; Du & Yang, 2000; Hull *et al.* 2006). In addition, $GABA_C$ receptors exhibit higher GABA affinity and a lower single-channel conductance (γ) than $GABA_A$ receptors (Feigenspan & Bormann, 1994; Qian & Dowling, 1995).

There is currently a lack of physiological evidence for the synaptic colocalization or segregation of $GABA_A$ and $GABA_C$ receptors in BC terminals. Immunolocalization studies in rat BCs suggest that the receptor subtypes are restricted to separate synaptic sites (Koulen *et al.* 1998), which would enable independent regulation of the

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transmission of light responses by $GABA_A$ and $GABA_C$ receptor pathways. In order to investigate the synaptic properties and functional localization of $GABA_A$ and $GABA_C$ receptors in BC terminals, I have analysed endogenous GABA receptor currents recorded directly from the synaptic terminals of BCs in goldfish retinal slices.

Methods

The experiments conformed with guidelines laid down by the animal welfare committee of Keele University. Retinal slices were prepared from goldfish (*Carassius auratus*; 8–14 cm) after 1 h dark-adaptation. Goldfish were killed by decapitation followed immediately by destruction of the brain and spinal cord. The eyeballs were removed and retinae dissected out and treated for 20 min with hyaluronidase to remove vitreous humor. Each retina was quartered, placed ganglion cell layer down on filter paper and kept until needed at 4°C in medium containing (mM): NaCl 127, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 1, Hepes 5 and glucose 12, pH adjusted to 7.45 with NaOH. Slices were cut at 250 μm intervals using a Narishige ST-20 slicer, transferred to the recording chamber and perfused (1 ml min^{-1}) with medium containing (mM): NaCl 108, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2.5, $NaHCO_3$ 24 and glucose 12, gassed with 95% O_2–5% CO_2, pH 7.4. Slice preparation and recordings were performed at room temperature (20–23°C), in daylight conditions. Drugs were bath applied in the perfusing medium. Picrotoxin, (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide (NBQX) and nifedipine were obtained from Tocris; bicuculline, strychnine and all other chemicals and salts were obtained from Sigma-Aldrich.

Whole-cell recordings were obtained from isolated BC terminals in retinal slices as previously described (Palmer *et al.* 2003). This technique maximizes the recording resolution of terminal GABA receptor currents and eliminates currents arising from somatodendritic receptors. Patch pipettes (5–8 MΩ) were pulled from borosilicate glass and filled with solution containing (mM): CsCl 115, Hepes 25, TEA-Cl 10, Mg-ATP 3, Na-GTP 0.5 and EGTA 0.5; pH 7.2. CsCl-based intracellular solution was used to increase the driving force through GABA receptors at a holding potential of −60 mV. The majority of recordings (32/39) were made in the presence of the AMPA receptor antagonist NBQX (5 μM) to reduce amacrine cell activity; however, no significant differences in GABA receptor properties were observed between recordings with and without NBQX.

Data acquisition was controlled by Heka Patchmaster software and signals were recorded via a Heka EPC-10 patch-clamp amplifier. Off-line analysis was performed using Wavemetrics IgorPro software. Miniature inhibitory postsynaptic currents (mIPSCs) were identified by rate of rise, aligned for averaging and analysed using IgorPro macros kindly provided by Dr H. Taschenberger. The peak amplitude of average mIPSCs was dependent on the mIPSC detection threshold, which could be lower in low-noise recordings. For comparison between different pharmacological conditions, the threshold was kept constant.

To estimate the frequency of $GABA_C$ mIPSCs underlying the tonic current, the plateau current evoked by summated mIPSC waveforms (instantaneous rise followed by exponential decay; amplitude, −10 pA; decay time constant (τ_{decay}), 54 ms) at frequencies of between 1 and 50 Hz was computed using Matlab software. The relationship between mean plateau current and frequency was linear and was approximately described by: mean current = frequency × amplitude × τ_{decay}.

