

# GABA<sub>B</sub> Receptors Regulate Chick Retinal Calcium Waves

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Correlated spiking activity and associated Ca<sup>2+</sup> waves in the developing retina are important in determining the connectivity of the visual system. Here, we show that GABA, via GABA<sub>B</sub> receptors, regulates the temporal characteristics of Ca<sup>2+</sup> waves occurring before synapse formation in the embryonic chick retina. Blocking ionotropic GABA receptors did not affect these Ca<sup>2+</sup> transients. However, when these receptors were blocked, GABA abolished the transients, as did the GABA<sub>B</sub> agonist baclofen. The action of baclofen was prevented by the GABA<sub>B</sub> antagonist *p*-3-aminopropyl-*p*-diethoxymethyl phosphoric acid (CGP35348). CGP35348 alone increased the duration of the transients, showing that GABA<sub>B</sub> receptors are tonically activated by endogenous GABA. Blocking the GABA transporter GAT-1 with 1-(4,4-diphenyl-3-butenyl)-3-piperidine carboxylic acid (SKF89976A) reduced the frequency of the transients. This reduction was prevented by CGP35348 and thus resulted from activation of GABA<sub>B</sub> receptors by an in-

crease in external [GABA]. The effect of GABA<sub>B</sub> receptor activation persisted in the presence of activators and blockers of the cAMP–PKA pathway. Immunocytochemistry showed GABA<sub>B</sub> receptors and GAT-1 transporters on ganglion and amacrine cells from the earliest times when Ca<sup>2+</sup> waves occur (embryonic day 8). Patch-clamp recordings showed that K<sup>+</sup> channels on ganglion cell layer neurons are not modulated by GABA<sub>B</sub> receptors, whereas Ca<sup>2+</sup> channels are; however, Ca<sup>2+</sup> channel blockade with  $\omega$ -conotoxin-GVIA or nimodipine did not prevent Ca<sup>2+</sup> waves. Thus, the regulation of Ca<sup>2+</sup> waves by GABA<sub>B</sub> receptors occurs independently of N- and L-type Ca<sup>2+</sup> channels and does not involve K<sup>+</sup> channels of the ganglion cell layer. GABA<sub>B</sub> receptors are likely to be of key importance in regulating retinal development.

**Key words:** retina; ganglion cells; Ca<sup>2+</sup>; spontaneous activity; GABA<sub>B</sub> receptors; patch-clamping

Spontaneous electrical activity plays a pivotal role in the development of the nervous system through the changes it produces in intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>). Electrical activity and associated changes in [Ca<sup>2+</sup>]<sub>i</sub> regulate the expression of ion channels and transmitter phenotype (Gu and Spitzer, 1995, 1997), dendritic geometry (Bodnarenko et al., 1995), neurite extension (Fields et al., 1990; Gomez and Spitzer, 1999), and synapse formation (Constantine-Paton et al., 1990; Kalil, 1990; Katz and Shatz, 1996). These experiments have shown that the frequency and kinetics of [Ca<sup>2+</sup>]<sub>i</sub> transients are key determinants of the development of the nervous system.

In the retina, spontaneous electrical activity takes the form of rhythmic bursts of action potentials that spread between adjacent ganglion and amacrine cells and produce transient elevations in [Ca<sup>2+</sup>]<sub>i</sub>. These waves have been described for both mammalian (Meister et al., 1991; Wong et al., 1993, 1995; Catsicas and Mobbs, 1995) and chick (Catsicas et al., 1998; Wong et al., 1998) retina and are necessary for the formation of the appropriate synaptic connections by ganglion cells within the lateral geniculate nucleus and optic tectum (Mooney et al., 1996; Wong, 1999). This activity is likely instructive in shaping cortical development (Weliky, 1999). However, the role of synchronized electrical activity and Ca<sup>2+</sup> waves within the retina is unknown.

At early developmental stages in the mammalian retina, initi-

ation and propagation of correlated activity requires cholinergic transmission (Feller et al., 1996; Penn et al., 1998), whereas at later stages, GABAergic, glycinergic, and glutamatergic transmission can modulate wave generation (Fischer et al., 1998; Wong, 1999). In contrast, in the chick retina, Ca<sup>2+</sup> wave production does not require cholinergic transmission, but endogenous ACh modulates the temporal pattern of the activity (Catsicas et al., 1998). Activation of other neurotransmitter receptors also changes the frequency with which Ca<sup>2+</sup> waves are produced; however, activation of GABA<sub>A</sub> receptors does not contribute to wave production at early times (Catsicas et al., 1998; Wong et al., 1998). This is surprising on several counts. First, GABA is released during early chick retinal development (Bonness et al., 1996). Second, at these ages, GABA is depolarizing and activation of GABA<sub>A</sub> receptors leads to Ca<sup>2+</sup> entry via voltage-gated channels (Yamashita and Fukuda, 1993). Third, in both mammalian and chick retina, the major source of ACh is a subpopulation of amacrine and displaced amacrine cells that also express and release GABA (O'Malley et al., 1992; Santos et al., 1998). Thus, GABA is a good candidate for modulating Ca<sup>2+</sup> waves.

Here, we show that endogenous GABA modulates the frequency and duration of [Ca<sup>2+</sup>]<sub>i</sub> waves in the chick retina from the earliest times at which such activity can be detected [embryonic day 8 (E8)]. This effect is mediated by GABA<sub>B</sub> receptors in a cAMP-independent manner, influenced by the activity of the GABA transporter 1 (GAT-1), and is independent of the modulation of Ca<sup>2+</sup> channels by GABA<sub>B</sub> receptors.

## MATERIALS AND METHODS

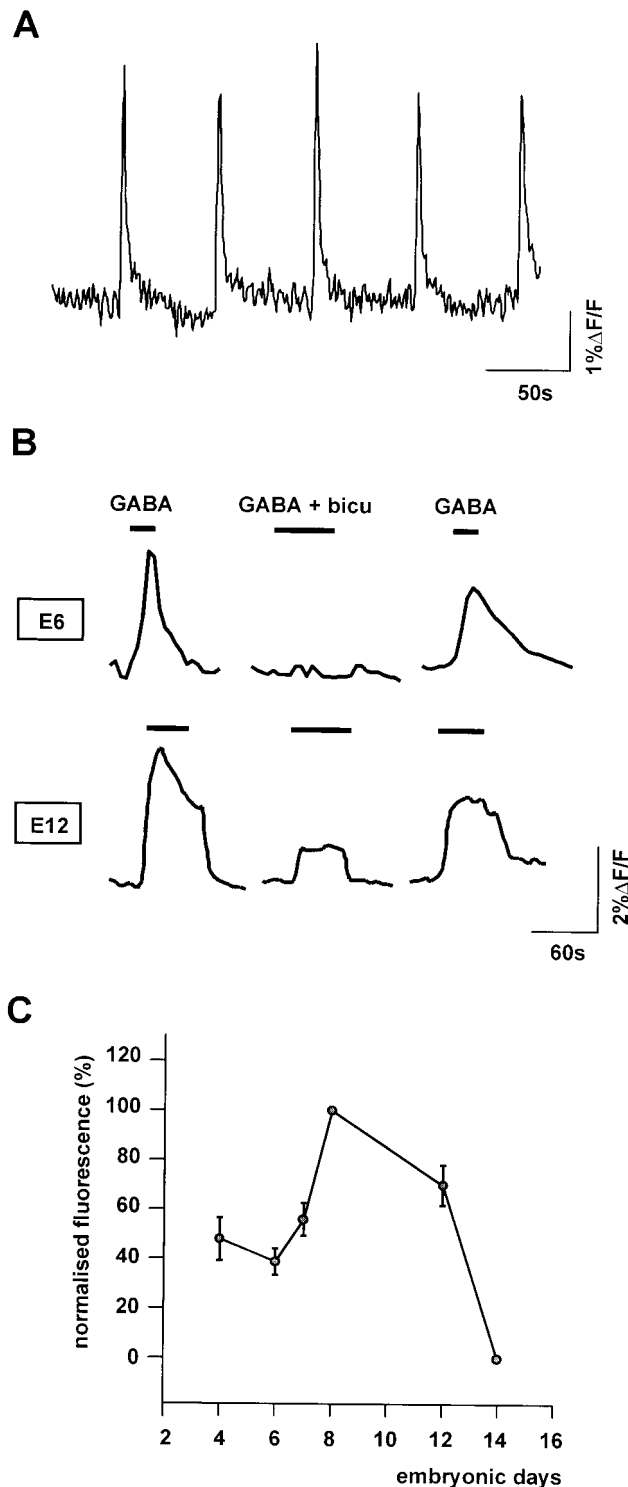
Chicken White Leghorn eggs were incubated at 37°C and 60% humidity. Chicken embryos between E8 and E12 were decapitated, and their eyes removed.

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**Figure 1.** Iontropic GABA receptors are expressed and depolarize retinal cells when  $\text{Ca}^{2+}$  waves can first be detected. *A*, Spontaneous  $\text{Ca}^{2+}$  transients from an E9 retina in control situation. The levels of fluorescence averaged over the field of view were normalized to the basal level at the beginning of the recording session. *B*, Traces plotting the changes in fluorescence in an E6 (top) and an E12 (bottom) retina. Application of GABA (100  $\mu\text{M}$ ) elicited a transient increase in  $[\text{Ca}^{2+}]_i$  at both ages (left traces), which was largely prevented by coapplication of the GABA<sub>A</sub> receptor antagonist bicuculline (50  $\mu\text{M}$ ; middle traces). The response to GABA recovered after 15 min washout of bicuculline (right traces). *C*, Developmental profile of the response to GABA. The amplitude of the responses is plotted as a percentage of the maximum response, observed at E8. All responses observed were increases in  $[\text{Ca}^{2+}]_i$ . GABA responses

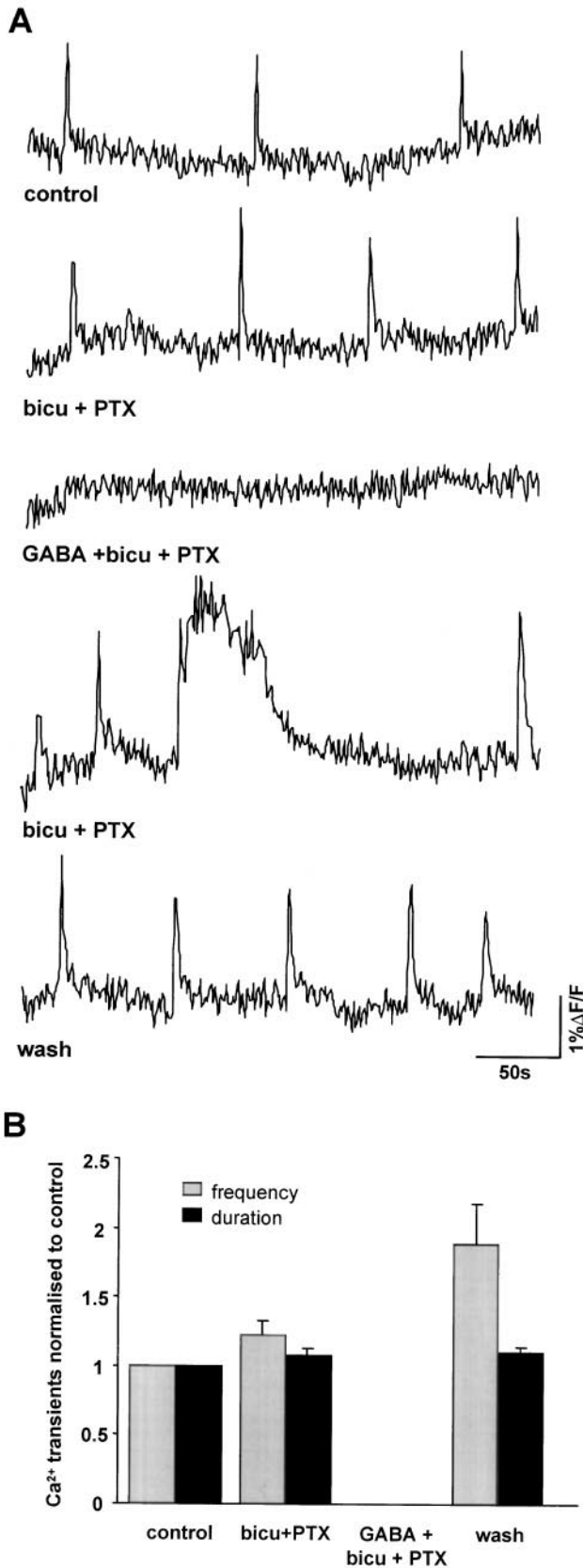
**Calcium imaging.** The retinas were dissected from the eyecup at room temperature in Krebs' solution containing (in mM): 100 NaCl, 30  $\text{NaHCO}_3$ , 6 KCl, 1  $\text{MgCl}_2$ , 1  $\text{CaCl}_2$ , 3  $\text{NaH}_2\text{PO}_4$ , and 20 glucose, pH 7.4 by gassing with 5%  $\text{CO}_2$ –95%  $\text{O}_2$ . After dissection, the retinas were loaded with the  $\text{Ca}^{2+}$ -sensitive dye Calcium Green-1 AM (10  $\mu\text{M}$ ; Molecular Probes, Eugene, OR) in Krebs' solution in the presence of the dispersant Cremophor-EL (0.03%; Sigma, Poole, UK) for 1 hr at room temperature. After loading, the retinas were maintained in fresh Krebs' solution at room temperature for up to 2 hr before use. The central piece of the retina ( $\sim 5 \times 5$  mm) was transferred to a perfusion chamber on the stage of an inverted microscope and imaged at 35°C, held flat with a nylon mesh glued to a platinum harp, and continually superfused with Krebs' solution delivered through a peristaltic pump.

**Imaging data acquisition and analysis.** Retinas were imaged as flat mounts, with the ganglion cell surface facing the objective, using an inverted epifluorescence microscope (Axiovert 100; Zeiss, Oberkochen, Germany) equipped with a fluorescein filter set (XF22; Omega Optical, Stanmore, UK) and a Zeiss Fluor 40 $\times$  lens (numerical aperture of 1.3). Fluorescence images were recorded using a slow-scan cooled CCD camera (model number 2000; Digital Pixell). Images were acquired at 1 Hz and analyzed off-line using Lucida 3.5 software (Kinetic Imaging Ltd., Liverpool, UK). The mean fluorescence (imaged field of view,  $\sim 100 \times 200 \mu\text{m}$ ) was calculated and normalized to its initial value at time 0. Increases in the fluorescence of Calcium Green-1 reflect increases in  $[\text{Ca}^{2+}]_i$ .

**Electrophysiology.** Membrane currents from amacrine and ganglion cells in the ganglion cell layer (GCL) of E9–E12 chick retinas were measured in the whole-cell clamp configuration using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Patch pipettes were pulled from thick-walled borosilicate glass with internal filament (GC150F; Clark Electromedical Instruments, Reading, UK) and had resistances of  $\sim 10$  M $\Omega$ . After dissection (see above), the extracellular matrix and major axon bundles overlying the GCL were removed mechanically in several small areas under a dissecting microscope using a pair of fine forceps. The central piece of the retina was transferred to a recording chamber (as above) on the stage of an upright microscope (BX50WI; Olympus Optical, London, UK) equipped with a 40 $\times$  water immersion lens. The ganglion cell surface faced the objective, and the cells were visualized under differential interference contrast optics or epifluorescence (see below).  $\text{K}^+$  currents were elicited with 200 msec voltage steps from  $-140$  to  $+50$  mV in 10 mV increments from a holding potential of  $-90$  mV. The steady-state current values were obtained by averaging the points between 30 and 130 msec into the voltage step. The effect of baclofen was determined by subtracting the currents evoked in baclofen from those in its absence.  $\text{Ca}^{2+}$  currents were elicited with 40 msec voltage steps to 0 mV from the holding potential ( $-90$  mV), alternated with  $-10$  mV steps. This was repeated five times, and the data were averaged. Leak subtraction was effected by scaling the trace resulting from the small step and subtracting it from the trace obtained in response to the large depolarizing step. The steady-state current values were obtained by averaging the points between 20 and 30 msec into the voltage step. The mean access resistance was  $30 \pm 2$  M $\Omega$ . In the case of  $\text{Ca}^{2+}$  currents, if the series resistance voltage errors exceeded 5 mV, data were discarded. Where outward  $\text{K}^+$  conductances were not blocked and the currents were large, voltages were corrected for the voltage error produced by current flow through the access resistance. The recordings were performed at room temperature. To investigate  $\text{K}^+$  currents, the external solution was Ringer's solution containing (in mM): 138 NaCl, 4 KCl, 0.41  $\text{MgCl}_2$ , 3 HEPES, 3  $\text{CaCl}_2$ , 1  $\text{NaH}_2\text{PO}_4$ , and 5.6 glucose, pH 7.4 with NaOH; 1  $\mu\text{M}$  TTX was added to block sodium currents. The internal solution consisted of (in mM): 108  $\text{KH}_2\text{PO}_4$ , 4.5  $\text{MgCl}_2$ , 9 HEPES, 14 creatine phosphate, 4  $\text{MgATP}$ , and 0.5  $\text{NaGTP}$ , pH 7.3 (with 100 mM KOH). To investigate  $\text{Ca}^{2+}$  currents, the external solution was Ringer's solution containing (in mM): 135 NaCl, 2.5 KCl, 0.41  $\text{MgCl}_2$ , 3  $\text{CaCl}_2$ , 5  $\text{BaCl}_2$ , 3 HEPES, 6 glucose, and 1  $\mu\text{M}$  TTX, pH 7.4 (with NaOH). The internal solution consisted of (in mM): 130 CsCl, 5 TEA-CI, 0.1  $\text{CaCl}_2$ , 1 NMDG-EGTA, 10 HEPES, 4  $\text{MgATP}$ , 0.5  $\text{NaGTP}$ , and 14

←

were present at E4 and peaked at E8. By E14, GABA no longer evoked increases in  $[\text{Ca}^{2+}]_i$  (see Results).  $n = 3$ –6 retinas for each age. The experiments in *B* and *C* were done in HEPES-buffered Ringer's solution and performed at room temperature to prevent spontaneous  $\text{Ca}^{2+}$  transients.



**Figure 2.** Blocking ionotropic GABA receptors does not affect spontaneous  $\text{Ca}^{2+}$  transients under control conditions. *A*,  $\text{Ca}^{2+}$  transients recorded from an E9 retina upon successive application of drugs, as indicated. The activity recovered in the presence of bicuculline and picrotoxin upon washout of GABA; some transients had an increased

creatine phosphate, pH 7.3 with CsOH. In half of the experiments, the CsCl in the internal solution was replaced by equal amounts of Cs-gluconate. This substitution did not affect the currents observed and helped to preserve the morphology of the cells. The internal solution was stored frozen, and Lucifer yellow (0.3% w/v; Sigma) was added just before use. At the end of the experiment, the morphology of the dye-filled cells was visualized using epifluorescence illumination, and images were acquired with a cooled CCD camera (C4880–80; Hamamatsu, Hamamatsu City, Japan). Two drugs, cAMP and adenosine 3',5'-cyclic phosphorothioate-Rp (Rp-cAMPS), were introduced into cells via the patch-pipette. The diffusion time for these drugs into cells was rapid because they have molecular weights similar to Lucifer yellow (~450 Da), which filled cells within a few seconds of moving into the whole-cell configuration.

**Drugs and statistical analysis.** During both imaging and electrophysiological experiments, drugs were applied by bath perfusion: GABA, bicuculline, forskolin, cAMP, 8-bromoadenosine 3':5'-cAMP (8-bromo-cAMP), and nifedipine (all from Sigma); picrotoxin (PTX), baclofen, and nimodipine (all from Tocris Cookson, Bristol, UK); *p*-3-aminopropyl-*p*-diethoxymethyl phosphoric acid (CGP35348) (gift from Novartis, Basel, Switzerland); 1-(4,4-diphenyl-3-butenyl)-3-piperidine carboxylic acid (SKF89976A) (SmithKline Beecham, Harlow, UK); and Rp-cAMPS (Calbiochem-Novabiochem, Nottingham, UK). Each sample trace shows the sequential application of drugs, as indicated, to a single retina.

All the quantitative data presented were tested using Student's *t* test, and differences were considered statistically significant at  $p < 0.05$ . The results are means  $\pm$  SEM.