Peak-scaled non-stationary noise analysis of $GABA_A$ mIPSCs was performed as previously described for synaptic currents (Traynelis *et al.* 1993; De Koninck & Mody, 1994). Baseline-subtracted mIPSCs exhibiting a fast rise time and no additional spontaneous activity were averaged, the mean mIPSC was peak-scaled to individual mIPSCs and the variance of the decay around the mean was measured. The average binned variance (σ^2) was plotted against mean mIPSC amplitude (I) and fitted with:

$$\sigma^2 = iI - I^2/N + b$$

to give estimates of single-channel current (i), the average number of channels open at the peak of the current (N) and baseline variance (b). For noise analysis of the $GABA_C$ tonic current, the variance of current traces (0.2–0.5 s duration) recorded during the current potentiation by the GAT-1 inhibitor NO-711 was measured, using only traces that were well fitted by a straight line. A plot of variance against mean current amplitude was fitted as above to yield an estimate of i. γ for $GABA_A$ and $GABA_C$ receptors was obtained from $\gamma = i/V$, with V being the driving force for Cl^-.

Pooled data are expressed as means ± S.E.M.; statistical significance was assessed using Student's paired t tests, with $P < 0.05$ considered significant.

Results

Fluctuating inward current was observed in isolated (axon-severed) BC terminals in retinal slices recorded with high intracellular [Cl^-] at −60 mV. The current consisted of a tonic component (−18 ± 2 pA, $n = 25$ terminals) plus mIPSCs (see below; Fig. 1*A*). The mIPSCs had a mean peak amplitude of −13.7 ± 0.9 pA, a 10–90% rise time of 0.29 ± 0.01 ms and a bi-exponential decay with time constants of 1.01 ± 0.03 and 16.7 ± 1.4 ms, with the fast time constant accounting for 78 ± 1% of the decay ($n = 25$, 195 ± 12 mIPSCs analysed per terminal; Fig. 1*B*). In the

presence of nifedipine (20 μM) to block voltage-gated Ca^{2+} channel activation, the mIPSCs reversed polarity at around 0 mV, which is approximately the Cl^- equilibrium potential ($n = 3$; Fig. 1*A*). Application of the glycine receptor antagonist strychnine (1 μM) had no effect on mIPSC amplitude or kinetics ($n = 4$; data not shown), consistent with an absence of glycine receptors in goldfish bipolar cells (Kaneko *et al.* 1991).

The $GABA_A$/$GABA_C$ receptor antagonist picrotoxin (50 μM) reduced the tonic current (from -27 ± 7 to -5 ± 3 pA, $n = 3$, $P < 0.05$) and abolished the mIPSCs (Fig. 1*C*). The tonic current was also sensitive to the $GABA_C$ receptor antagonist TPMPA (50 μM; current reduced from -23 ± 4 to -7 ± 2 pA, $n = 8$, $P < 0.05$) but the mIPSCs were not abolished (Fig. 1*D*). By contrast, the $GABA_A$ receptor antagonist bicuculline (50 μM) inhibited