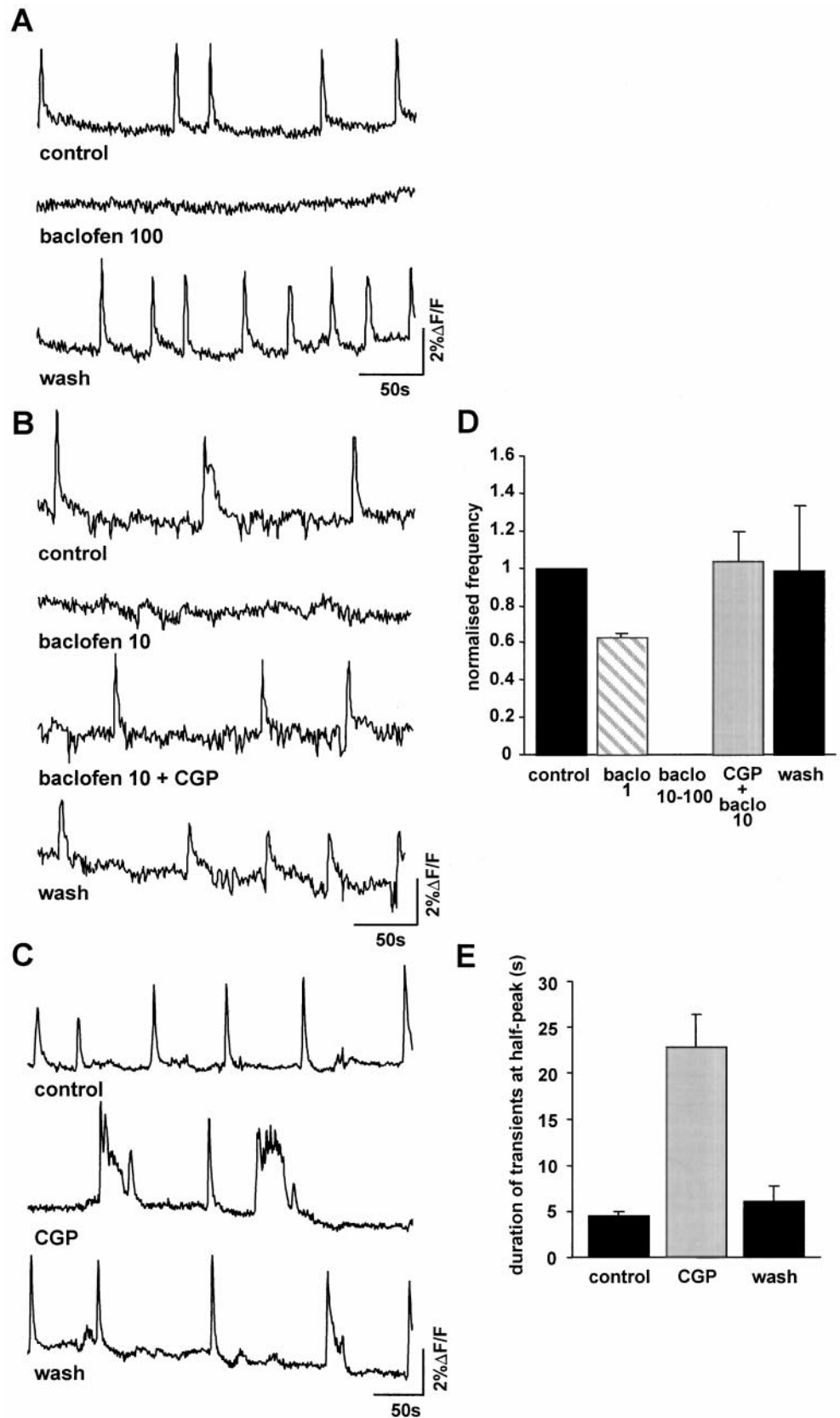
**Immunocytochemistry.** Eyes from E6, E8, E10, and E18 chicks were isolated and fixed by immersion in 4% paraformaldehyde–PBS overnight at 4°C. After three rinses in PBS, the eyes were stored in PBS at 4°C. For sectioning, the eyes were embedded in liquid 4% agar–PBS in brass molds that ensured rapid heat dissipation from the agar block. Transverse sections 100- $\mu\text{m}$ -thick were cut using a vibratome (Campden Instruments, Loughborough, UK) and kept in PBS at 4°C until processed for immunocytochemistry. The sections were permeabilized with 0.05% Triton X-100 in 0.05% BSA and PBS (blocking solution) and then incubated free-floating overnight at 4°C in blocking solution containing a polyclonal primary antibody raised against synthetic peptides of the rat sequences of either GABA<sub>B</sub> receptors (PC300; 1:500) or the neuronal GABA transporter GAT-1 (PC250L; 1:125) (both primary antibodies from Calbiochem-Novabiochem). After rinsing in PBS, the sections were incubated in either anti-guinea pig (GABA<sub>B</sub>) or anti-rabbit (GAT-1) biotinylated secondary antibodies for 2 hr at room temperature (both 1:150; Vector Laboratories, Peterborough, UK). The slices were then rinsed and incubated in Texas Red- or FITC-tagged streptavidin (1:25; Vector Laboratories) for 2 hr at room temperature. After rinsing off the excess streptavidin with PBS, the sections were mounted in Citifluor (Citifluor Ltd., London, UK) on glass slides and imaged on a confocal microscope (LSM510; Zeiss). Negative controls consisted of sections processed as described above but in the absence of primary antibody.

## RESULTS

### When spontaneous calcium activity first occurs, activating GABA<sub>A</sub> receptors depolarizes cells

As described previously, when experiments were performed at ~35°C, regular spontaneous  $\text{Ca}^{2+}$  transients propagated as waves that could be detected from E8 onward (Catsicas et al., 1998). These occurred in 50% of the retinas at E8 ( $n = 20$  retinas) and in ~80% of retinas from E9 onward ( $n = 86$ ). The  $\text{Ca}^{2+}$  tran-

duration under these conditions. *B*, Histogram of the results pooled from four retinas at E9. The frequency (gray bars) and duration (black bars) of  $\text{Ca}^{2+}$  transients in the presence of drugs is shown as a percentage change compared with the control condition. Application of the ionotropic GABA receptor antagonists bicuculline (*bicu*; 50  $\mu\text{M}$ ) and PTX (100  $\mu\text{M}$ ) did not affect the frequency of the transients. In contrast, application of GABA (50  $\mu\text{M}$ ) in the presence of bicuculline and PTX abolished the transients. After a 15 min wash of the drugs, the transients recovered with a frequency initially higher than in control ( $189 \pm 29\%$ ;  $p = 0.09$ ) and a duration comparable with that in control.



**Figure 3.** GABA<sub>B</sub> receptors modulate spontaneous Ca<sup>2+</sup> transients. *A, B, D*, Activating GABA<sub>B</sub> receptors with the agonist baclofen (100 μM) reversibly abolished the transients. The effect of baclofen was dose-dependent (*D*) and prevented by application of the GABA<sub>B</sub> receptor antagonist CGP35348 (*CGP*, 100 μM; *B, D*, *n* = 3–4 retinas per condition). *C, E*, Blocking GABA<sub>B</sub> receptors prolongs spontaneous Ca<sup>2+</sup> transients. Spontaneous Ca<sup>2+</sup> transients in an E9 retina under control conditions (*top trace*), in the presence of CGP35348 (100 μM; *middle trace*), and after 10 min washout of CGP35348 (*bottom trace*). *E*, Pooled data taken from four retinas (comprising 4–10 transients for each condition) showing the duration of Ca<sup>2+</sup> transients as measured at half-peak.

sients had a mean duration (measured at half-maximal amplitude) of  $5 \pm 1$  sec and occurred with a mean frequency of  $1 \pm 0.1$  min<sup>-1</sup> (Fig. 1*A*). Yamashita and Fukuda (1993) have shown that

activation of GABA<sub>A</sub> receptors very early in development (E3) leads to depolarization of chick retinal cells and a transient increase in [Ca<sup>2+</sup>]<sub>i</sub> via voltage-gated L-type Ca<sup>2+</sup> channels. This



raises the possibility that ionotropic GABA receptors regulate Ca<sup>2+</sup> waves early in development.

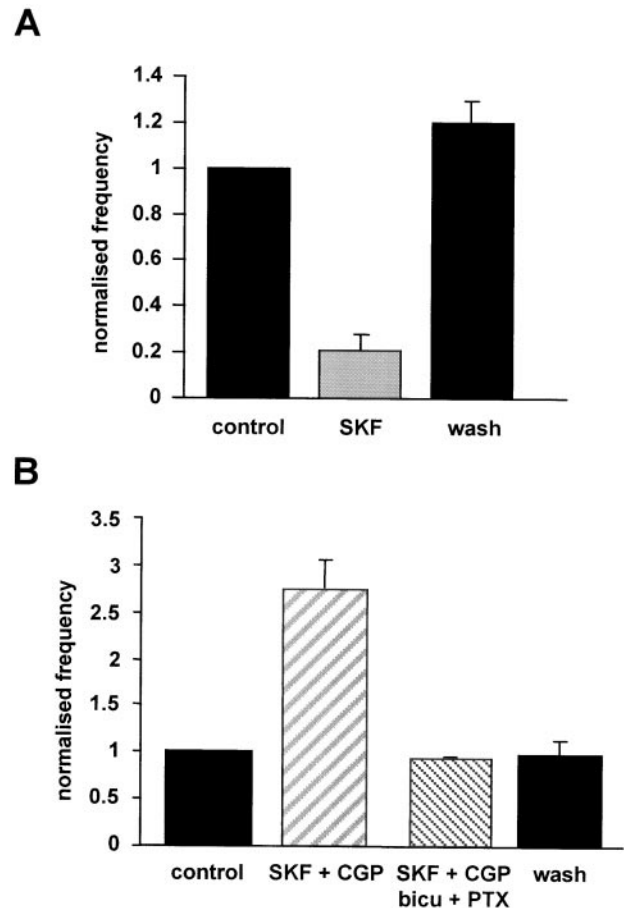
We first investigated whether activation of GABA<sub>A</sub> receptors leads to an increase in [Ca<sup>2+</sup>]<sub>i</sub> at the time waves first occur. These experiments were performed at room temperature to prevent spontaneous Ca<sup>2+</sup> waves (such as in Fig. 1A). Between E4 (the earliest time investigated) and E12, application of GABA (100 μM) induces a transient increase in Ca<sup>2+</sup> that is greatly and reversibly reduced by the GABA<sub>A</sub> receptor antagonist bicuculline (50 μM) (Fig. 1B,C). The developmental profile of this response is shown in Figure 1C, in which the fluorescence changes evoked by 100 μM GABA were normalized to the mean of the data at E8, the stage when the responses were largest. At E4, five of six retinas responded to GABA. On subsequent days and up until E12, GABA produced increases in [Ca<sup>2+</sup>]<sub>i</sub> in all retinas tested (*n* = 23). From E14 (*n* = 3) onward, GABA-evoked changes in [Ca<sup>2+</sup>]<sub>i</sub> were no longer apparent. GABA-evoked currents can be recorded by whole-cell patch clamping at this age and beyond (our unpublished observation), and GABA is the major inhibitory neurotransmitter in the mature retina (Freed, 1992; Luckasiewicz and Shields, 1998), so the absence of a Ca<sup>2+</sup> response at late times presumably reflects a switch in the action of GABA from depolarizing to hyperpolarizing or shunting. Thus, from E8 to E12, the period during which we have studied retinal Ca<sup>2+</sup> waves, activation of GABA<sub>A</sub> receptors leads to transient increases in [Ca<sup>2+</sup>]<sub>i</sub>.

#### GABA affects spontaneous calcium transients independently of ionotropic GABA receptors

The data in Figure 1 raise the possibility that ionotropic GABA receptors are involved in the generation of Ca<sup>2+</sup> waves. To address this, we investigated whether or not blocking ionotropic GABA receptors affects spontaneous Ca<sup>2+</sup> transients. Application of bicuculline (50 μM) and PTX (100 μM), to block GABA<sub>A</sub> and GABA<sub>C</sub> receptors, in retinas maintained at 35°C was without effect on the frequency or duration of the transients (*p* = 0.18 for the frequency; *p* = 0.5 for the duration; *n* = 4) (Fig. 2A,B). Thus, under control conditions, ionotropic GABA receptors do not regulate the temporal characteristics of spontaneous Ca<sup>2+</sup> transients at this stage in development. In contrast, application of GABA (50 μM) in the presence of bicuculline and PTX reversibly abolished the Ca<sup>2+</sup> transients (*n* = 4) (Fig. 2A,B). Upon washout of the drugs, the transients recovered with a frequency initially higher than, and a duration similar to, that in control situation. These results show that GABA can modulate Ca<sup>2+</sup> waves independently of ionotropic GABA receptors.

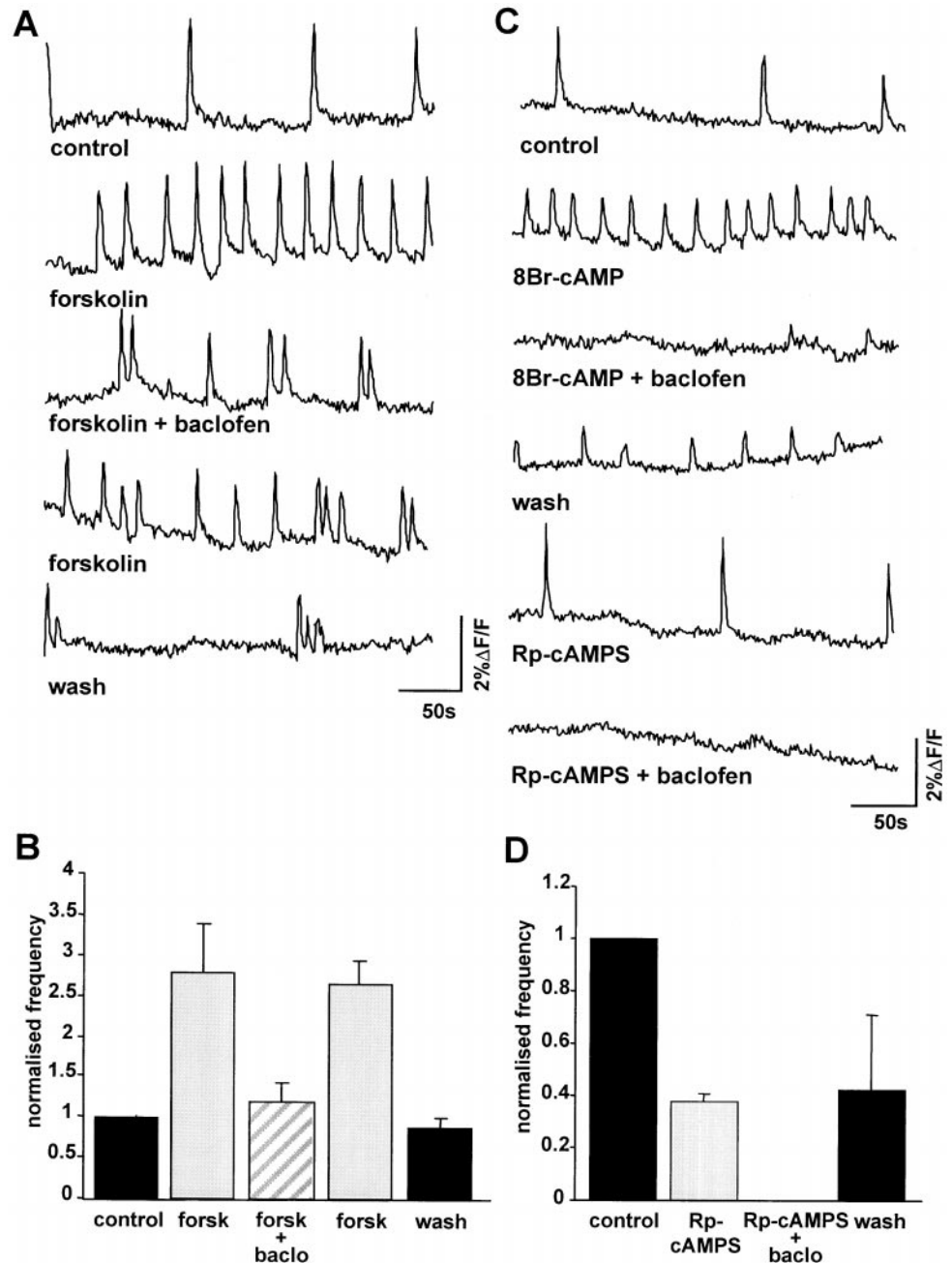
#### GABA<sub>B</sub> receptors modulate spontaneous calcium transients

In addition to ionotropic receptors, GABA can exert its actions through activation of GABA<sub>B</sub> receptors. Applying the GABA<sub>B</sub> receptor agonist baclofen (100 μM) reversibly blocked the Ca<sup>2+</sup> transients (*n* = 4) (Fig. 3A). This effect was dose-dependent and prevented by coapplication of CGP35348 (100 μM), an antagonist at GABA<sub>B</sub> receptors (1 μM baclofen, *p* = 0.01, *n* = 5; 10 μM baclofen, *n* = 3) (Fig. 3B,D). To determine whether or not GABA<sub>B</sub> receptors are endogenously activated during spontaneous activity, we applied CGP35348 alone. Figure 3C shows a sample trace from an E9 retina before (*top*), during (*middle*), and after (*bottom*) application of CGP35348. Although some transients with kinetics similar to those in



**Figure 4.** Blocking neuronal GABA transport reduces the frequency of spontaneous Ca<sup>2+</sup> transients. *A*, The frequency of transients was reversibly reduced with application of the GAT-1 transporter blocker SKF89976A (SKF; 100 μM; *p* < 0.001; *n* = 7). *B*, The effect of SKF was prevented by coapplication of CGP35348 (CGP; 100 μM). In the presence of SKF89976A and CGP35348, the frequency of Ca<sup>2+</sup> transients increased approximately threefold compared with control levels (*p* < 0.01). This overshoot in the frequency was prevented if bicuculline (bicu; 50 μM) and PTX (100 μM) were applied together with SKF89976A and CGP35348 (*n* = 5 retinas). The duration of the transients in the presence of SKF89976A and CGP35348 was similar to that in control (data not shown).

control situation were seen in the presence of CGP35348 (*middle of the trace*), most had a greatly prolonged duration. The mean duration of the transients was measured at half-maximal amplitude over a 6 min period in seven retinas in the presence of CGP35348 and compared with that in control (Fig. 3E). CGP35348 reversibly prolonged the duration of the transients approximately fivefold: *control*, 4.49 ± 0.57 sec; *CGP*, 22.79 ± 3.53 sec (*p* < 0.005); *wash*, 5.95 ± 1.73 sec. We cannot exclude the possibility that the Ca<sup>2+</sup> transients observed in the presence of CGP35348 resulted from the merging of several unitary events and thus reflect an increase in transient frequency. However, it is more likely that they correspond to an increased duration of single events, because agents such as forskolin (see Fig. 5), which greatly increased the frequency of Ca<sup>2+</sup> transients, never gave rise to longer lasting transients comparable with those seen in the presence of CGP35348. These results show that GABA<sub>B</sub> receptors are endogenously activated and regulate the duration (and/or frequency) of transients.



**Figure 5.** GABA<sub>B</sub> receptors act in push-pull with a cAMP-dependent pathway. *A*, Spontaneous Ca<sup>2+</sup> transients recorded from an E9 retina upon sequential application of forskolin and baclofen, as indicated. *B*, Pooled data taken from six retinas of the Ca<sup>2+</sup> transient frequency normalized to control in each condition. Activating adenylate cyclase with forskolin (1 μM) increased the frequency of transients. This effect could be reversibly prevented by coapplication of baclofen (50 μM). The effect of both drugs reversed after 20 min wash. *C*, Bath application of 8-bromo-cAMP (500 μM, 30 min) mimicked the effect of forskolin (*n* = 2) and was antagonized by coapplication of baclofen (50 μM). Blocking protein kinase A with bath application of Rp-cAMPS (500 μM, 20–30 min) reduced the frequency of transients, which could be further decreased by baclofen (50 μM). The activity recovered only partially (*D*). *D*, Pooled data from three retinas showing the frequency of Ca<sup>2+</sup> transients normalized to control.

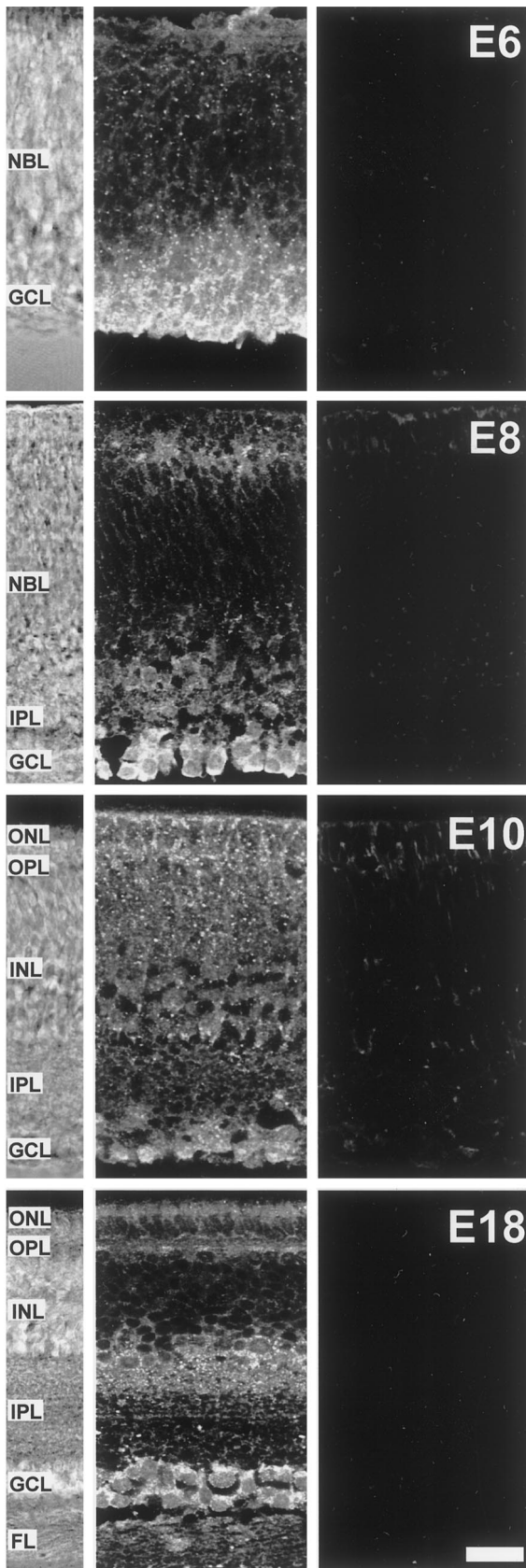
### Modulation of spontaneous calcium transients by GAT-1 transporters

To better understand the mechanisms regulating the concentration of ambient GABA, we next examined the effect of SKF89976A, a selective antagonist of the neuronal GABA transporter GAT-1 (Fig. 4). Application of SKF89976A (100 μM) reversibly reduced the frequency of Ca<sup>2+</sup> transients by ~80% (*p* < 0.001; *n* = 7) (Fig. 4*A*). This is likely to occur via activation of GABA<sub>B</sub> receptors, because it mimics the effect of the GABA<sub>B</sub> receptor agonist baclofen (Fig. 3*A,B,D*). In support of this interpretation, the effect of SKF89976A was prevented by coapplication of CGP35348 (100 μM); however, in the presence of SKF89976A and CGP35348, the frequency of transients exceeded that in control (*p* < 0.01; *n* = 5) (Fig. 4*B*). This “overshoot” in frequency could be prevented by application of the ionotropic GABA receptor antagonists bicuculline (50 μM) and PTX (100 μM) together with SKF89976A and

CGP35348 (*p* = 0.19; *n* = 3) (Fig. 4*B*). These results suggest that, in basal conditions, GAT-1 transporters take up GABA maintaining it at a low concentration in the extracellular space thereby limiting the activation of GABA<sub>B</sub> receptors. However, when GAT-1 is inhibited, the rise in GABA concentration is sufficient to reduce the frequency of Ca<sup>2+</sup> transients through increased activation of GABA<sub>B</sub> receptors. In the presence of CGP35348, this negative regulation is prevented, and the elevated levels of GABA increase the frequency of Ca<sup>2+</sup> transients via activation of ionotropic GABA receptors.