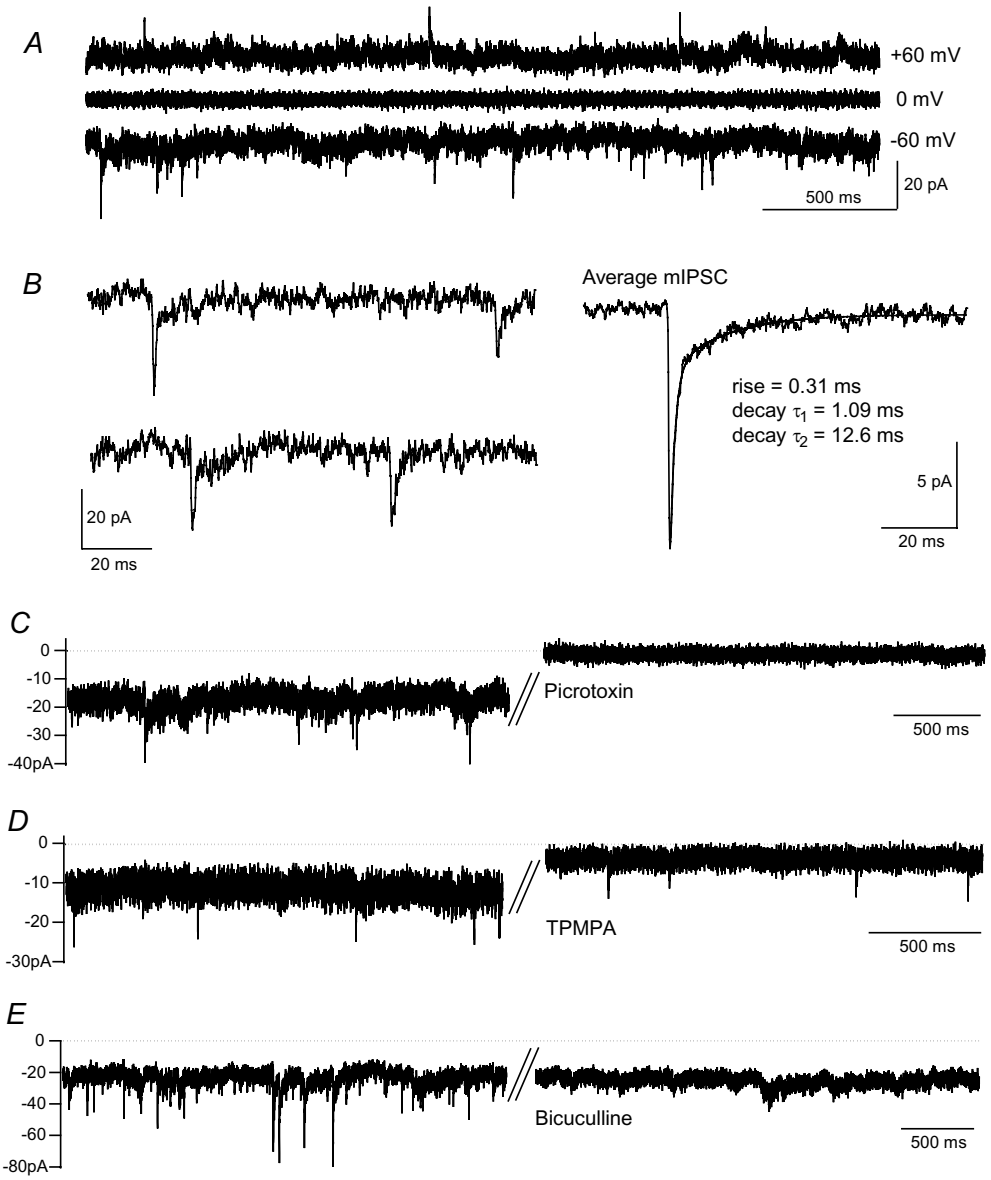

Figure 1. $GABA_A$ and $GABA_C$ receptors mediate phasic and tonic components of the endogenous GABA current in BC terminals

A, spontaneous membrane currents recorded with CsCl-based intracellular solution reversed polarity at around 0 mV. Nifedipine (20 μM) was present to inhibit L-type Ca^{2+} channel activation. *B*, individual mIPSCs from the terminal in *A* on an expanded time scale, and the average mIPSC in this terminal ($n = 158$). *C*, the mIPSCs and the tonic current were inhibited by the $GABA_A$/$GABA_C$ receptor antagonist picrotoxin (50 μM). *D*, the tonic current alone was inhibited by the $GABA_C$ receptor antagonist TPMPA (50 μM). *E*, the mIPSCs alone were inhibited by the $GABA_A$ receptor antagonist bicuculline (50 μM).

the mIPSCs but left the tonic current intact (control, -19 ± 3 pA; bicuculline, -20 ± 2 pA, $n = 3$; Fig. 1*E*). $GABA_A$ receptors therefore mediate fast, transient synaptic currents in BC terminals whereas $GABA_C$ receptors underlie a slow baseline conductance.

If $GABA_A$ and $GABA_C$ receptors are present at the same synapses in BC terminals, mIPSCs would be expected to exhibit both receptor components. The kinetics of mIPSCs were therefore compared before and after application of TPMPA (50 μM). TPMPA was found to have no effect on mIPSC decay times, as shown in Fig. 2*A* and *B*, or on mIPSC amplitude (control: -13.3 ± 1.3 pA, 195 ± 33 mIPSCs; TPMPA: -13.3 ± 1.4 pA, 183 ± 33 mIPSCs; $n = 8$ terminals). Inhibition of the GABA transporter GAT-1 has recently been shown to increase the $GABA_C$ tonic current in BC terminals (Hull *et al.* 2006). To determine whether GAT-1 may limit the activation of perisynaptic $GABA_C$ receptors at $GABA_A$ synapses, mIPSCs were compared in the absence and presence of the GAT-1 inhibitor NO-711 (3 μM). As shown in Fig. 2*C* and *D*, mIPSC decay kinetics were unaffected by NO-711 (control: 80 ± 11 mIPSCs; NO-711: 38 ± 8 mIPSCs; $n = 8$ terminals). Comparison of average mIPSC amplitudes was not meaningful because of the difficulty in detecting small mIPSCs within the increased current noise in the presence of NO-711. The increase in the tonic current was subsequently reversed to baseline with TPMPA (50–100 μM), again with no change in mIPSC kinetics ($n = 8$; data not shown). Spontaneous exocytosis at $GABA_A$ receptor synapses therefore does not appear to activate $GABA_C$ receptors, even under conditions of GAT-1 inhibition.