### GABA<sub>B</sub> receptors oppose the effects of cAMP-activating pathways to set the frequency of calcium waves

GABA<sub>B</sub> receptors commonly exert their actions through coupling to G-proteins of the G<sub>i/o</sub> type. Their activation may lead to direct



**Figure 6.** Expression of GABA<sub>B</sub> receptors in the developing chick retina. Confocal microscope sections of GABA<sub>B</sub> receptor immunostaining of transverse vibratome slices from E6, E8, E10, and E18 chick retina. *Left panels*, White-light micrographs from the slices in the *middle panels* showing the structure of the retina for each age. *Middle panels*, Sections

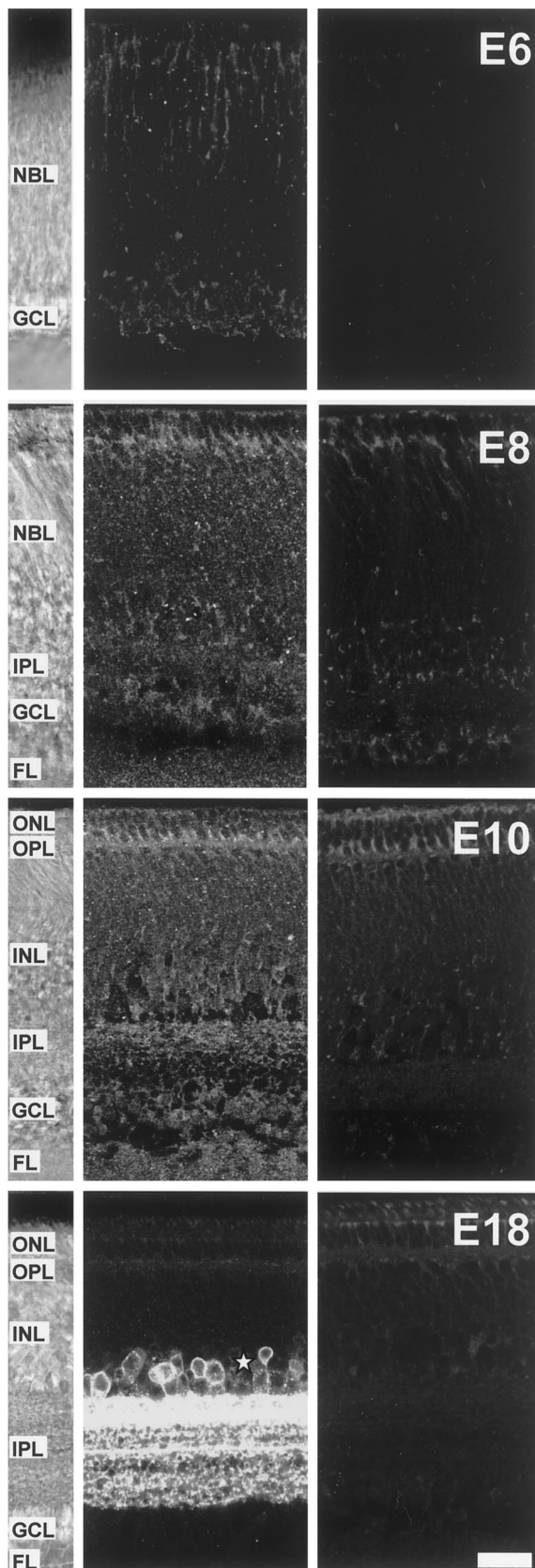
modulation of K<sup>+</sup> and Ca<sup>2+</sup> channels by the G<sub>βγ</sub> subunit or to modulation, generally a downregulation, of adenylate cyclase via the G<sub>α</sub> subunit (Bettler et al., 1998, and references therein). Thus, GABA<sub>B</sub> receptors could modulate calcium waves by reducing cAMP levels. We have shown previously that forskolin, a constitutive activator of adenylate cyclase that raises cAMP levels in cells, increases the frequency of calcium waves in the embryonic chick retina (Catsicas et al., 1998); similar results were obtained by Stellwagen et al. (1999) in developing ferret retina. Here we show that 1 μM forskolin is sufficient to raise the frequency of calcium waves ~2.5-fold ( $p < 0.05$ ;  $n = 6$ ) (Fig. 5*A,B*) and that coapplication of 50 μM baclofen, a dose that in isolation would block calcium waves (Fig. 3), reversibly counteracted the effect of forskolin, resulting in a frequency similar to control ( $n = 6$ ). The effect of forskolin could be mimicked by long perfusion (25 min) of 8-bromo-cAMP, a membrane-permeable analog of cAMP (500 μM; a fourfold increase in two retinas) (Fig. 5*C*), showing that the effect of forskolin on calcium waves is attributable to the activation of adenylate cyclase. As was the case for forskolin, coapplication of baclofen (50 μM) reduced the frequency of transients in the presence of 8-bromo-cAMP (by 80% compared with 8-bromo-cAMP alone in two retinas) (Fig. 5*C*).

Many effects of cAMP are attributable to a signaling cascade involving the activation of protein kinase A (PKA). Thus, to investigate the participation of PKA in regulating spontaneous calcium waves, we used the membrane-permeable, specific inhibitor Rp-cAMPS to block its endogenous activation. Application of Rp-cAMPS (500 μM, 15–30 min) resulted in a decrease in transient frequency that could be further reduced by application of 50 μM baclofen ( $p = 0.001$  comparing Rp-cAMPS with control;  $n = 3$ ) (Fig. 5*C,D*). Upon washout of both drugs, the activity recovered only partially, except for one retina that could be imaged long enough (80 min wash) for the activity to recover to control level. The concentration of Rp-cAMPS used here is well above its  $k_i$  for PKA (11 μM) and should result in a complete block of the kinase and, indeed, similar doses of Rp-cAMPS (100–500 μM) applied extracellularly block forskolin-induced, cAMP-dependent enhancement of synaptic transmission in neostriatal slices (Colwell and Levine, 1995) and vesicular recycling in dissociated granule cells (Chavis et al., 1998). Our results show that the cAMP–PKA signaling cascade is constitutively activated in this system and promotes calcium transients. Activating

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immunoreacted with the anti-GABA<sub>B</sub> receptor antibody. *Right panels*, Negative controls in which the primary antibody was omitted. At E6, GABA<sub>B</sub> receptor-immunoreactive cells, presumptive ganglion cells, were present at the inner margin of the NBL in which the GCL forms. Punctate immunostaining was also present through the thickness of the NBL. At E8, the GCL is separated from the NBL by a layer of processes, the IPL. GABA<sub>B</sub> receptor immunoreactivity was prominent in cells of the GCL. Cells at the inner margin of the NBL, presumptive amacrine cells, were also positive, as well as fibers of the IPL. Immunoreactivity was still present in the rest of the NBL, in particular surrounding distal cell bodies of the NBL outlining the prospective OPL (presumptive horizontal cells). At E10, the pattern of staining was essentially the same as at E8, except there were more immunoreactive plaques in the outer retina at later times. By E18, the retina has reached its mature morphology. GABA<sub>B</sub> receptor immunoreactivity was present in cells of the GCL (ganglion and displaced amacrine cells), of the inner part of the INL (amacrine and displaced ganglion cells), and bordering the OPL. The pattern of staining showed a clear lamina distribution in both OPL and IPL, and immunoreactivity was present in the FL. Some autofluorescence is apparent in the outer retina at the level of the outer nuclear layer (ONL) and OPL (see negative controls). Scale bar, 25 μm.





**Figure 7.** Expression of GAT-1 transporters in the developing chick retina. Confocal microscope sections of GAT-1 transporter immunostaining of transverse vibratome slices from E6, E8, E10, and E18 chick retina. *Left panels*, White-light micrographs from the slices in the *middle panels* showing the structure of the retina for each age. *Middle panels*, Sections

GABA<sub>B</sub> receptors with baclofen has an effect that counteracts that of cAMP pathway activators, such as forskolin and 8-bromo-cAMP, and is additive to that of blocking PKA with Rp-cAMPS. This suggests that GABA<sub>B</sub> receptors do not decrease the frequency of calcium waves by blocking adenylate cyclase and lowering cAMP levels.

#### Expression of GABA<sub>B</sub> receptors and GAT-1 transporters in developing chick retina

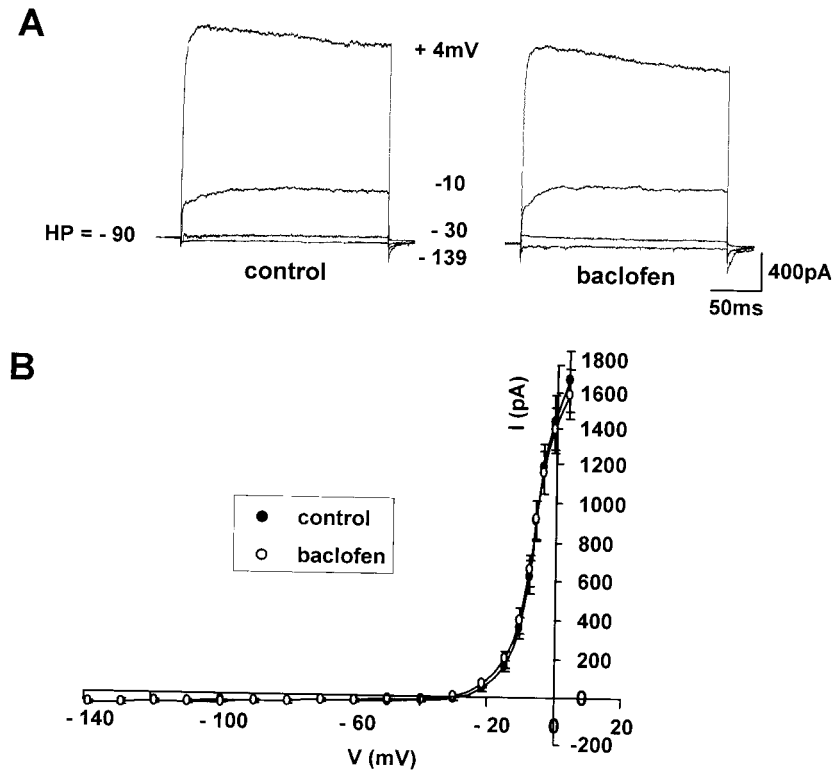
At times when we are first able to detect spontaneous Ca<sup>2+</sup> waves (E8), the layers of processes [inner plexiform layer (IPL) and outer plexiform layer (OPL)] in the chick retina have yet to mature, and morphologically identifiable synapses are absent (Hughes and LaVelle, 1974; Hering and Kröger, 1996). Little is known of the distribution of GABA<sub>B</sub> receptors and GAT-1 transporters at these times. We thus examined their expression patterns between E6 and E18 using polyclonal antibodies raised against their mammalian counterparts (Figs. 6, 7). At E6, GABA<sub>B</sub> receptor immunoreactivity was already apparent in the developing chick retina (Fig. 6). Presumptive ganglion cells in the emerging GCL were labeled. Plaques of immunoreactivity were also apparent throughout the thickness of the tissue. At E8, cells in the GCL, as well as in the inner part of the neuroblastic layer (NBL) in which amacrine and displaced ganglion cells are located, were immunopositive for GABA<sub>B</sub> receptors. Punctate staining was detected in the IPL, in the outer NBL, the location of presumptive horizontal cells, and in the nascent OPL. At E10, the staining was very similar to that at E8, but more immunoreactive plaques were present in the outer retina at the later time. By the time the retina has reached a mature morphology, at E18, the lamina-specific staining had become apparent in both IPL and OPL. Differential staining of cell bodies in the inner nuclear layer (INL) was also apparent, most cells in the amacrine sublayer of the INL, bordering the IPL, were heavily immunopositive, as well as putative horizontal cells bordering the inner part of the OPL. Cell bodies in the GCL and ganglion cell axons in the fiber layer (FL) were heavily labeled.

At E6, GAT-1 transporter immunoreactivity (Fig. 7) was limited to a punctate staining in the outer NBL and in the GCL. At E8 and E10, this punctate staining was superimposed on a diffuse labeling present throughout the retina. By E10, staining in the IPL was apparent and lamina-specific, the laminae closer to the INL being more strongly labeled than the laminae closer to the GCL. This lamina-specific staining was conserved and striking at E18. By then, GAT-1 immunoreactivity had reached its mature pattern, similar to that described for the rat retina (Johnson et al., 1996), in which the staining is restricted to the plasma membrane of cells in the amacrine sublayer of the INL (*asterisk*) and the IPL. Immunoreactivity was absent from the GCL. These results show that both GABA<sub>B</sub> receptors and GAT-1 transporters are expressed in regions in which amacrine and ganglion cells and

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immunoreacted with the anti-GAT-1 antibody. *Right panels*, Negative controls in which the primary antibody was omitted. At E6, GAT-1 immunostaining was punctate and restricted to the outer NBL and the GCL. At E8, immunoreactive puncta were distributed throughout the retina. By E10, GAT-1 lamina-specific staining was apparent in the IPL, with laminae closer to the INL more strongly labeled. At E18, GAT-1 staining is restricted to the IPL, in which it has a clear laminar distribution, and to somata in the proximal INL, presumptive amacrine cells (*asterisk*). Some autofluorescence is apparent in the outer retina at the level of the ONL and OPL (see negative controls). Scale bar, 20  $\mu$ m.





**Figure 8.** GABA<sub>B</sub> receptors do not modulate K<sup>+</sup> currents in ganglion cell layer neurons. *A*, Currents evoked in an E9 neuron by 200 msec voltage steps to the indicated potentials from a holding potential of -90 mV in control solution (*left*) and in the presence of 100 μM baclofen (*right*). *B*, Current-voltage relationship from six neurons evoked by voltage steps in 10 mV increments from -139 to +4 mV from a holding potential of -90 mV in the absence (*control*, filled circles) and presence of 100 μM baclofen (*open circles*). These data have been corrected for any voltage error produced by current flow through the access resistance in the steady state.

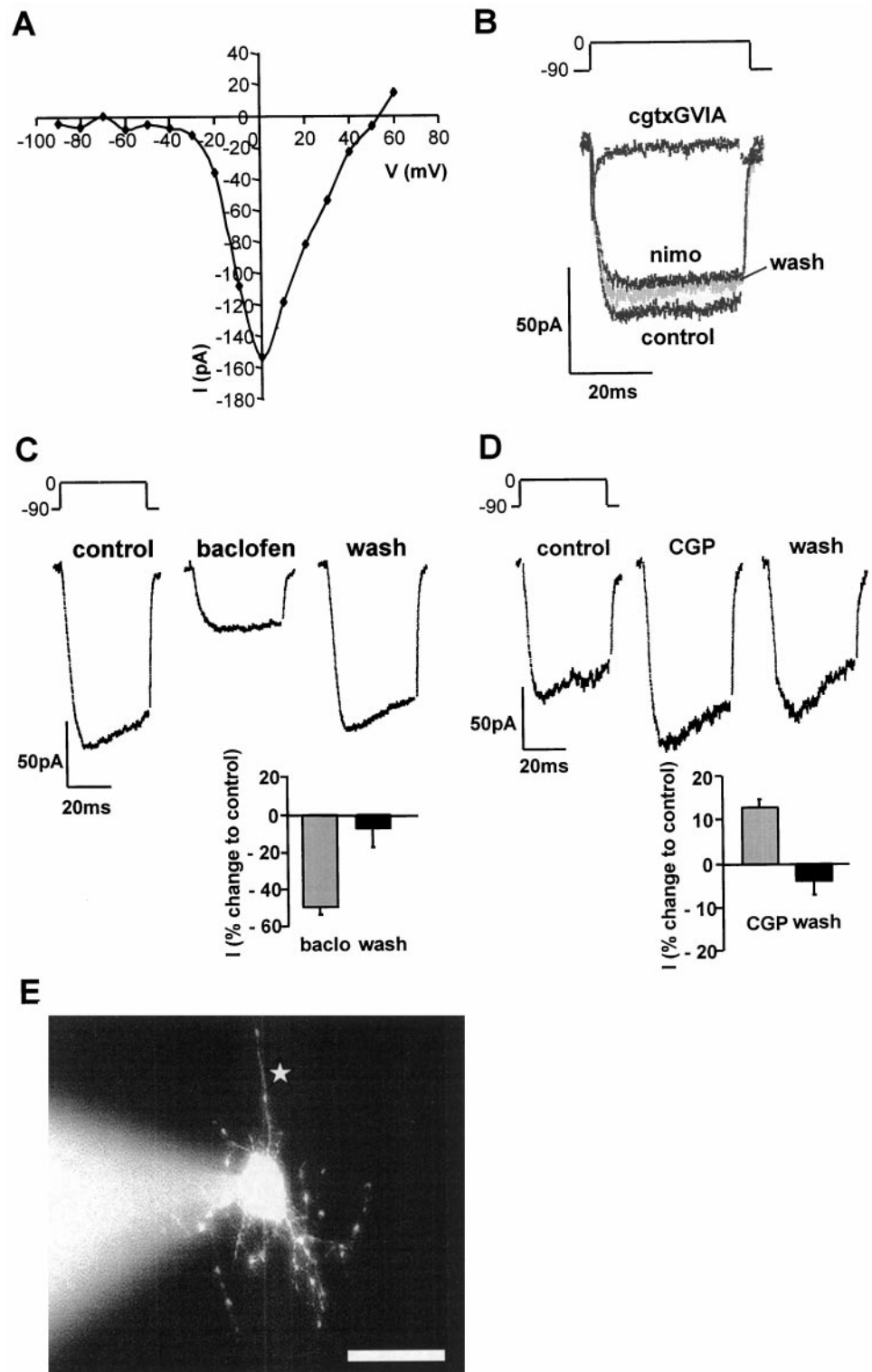
their processes are located, at a time and in a pattern that is compatible with their ability to regulate spontaneous calcium waves.

### GABA<sub>B</sub> receptors modulate calcium currents in ganglion cells and displaced amacrine cells

GABA<sub>B</sub> receptors usually act to inhibit cells by increasing a K<sup>+</sup> conductance or reduce neurotransmission by decreasing a Ca<sup>2+</sup> conductance (Bettler et al., 1998). We first investigated the presence of baclofen-induced K<sup>+</sup> currents in the developing retina using the whole-cell patch-clamp technique to record from ganglion and amacrine cells in the GCL (Fig. 8). K<sup>+</sup> currents were evoked by voltage steps between -140 and +50 mV (holding potential of -90 mV). Baclofen had no effect on the currents evoked at voltages between -140 and -50 mV ( $n = 6$ ) (Fig. 8*A,B*), showing that GABA<sub>B</sub> receptors do not activate K<sup>+</sup> channels in GCL neurons. However, at more positive potentials, baclofen suppressed a small inward current described in more detail below.

Sun and Chiu (1999) have shown that N-type Ca<sup>2+</sup> channels in neonatal rat optic nerve are regulated by GABA<sub>B</sub> receptors and control the magnitude of the Ca<sup>2+</sup> influx associated with action potentials. We examined the possibility that GABA<sub>B</sub> receptors exert their action on spontaneous Ca<sup>2+</sup> waves through a similar mechanism. Ca<sup>2+</sup> currents were recorded from GCL neurons (Fig. 9) at room temperature in an external solution containing 3 mM Ca<sup>2+</sup> plus 5 mM Ba<sup>2+</sup> and 1 μM TTX. The presence of Ba<sup>2+</sup> in the external solution together with 5 mM TEA in the patch pipette ensured K<sup>+</sup> currents were minimal. Under these conditions, a depolarizing voltage step to 0 mV from a holding potential of -90 mV elicited an inward Ca<sup>2+</sup> current. This current was evoked by voltage steps positive to -40 mV and reached maximum amplitude between 0 and +10 mV (Fig. 9*A*). We investigated the nature of this Ca<sup>2+</sup> current using the dihydropyridines

(DHP) nifedipine and nimodipine, blockers of L-type Ca<sup>2+</sup> channels, and the peptide ω-conotoxin-GVIA (cgtxGVIA), a blocker of N-type Ca<sup>2+</sup> channels (Fig. 9*B*). A saturating dose of either DHP (10 μM) blocked a relatively small portion, ~15%, of the Ca<sup>2+</sup> current (in DHP, 85 ± 6% of control;  $p = 0.03$ ;  $n = 8$ ). On the other hand, cgtxGVIA (5 μM) blocked ~90% of the total current (in cgtxGVIA, 10 ± 1% of control;  $n = 3$ ), showing that most of the Ca<sup>2+</sup> current present in GCL neurons is of the N-type. As observed previously (Amico et al., 1995), the effect of cgtxGVIA was irreversible even after a prolonged wash (30 min). This high-voltage activated Ca<sup>2+</sup> current was reduced by 100 μM baclofen in all cells tested (26 cells) (Fig. 9*C*). This dose of baclofen, which abolished spontaneous Ca<sup>2+</sup> transients (Fig. 3), reversibly reduced the Ca<sup>2+</sup> current by half ( $p < 0.001$ ;  $n = 15$  cells in which baclofen alone was tested; current, 51 ± 7% of control in six ganglion cells and 51 ± 6% in nine amacrine cells) (Fig. 9*C*, *inset*). The magnitude of the block by baclofen in the presence of DHP was comparable with that in the absence of DHP: baclofen plus DHP, 50 ± 5% of DHP alone current ( $n = 6$ ); baclofen alone, 51 ± 4% of control ( $n = 15$ ). This could be taken to suggest that baclofen affects equally the N- and L-type current. However, given the small size of the L-type current, this requires further investigation. Blocking GABA<sub>B</sub> receptors with the specific antagonist CGP35348 (100 μM) reversibly increased the Ca<sup>2+</sup> current by ~10% ( $p < 0.01$ ;  $n = 6$ ) (Fig. 9*D*, *inset*). Twenty of the cells recorded were filled with Lucifer yellow introduced via the patch pipette of which 10 were ganglion cells, as shown by the presence of an axon that ran in the fiber layer and the large diameter of their cell body (≥15 μm); an example of such a cell is shown in Figure 9*E*. It is likely, because of their smaller cell bodies (≤10 μm), their position on the outer margin of the GCL and the absence of an axon, that the remaining cells were displaced amacrine cells (Zhou, 1998). These results show that both ganglion