Amplitude histograms of mIPSCs recorded in the presence of TPMPA (50 μM) consisted of a single peak with a tail of larger values and had a mode of -7.9 ± 0.4 pA ($n = 8$, 386 ± 29 mIPSCs per terminal; Fig. 3*A*). The histograms were best fitted by the sum of two Gaussians: the mean of the first Gaussian was -7.3 ± 0.3 pA and showed little variability between recordings (coefficient of variation (CV) = 0.10; Fig. 3*B*); the mean of the second Gaussian was -12.5 ± 1.1 pA ($n = 8$; Fig. 3*A*). The histogram peak therefore gives a quantal amplitude of approximately -7 pA for $GABA_A$ synapses. The tail of larger amplitude mIPSCs could have a variety of causes, including variability in the number of synaptic $GABA_A$ receptors (Nusser *et al.* 1997) or simultaneous release at several amacrine cell–BC terminal synapses.

An estimate of the single-channel conductance (γ) of the $GABA_A$ receptors was obtained from peak-scaled non-stationary noise analysis of mIPSCs recorded in the presence of TPMPA (Fig. 3*C* and *D*). The mean single-channel current obtained from variance *versus* amplitude plots was -1.0 ± 0.1 pA, equating to γ of 17 ± 1 pS ($n = 7$, 113 ± 13 mIPSCs analysed per terminal). From this estimate, a BC terminal quantal $GABA_A$ response is mediated by, on average, seven receptors.

For comparison, I investigated whether quantal $GABA_C$ receptor events could be observed in the presence of $GABA_A$ receptor antagonism. Application of bicuculline (25–50 μM) often evoked or potentiated slow oscillations in the tonic current (Fig. 4*A*), which were variable in amplitude and duration between recordings (-20 to -130 pA, 0.5–9 s; $n = 9$). The oscillations were reduced or blocked by a high concentration of TPMPA (100–200 μM, $n = 5$; Fig. 4*A*) or by picrotoxin (50 μM, $n = 2$). In some terminals, smaller TPMPA-sensitive events that resembled postsynaptic currents were occasionally observed within the tonic current (arrows in Fig. 4*B*). A subpopulation of these events exhibited a single, fast rising phase and were identified as $GABA_C$ mIPSCs (Fig. 4*C*). Average $GABA_C$ mIPSCs had a peak amplitude of -10.0 ± 0.4 pA, which showed little variability between terminals (CV = 0.10; Fig. 4*D*), a 10–90% rise time of 1.0 ± 0.1 ms and a mono-exponential decay with a time constant of 54 ± 6 ms ($n = 6$, 13 ± 2 mIPSCs per terminal). This decay time is very similar to the value of 51 ms reported for putative $GABA_C$ IPSCs in mouse rod BCs (Frech & Backus, 2004). $GABA_C$ mIPSCs therefore exhibit significantly slower decay kinetics than $GABA_A$ mIPSCs (Fig. 4*C*). Assuming that the $GABA_C$ tonic current arises from the summation of spontaneous mIPSCs, a simple convolution model of the $GABA_C$ mIPSC waveform was used to estimate the frequency of those events. The average TPMPA-sensitive tonic current of -17 ± 2 pA ($n = 8$) would be evoked by mIPSCs at a frequency of approximately 30 Hz.

$GABA_C$ receptor γ was estimated from noise analysis of the tonic current during potentiation by the GAT-1 inhibitor NO-711 (3 μM), in the presence of bicuculline (25–50 μM; Fig. 4*E*). Plots of current variance *versus* amplitude gave a mean single-channel current of -0.24 ± 0.05 pA, equating to a γ value of 4.0 ± 0.7 pS ($n = 4$; Fig. 4*F*). From this estimate, the average maximum current in the presence of NO-711 (-325 ± 86 pA, $n = 4$) is mediated by approximately 1350 $GABA_C$ receptors, which may reflect the total number of $GABA_C$ receptors per terminal, and a $GABA_C$ mIPSC is mediated by approximately 42 receptors.

Discussion

The results of this study demonstrate that $GABA_A$ and $GABA_C$ receptors in BC terminals are activated independently by spontaneous GABA release and mediate currents with very different kinetics. $GABA_A$ mIPSCs are rapid and transient whereas $GABA_C$ mIPSCs decay slowly and give rise to a tonic current. $GABA_A$ mIPSCs exhibit no $GABA_C$ component, either in control conditions or following inhibition of GAT-1.

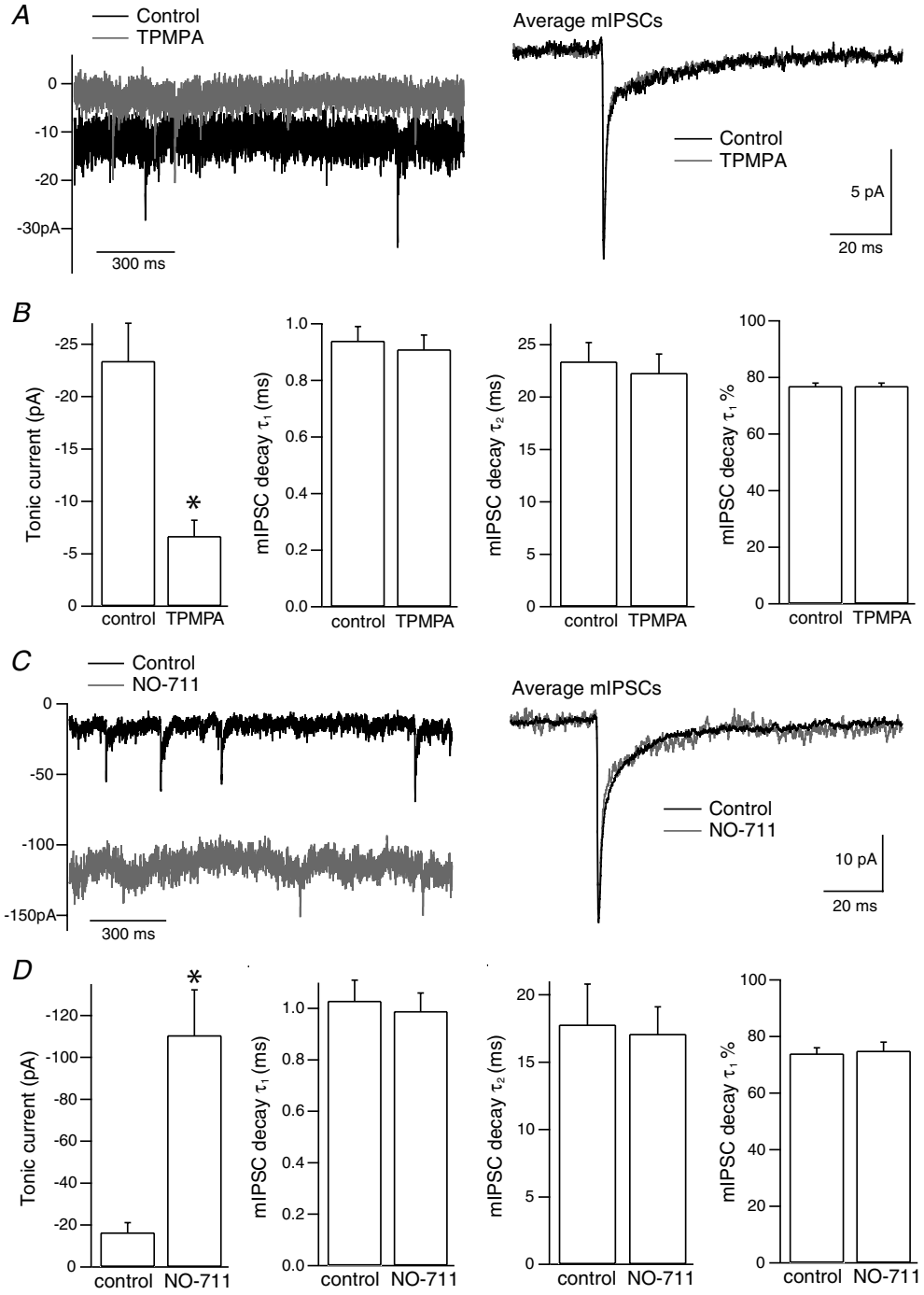

Figure 2. GABA$_C$ receptors are not activated by spontaneous release at GABA$_A$ synapses
A, current traces from a terminal before and during application of TPMPA (50 μM), with the superimposed average mIPSCs in the two conditions (control, $n = 100$; TPMPA, $n = 102$). *B*, mean data from eight terminals showing that TPMPA reduced the tonic current but had no significant effect on mIPSC decay kinetics (τ_1, fast time constant; τ_2, slow time constant; τ_1%, percentage contribution of τ_1 to the decay). *C*, current traces from a terminal before and during application of the GAT-1 inhibitor NO-711 (3 μM), with the superimposed average mIPSCs in the two conditions (control, $n = 74$; NO-711, $n = 43$). *D*, mean data from eight terminals showing that NO-711 greatly potentiated the tonic current but had no effect on mIPSC decay kinetics. In *B* and *D*, error bars represent S.E.M., $^*P < 0.05$.

The results are consistent with a model in which $GABA_C$ receptors are excluded from $GABA_A$ synapses in BC terminals. $GABA_C$ receptors are therefore located at separate synapses and/or extrasynaptically. The strong punctate staining of $GABA_C$ receptor ρ subunits in BC terminals (Enz *et al.* 1996; Koulen *et al.* 1997, 1998; Fletcher *et al.