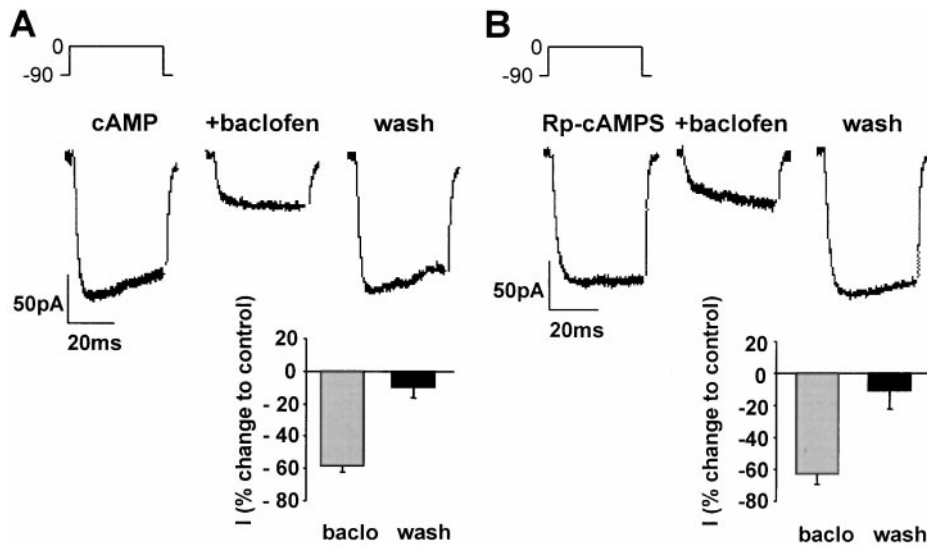
**Figure 9.** Effect of GABA<sub>B</sub> receptors on the Ca<sup>2+</sup> current in ganglion cell layer neurons. *A*, Current–voltage relationship from an E11 cell in response to depolarizing steps in 10 mV increments from a holding potential of –90 to +60 mV in control solution. *B*, Current traces in response to a depolarizing step to 0 mV from a holding potential of –90 mV in control solution and during the sequential application of the L-type Ca<sup>2+</sup> channel blocker nimodipine (10 μM) and of the peptide N-type Ca<sup>2+</sup> channel blocker cgtxGVIA (5 μM). *wash* indicates washout of nimodipine before application of cgtxGVIA. The effect of cgtxGVIA was irreversible. *C*, Current traces in response to a depolarizing step to 0 mV from a holding potential of –90 mV in control solution (5 mM Ba<sup>2+</sup> and 1 μM TTX; *left*) in the presence of baclofen (100 μM; *middle*) and after wash to control (*right*). The histogram shows pooled data from 15 cells between E9 and E12 in which the amplitude of the currents was normalized to the control current and plotted as percentage change from control. Baclofen reversibly decreased the current by ~50% ( $p < 0.001$ ). *D*, Current traces in response to a depolarizing step to 0 mV from a holding potential of –90 mV in control solution (*left*), in the presence of CGP35348 (100 μM; *CGP*; *middle*) and after wash (*right*). The histogram shows pooled data from six cells at E10–E11 in which the amplitude of the currents was normalized to the control current and plotted as percentage change from control. CGP35348 reversibly increased the current by ~10% ( $p < 0.01$ ). *E*, Example of a Lucifer yellow-filled ganglion cell in an E10 retina photographed after whole-cell patch-clamp recording *in situ*. The patch pipette, still attached to the cell, is visible to the *left*. The image is an overlay of four photographs taken at different focal planes to reveal the extent of the dendritic tree. An *asterisk* marks the axon, running more superficial than the dendrites in the fiber layer. Scale bar, 20 μm.

cells and displaced amacrine cells possess N- and L-type Ca<sup>2+</sup> channels that are negatively regulated by GABA<sub>B</sub> receptors.

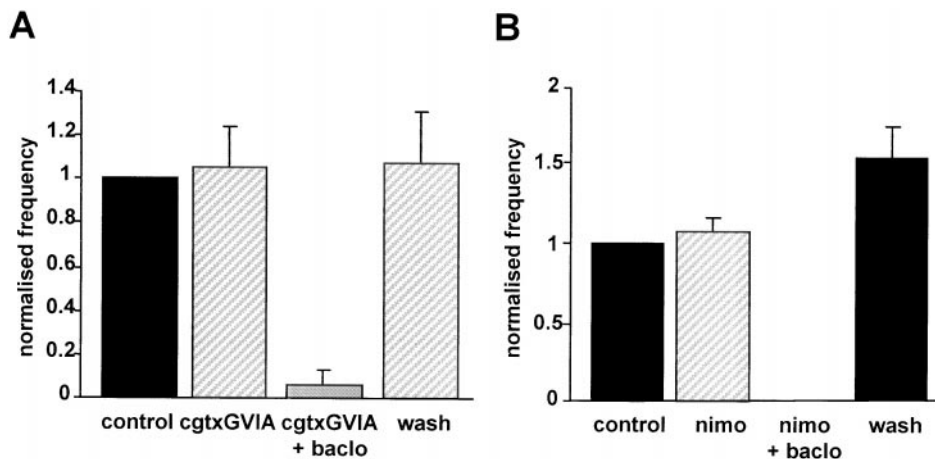
#### GABA<sub>B</sub> modulation of the calcium current in GCL neurons is independent of cAMP and the activation of PKA

The imaging experiments described above (Fig. 5) show that GABA<sub>B</sub> receptors can modulate spontaneous calcium waves irrespective of whether the cAMP–PKA signaling pathway is activated or blocked. To determine whether GABA<sub>B</sub>-mediated inhi-

bition of Ca<sup>2+</sup> currents is also independent of this signaling cascade, we next used whole-cell patch clamping of GCL neurons (Fig. 10), including in the patch pipette either cAMP (2 mM, to clamp intracellular cAMP levels) (Fig. 10*A*) or Rp-cAMPS (500 μM, to block PKA) (Fig. 10*B*). Under both conditions, baclofen produced a reduction in the Ca<sup>2+</sup> current comparable with that in control conditions: change in control, 49 ± 4% ( $n = 15$ ); in cAMP, 58 ± 4% ( $n = 10$ ;  $p = 0.2$ ); in Rp-cAMPS, 63 ± 6% ( $n =$



**Figure 10.** GABA<sub>B</sub> receptors modulate Ca<sup>2+</sup> currents in ganglion cell layer neurons in a cAMP–PKA-independent manner. *A, B*, Current traces in response to a depolarizing step to 0 mV from a holding potential of –90 mV in the presence of either cAMP (2 mM; *A*) or the PKA blocker Rp-cAMPS (500 μM; *B*, left traces) in the patch pipette, upon application of baclofen (100 μM; middle traces) and after wash of baclofen (right traces). The histograms show pooled data from 10 (*A*) or six (*B*) cells at E9–E11 in which the amplitude of the currents in baclofen was normalized to the currents in the presence of cAMP or Rp-cAMPS, respectively, and plotted as percentage change.



**Figure 11.** Blocking N- or L-type Ca<sup>2+</sup> channels does not block spontaneous Ca<sup>2+</sup> transients. Application of cgtxGVIA (5 μM; *A*) or nimodipine (10 μM; *B*) to block N- and L-type Ca<sup>2+</sup> channels, respectively, did not affect the frequency of transients (*n* = 4 retinas). Coapplication of baclofen (10 μM) reversibly abolished the transients in both cases.

6; *p* = 0.07). The density of Ca<sup>2+</sup> current in cells exposed to Rp-cAMPS was smaller than that in control cells (control, 17.5 ± 1.5 pA/pF; Rp-cAMPS, 11 ± 1 pA/pF; *p* = 0.01), suggesting the existence of a PKA-sensitive fraction in this current. How GABA<sub>B</sub> receptors modulate the Ca<sup>2+</sup> current remains to be determined, but our results are consistent with the GABA<sub>B</sub> receptor exerting its effects on the Ca<sup>2+</sup> current via a direct G-protein modulation of the channels, a mechanism by which these receptors are known to operate in other circumstances (Herlitze et al., 1996; Ikeda, 1996).

#### The effect of GABA<sub>B</sub> receptors on Ca<sup>2+</sup> waves is independent of their action on Ca<sup>2+</sup> channels

The effect the activation of GABA<sub>B</sub> receptors has on the Ca<sup>2+</sup> channels could account for their effect on Ca<sup>2+</sup> waves. However, blocking N- or L-type channels with cgtxGVIA (5 μM) or nimodipine (10 μM), respectively, had no effect on the frequency of Ca<sup>2+</sup> waves (*n* = 4) (Fig. 11*A, B*). Furthermore, baclofen (10 μM) still blocked Ca<sup>2+</sup> waves in the presence of these antagonists (Fig. 11). This indicates that the action of GABA<sub>B</sub> receptors in modulating Ca<sup>2+</sup> waves is independent of its action on N- and L-type channels. In addition, we have shown that there is no effect of GABA<sub>B</sub> receptor activation on the K<sup>+</sup> currents of cells in the GCL (Fig. 8). This suggests that, if K<sup>+</sup> channels mediate the effect of GABA<sub>B</sub> receptors on Ca<sup>2+</sup> waves, they must do so at a site outside of the GCL.