* 1998) and the occurrence of fast-rising $GABA_C$ mIPSCs are most consistent with a synaptic localization. Conversely, the strong regulation of the $GABA_C$ current by GAT-1 would seem to suggest an extrasynaptic localization. However, due to the complete lack of desensitization of $GABA_C$ receptor currents (Hull *et al.* 2006), $GABA_C$ receptors within synapses would also be regulated by the activity of GABA transporters. Indeed, the rate of decay of the $GABA_C$ mIPSCs (τ_{decay} ~54 ms) may reflect the rate of clearance of GABA from the synaptic cleft by diffusion and uptake. This may explain some of the variability in τ_{decay} between terminals (Fig. 4*D*).

The estimated γ values for $GABA_A$ and $GABA_C$ receptors in BC terminals (17 and 4 pS, respectively) are similar to values previously obtained from exogenous GABA application to isolated BCs. Estimates of γ for $GABA_A$ and $GABA_C$ were, respectively, 10 and 4 pS in hybrid bass BCs (Qian & Dowling, 1995) and 30 and 8 pS in rat BCs (Feigenspan & Bormann, 1994). It is interesting that the estimated γ of $GABA_A$ receptors mediating a tonic current in hippocampal neurons was ~6 pS, significantly lower than that of $GABA_A$ receptors mediating fast mIPSCs in the same neurons (Bai *et al.* 2001). In BC terminals, the small γ of $GABA_C$ receptors appears to be compensated by a greater number of activated receptors per synapse, resulting in a similar quantal amplitude for $GABA_A$ and $GABA_C$ receptor synapses.

The apparent segregation of $GABA_A$ and $GABA_C$ receptors to different synapses in BC terminals will enable independent functioning and regulation of these kinetically distinct forms of inhibition. It will be interesting to determine whether particular classes of amacrine cell form only $GABA_A$ or $GABA_C$ receptor synapses. The specific roles of $GABA_A$ and $GABA_C$ receptor inhibition in retinal processing are at present

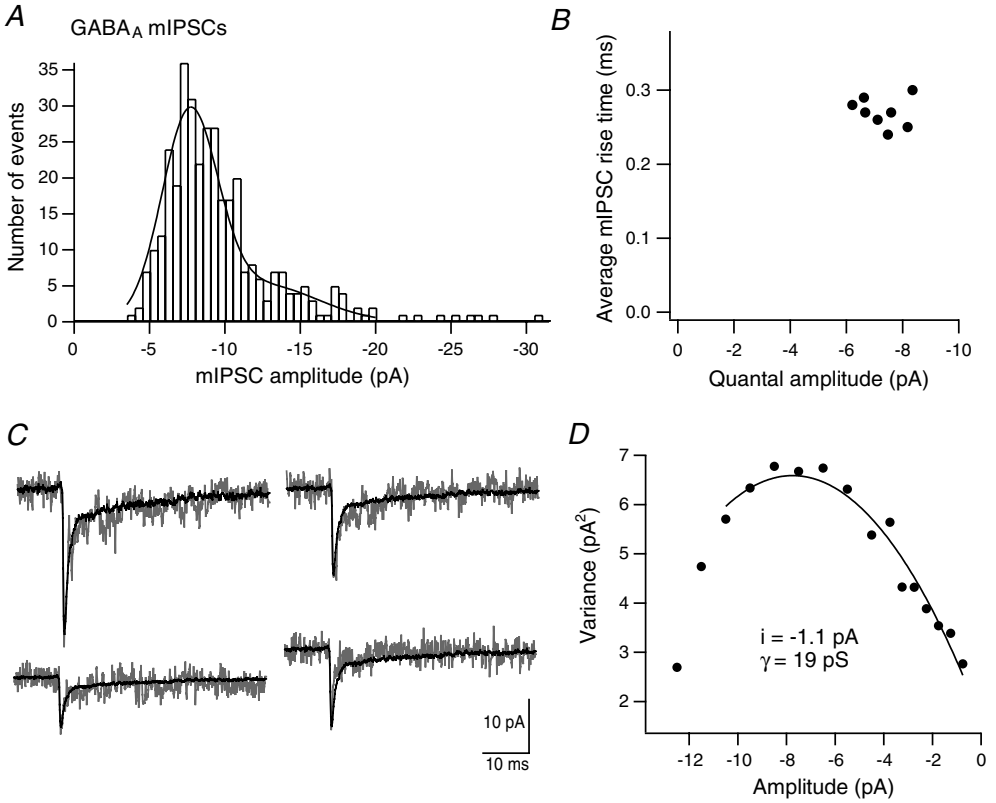

Figure 3. Single-channel properties underlying $GABA_A$ receptor mIPSCs
A, the mIPSC amplitude histogram for one terminal in the presence of TPMPA (50 μM) fitted with the sum of two Gaussians (first mean, −7.6 pA; second mean, −13.6 pA). *B*, average mIPSC 10–90% rise time *versus* quantal amplitude (peak of mIPSC amplitude histogram) for eight terminals. *C*, example mIPSCs from the terminal in *A*, with the peak-scaled average mIPSC superimposed for noise analysis. *D*, plot of mean current variance *versus* amplitude for this terminal. The curve was fitted to yield an estimate of single-channel current (*i*).