## DISCUSSION

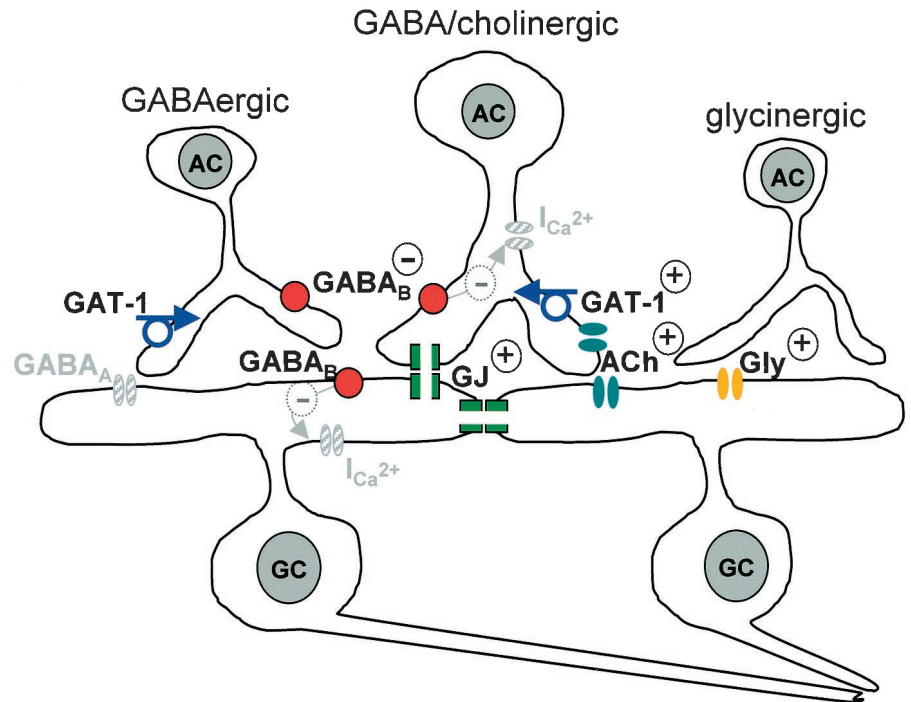
### Developmental changes in the regulation of Ca<sup>2+</sup> waves by neurotransmitters

The pharmacology of Ca<sup>2+</sup> waves in the chick retina is developmentally regulated. At late times (E16), glutamatergic inputs, perhaps from bipolar cells, may initiate or drive spontaneous activity in the GCL (Wong et al., 1998). The results of Wong et al. also suggest that glycinergic and GABAergic inputs from amacrine cells inhibit this activity at times when these neurotransmitters are hyperpolarizing or shunting and that cholinergic amacrine cells, while present appear, unlike in mammalian retina (Zhou, 1998), to exert little effect on the activity. We have shown, in contrast to Wong et al. (1998), that calcium waves are a robust phenomenon before synaptogenesis. At these times (E8–E12) (Fig. 12), unlike later on, blockade of glutamate is without effect, cholinergic and glycinergic antagonists reduce activity (Catsicas et al., 1998), and, as we show here, GABA exerts its action via GABA<sub>B</sub> receptors rather than ionotropic receptors. These changing influences of neurotransmitters may result from differences in the level, pattern of expression, or type of receptor expressed, or from changes in the ionic permeability or the equilibrium potential of the permeant ions. At both E8 and E16, blocking gap junctions suppresses Ca<sup>2+</sup> waves (Catsicas et al., 1998; Wong et al., 1998).

Recently, Stellwagen et al. (1999) have shown that, in ferret



**Figure 12.** Mechanisms regulating calcium waves during early development (E8–E12). Before synaptogenesis, the frequency of the calcium waves that propagate between ganglion and amacrine cells (*GC* and *AC*, respectively) is regulated by a number of the transmitter molecules released by amacrine cells, including ACh acting at nicotinic receptors, glycine (*Gly*), adenosine (data not shown), and GABA acting at GABA<sub>B</sub> receptors. The GABA transporter GAT-1 acts to keep GABA in the extracellular space at low levels. Glutamate receptors appear not to be involved in the regulation of calcium waves until after E12, at which time glutamate antagonists block wave activity probably through an action at the newly formed synapses between bipolar and ganglion cells (not shown). At both early and late times, gap junctions (*GJ*) are important for wave propagation because agents that block the junctions inhibit the waves, whereas agents known to influence coupling through modulation of intracellular levels of cAMP (forskolin, dopamine, and adenosine) increase wave frequency (see Discussion). Positive and negative signs indicate wave-promoting and wave-inhibiting signals, respectively. *Shaded figures* and *pathways* indicate that they are present but not instrumental in wave activity.



retina, adenosine strongly affects wave dynamics via A2 receptors and an elevation of cAMP and activation of PKA. We have shown here that, in chick, a similar cAMP–PKA-activated pathway exists in which elevation of cAMP with forskolin or 8-bromo-cAMP increases wave frequency, whereas blockade of PKA reduces it. We have shown that GABA<sub>B</sub> receptor activation opposes cAMP-activating pathways, which raises the possibility that the net frequency of Ca<sup>2+</sup> waves, and possibly their overall dynamics, results from the balance of release of GABA, acting at GABA<sub>B</sub> receptors, and transmitters such as adenosine activating the cAMP pathway. Interestingly, a subpopulation of starburst amacrine cells displaced to the ganglion cell layer coexpresses ACh, GABA, and adenosine in the adult mammalian retina (Blazynski, 1989). If this were true also in the developing retina, it would make displaced starburst amacrine cells ideally suited for the fine-tuning of the properties of Ca<sup>2+</sup> waves. These same authors (Stellwagen et al., 1999) have shown that, although present, ionotropic GABA receptors do not modulate wave dynamics in neonatal ferret retina, a result similar to what we see in early chick retina (but see Fischer et al., 1998).

### The source of GABA and its actions on Ca<sup>2+</sup> waves

One of the major sources of GABA within the developing retina is the amacrine cell population (Massey and Redburn, 1987; Freed, 1992). The transporter GAT-1 is present in the chick retina throughout the period during which Ca<sup>2+</sup> waves occur and is strongly expressed by amacrine cells (Fig. 7). GAT-1 is also the predominant transporter of amacrine cells in the rat retina (Johnson et al., 1996), although GAT-3 is also found in some amacrine cells and is strongly expressed by Müller cells. The GAT-1 inhibitor SKF89976A reduces the frequency of the transients presumably because it induces an increase in the extracellular concentration of GABA. Because SKF89976A is not transported and thus cannot increase extracellular concentrations of the transmitter via heteroexchange, GABA release in its presence could occur through reversal of

another GABA transporter or via vesicular release. The fact that the available blockers of GAT-3 transporters are translocated, and so lead to GABA release by heteroexchange, prevented us from investigating their role in regulating the extracellular GABA concentration.

When CGP35348 is coapplied with SKF89976A to prevent the activation of GABA<sub>B</sub> receptors, the frequency of the transients is elevated beyond that in control conditions but is restored to control levels when ionotropic GABA receptors are blocked with a mixture of bicuculline and PTX. This result may be explained by the depolarizing activity of GABA at ionotropic receptors, which one might expect to increase spontaneous activity, and a “damping” action at the GABA<sub>B</sub> receptor. The fact that blockers of ionotropic GABA receptors have no effect on transient frequency when the GAT-1 transporter operates probably reflects the low levels of GABA in the extracellular space under these conditions; although the affinity of GABA<sub>A</sub> receptors for GABA varies greatly with the subunits expressed, and we do not know which subunits are expressed at early times, our results suggest a higher affinity of GABA<sub>B</sub> receptors than GABA<sub>A</sub> receptors for GABA in the developing retina, as has been described previously (Sodickson and Bean, 1996). Although GABA<sub>C</sub> receptors are more sensitive to GABA than GABA<sub>A</sub> receptors (Bormann and Feigenspan, 1995), our observations show that endogenous activation of GABA<sub>C</sub> receptors does not modulate Ca<sup>2+</sup> waves. Thus, under physiological conditions, GABA reaches the critical concentration required for the activation of GABA<sub>B</sub> receptors and may be a major factor determining the frequency and duration of Ca<sup>2+</sup> waves at early times in the developing retina. A similar role for GABA<sub>B</sub> receptors has been proposed in the control of the GABA<sub>A</sub>-dependent network activity seen in rat hippocampus (McLean et al., 1996).

We have shown that GABA<sub>B</sub> receptors are expressed in chick retina in regions of ganglion and amacrine cells throughout the period when Ca<sup>2+</sup> waves occur. Koulen et al. (1998)

have described a similar distribution in developing rat retina. GABA<sub>B</sub> receptors, through coupling to G-proteins, can either directly modulate ion channels or negatively couple to adenylate cyclase (Bettler et al., 1998). Thus, the inhibitory effect of GABA<sub>B</sub> receptors on calcium waves could result from inhibition of Ca<sup>2+</sup> channels, activation of K<sup>+</sup> channels, or inhibition of cAMP production and its signaling cascade. Our results show that GABA<sub>B</sub> receptors do not act on K<sup>+</sup> channels of GCL neurons but do affect their Ca<sup>2+</sup> channels directly, downregulating Ca<sup>2+</sup> influx in a cAMP-independent manner. This Ca<sup>2+</sup> current and its modulation by GABA<sub>B</sub> receptors were observed in all the cells we recorded in the GCL and thus are present in both ganglion and amacrine cells. Surprisingly, blocking calcium channels with cgtxGVIA and nimodipine does not prevent the occurrence of calcium waves. Thus, GABA<sub>B</sub> receptors do not shut down wave activity via an action on GCL neuron K<sup>+</sup> currents or via an action on N- or L-type Ca<sup>2+</sup> channels in the GCL or elsewhere. The route by which Ca<sup>2+</sup> enters cells involved in wave activity is unknown for any species so far examined. Further experiments are required to determine the mechanisms of the [Ca<sup>2+</sup>]<sub>i</sub> increase. It is possible that these include both ligand- and voltage-gated channels.

Starburst amacrine cells are thought to drive wave activity in ganglion cells via ACh release and also possibly gap junctions. If GABA<sub>B</sub> receptors are present in starburst amacrine cells, it is possible they could reduce ACh release by clamping their membrane voltage to the resting potential through the activation of a K<sup>+</sup> conductance. Thus, one might expect blocking the GABA<sub>B</sub> receptor to have a similar effect to that of raising extracellular ACh. Interestingly, we have shown previously that eserine, which blocks ACh breakdown by acetylcholinesterase, increases wave frequency (Catsicas et al., 1998). However, eserine raises the steady-state concentration of ACh rather than increasing the frequency of its release as might result from blocking the GABA<sub>B</sub> receptor. Zhou and Fain (1995) examined the actions of GABA on rabbit starburst cells and showed that the GABA-evoked currents found are produced by the activation of GABA<sub>A</sub>, and not GABA<sub>B</sub> or GABA<sub>C</sub>, receptors. However, these authors did not study the possible modulation of voltage-dependent channels by GABA<sub>B</sub> receptors.

The results we present here demonstrate the importance of GABA and GABA<sub>B</sub> receptors in the control of Ca<sup>2+</sup> waves in the developing retina and point to substantial changes in the roles played by GABA in the regulation of this activity during development. Ca<sup>2+</sup> waves appear before anatomically distinct synapses are established and during the period that ganglion and amacrine cell dendrites are forming. They are robust through the time when these cells undergo a period of massive cell death. Our results show that baclofen, CGP35348, and SKF89976A are useful tools with which to manipulate the frequency of Ca<sup>2+</sup> waves at these crucial times in development. Experiments using these agents will help clarify the role of Ca<sup>2+</sup> waves in the regulation of dendritic maturation, synapse formation, and cell death.

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