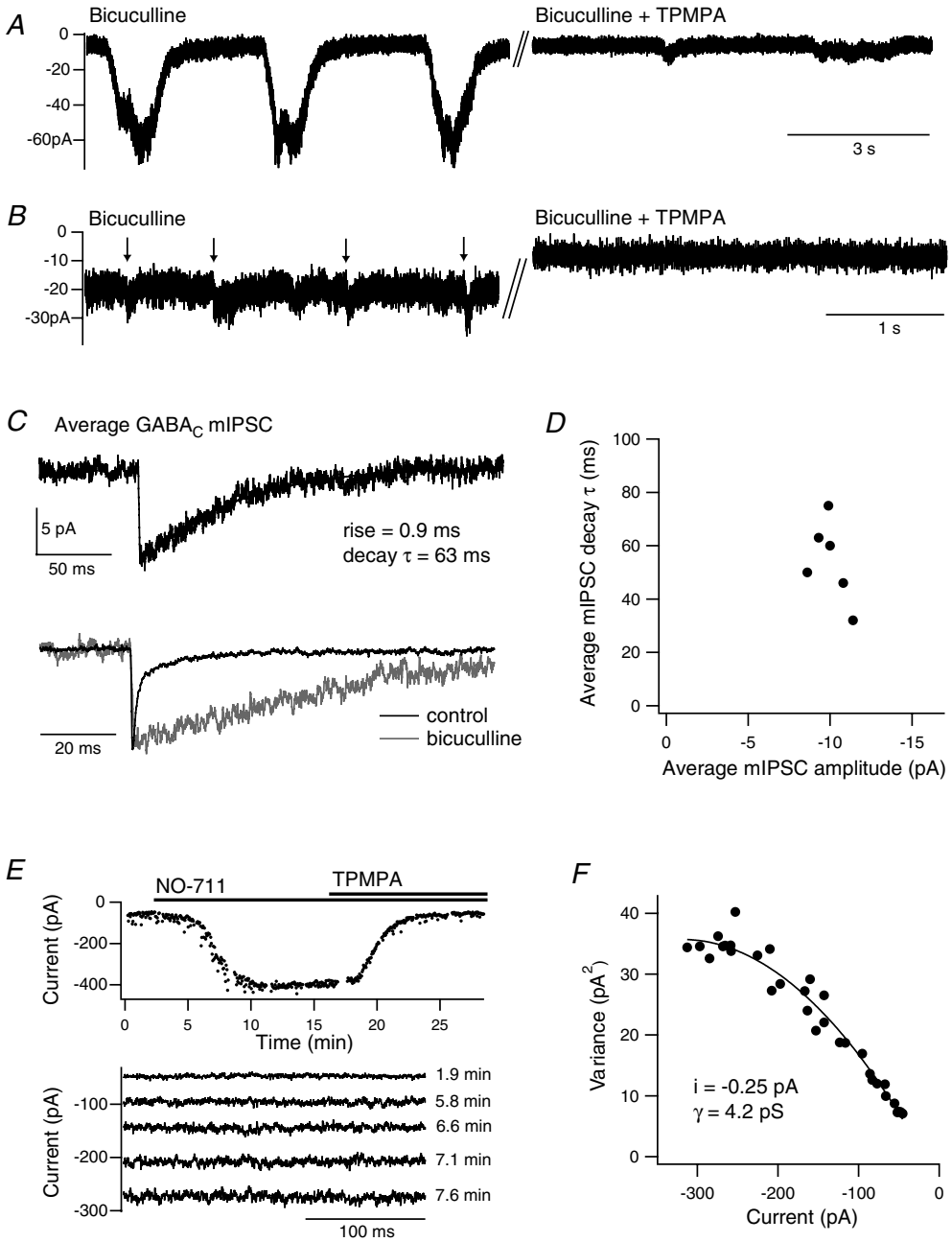

Figure 4. Single-channel properties underlying $GABA_C$ receptor currents
A, example traces showing the slow current oscillations in the presence of bicuculline (25 μM) and their inhibition by a high concentration of TPMPA (200 μM). *B*, in some terminals, small synaptic currents (marked by arrows) were observed within the tonic current. They were inhibited by TPMPA (50 μM). *C*, the average $GABA_C$ mIPSC in the presence of bicuculline (50 μM) in one terminal ($n = 16$). Only events with a single, fast rising phase were included. Below, the $GABA_C$ mIPSC is peak scaled and superimposed with the $GABA_A$ mIPSC from the same terminal prior to application of bicuculline ($n = 180$). *D*, average mIPSC decay time constant *versus* peak amplitude for six terminals. *E*, mean current amplitude *versus* time for a terminal in the presence of bicuculline (25 μM), showing the potentiation of the tonic current by NO-711 (3 μM) and subsequent inhibition by TPMPA (100 μM). Below are example current traces from selected time points during the potentiation. *F*, current variance *versus* amplitude for the terminal in *E*, fitted to yield an estimate of single-channel current (*i*).

unclear, although $GABA_C$ receptors are known to limit BC exocytosis during light responses. The prolonged time course of $GABA_C$ feedback inhibition is particularly suited to regulating sustained exocytosis from BCs (Vigh & von Gersdorff, 2005). $GABA_C$ receptors also have the potential to control regenerative potentials in BC terminals via effects on membrane conductance (Hull *et al.* 2006). The large slow oscillations in the $GABA_C$ tonic current observed in the present study suggest that membrane conductance may be continuously modulated by networked amacrine cell activity. By contrast, the rapid time course of the $GABA_A$ feedback current is suited to regulating phasic exocytosis from BCs. $GABA_A$ receptors have recently been shown to inhibit exocytosis from rod BCs during light responses, although to a lesser extent than $GABA_C$ receptors (Eggers & Lukasiewicz, 2006). Building on the current evidence for synaptic segregation of $GABA_A$ and $GABA_C$ receptors in BC terminals, further work will determine their mechanisms of regulation and specific functions in retinal processing.

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