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Clustering of Extrasynaptic $GABA_A$ Receptors Modulates Tonic Inhibition in Cultured Hippocampal Neurons*

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Tonic inhibition plays a crucial role in regulating neuronal excitability because it sets the threshold for action potential generation and integrates excitatory signals. Tonic currents are known to be largely mediated by extrasynaptic γ-aminobutyric acid type A ($GABA_A$) receptors that are persistently activated by submicromolar concentrations of ambient GABA. We recently reported that, in cultured hippocampal neurons, the clustering of synaptic $GABA_A$ receptors significantly affects synaptic transmission (Petrini, E. M., Zacchi, P., Barberis, A., Mozrzymas, J. W., and Cherubini, E. (2003) *J. Biol. Chem.* 278, 16271–16279). In this work, we demonstrated that the clustering of extrasynaptic $GABA_A$ receptors modulated tonic inhibition. Depolymerization of the cytoskeleton with nocodazole promoted the disassembly of extrasynaptic clusters of δ and γ_2 subunit-containing $GABA_A$ receptors. This effect was associated with a reduction in the amplitude of tonic currents and diminished shunting inhibition. Moreover, diffuse $GABA_A$ receptors were less sensitive to the GAT-1 inhibitor NO-711 and to flurazepam. Quantitative analysis of GABA-evoked currents after prolonged exposure to submicromolar concentrations of GABA and model simulations suggest that clustering affects the gating properties of extrasynaptic $GABA_A$ receptors. In particular, a larger occupancy of the singly and doubly bound desensitized states can account for the modulation of tonic inhibition recorded after nocodazole treatment. Moreover, comparison of tonic currents recorded during spontaneous activity and those elicited by exogenously applied low agonist concentrations allows estimation of the concentration of ambient GABA. In conclusion, receptor clustering appears to be an additional regulating factor for tonic inhibition.

Similar to many other neurotransmitter receptors, γ-aminobutyric acid type A ($GABA_A$)[1] receptors are localized at synaptic and extrasynaptic levels. Whereas synaptic $GABA_A$ receptors are involved in phasic inhibition (1), extrasynaptic ones are responsible for tonic inhibition (2–9). Tonic inhibition is due to persistent inhibitory conductance that contributes to "signal integration" in the brain since it sets the threshold for action potential generation (10, 11) and shunts excitatory synaptic inputs (2, 12–15). This conductance is maintained by "ambient" GABA, which represents the amount of neurotransmitter present in the extracellular space. Ambient GABA originates from spillover of the neurotransmitter released at neighboring synapses (3, 5, 11), from astrocytes (16, 17), or from non-vesicular release (18, 19). Tonic inhibition has been well characterized in the cerebellum, where α_6 subunit-containing receptors act as high affinity sensors for GABA (4, 11, 20, 21). Persistent GABA conductance has also been identified in other brain regions, including the hippocampus (6, 8, 9, 22, 23). However, in this structure, the subunit composition of the receptors involved has not been fully elucidated. In the past years, immunocytochemical and *in situ* hybridization approaches (20, 24–26) have demonstrated that $GABA_A$ receptors are clustered not only at the synaptic level, but also at the extrasynaptic level. However, at present, the influence of the clustering of extrasynaptic $GABA_A$ receptors on tonic current is still unclear, although it is well established that clustering of synaptic receptors ensures proper synaptic signaling. In line with a previous study (27), we have pharmacologically induced the declusterization of $GABA_A$ receptors using nocodazole, a microtubule-disrupting agent, and we have analyzed tonic inhibition in cultured hippocampal neurons. With immunocytochemical experiments, we found clusters of δ subunit-containing $GABA_A$ receptors exclusively at extrasynaptic locations, whereas clusters of γ_2 subunit-containing receptors were detectable at both synaptic and extrasynaptic sites. Nocodazole treatment induced the disassembly of all the clusters, thus promoting a uniform distribution of δ and γ_2 subunit-containing $GABA_A$ receptors on the cell surface. This effect was associated with a reduced tonic current. Moreover, diffuse $GABA_A$ receptors were less sensitive to the GAT-1 inhibitor NO-711 and to flurazepam (FZP). Model simulations suggest that a larger occupancy of a singly bound desensitized state of declustered receptors may account for the reduction of the tonic current after nocodazole treatment.

EXPERIMENTAL PROCEDURES

Cell Culture—Hippocampal cell cultures were prepared as described previously (28). Briefly, 2–4-day-old Wistar rats were decapitated after being anesthetized with an intraperitoneal injection of urethane (2 mg/kg). The hippocampus was dissected free, sliced, digested with trypsin, mechanically triturated, centrifuged twice at $40 \times g$, plated in Petri dishes, and cultured for up to 14 days. Experiments were performed on cells cultured for at least 7 days.

Nocodazole Treatment—Nocodazole (purchased from Sigma, Milan, Italy) was used to disrupt microtubules. It was applied at a concentration of 10 μg/ml (29) from a 100% Me_2SO stock solution. The final concentration of Me_2SO in the working solutions was 0.1% (v/v). Nocodazole treatment consisted of incubating the neurons with the drug for at least 2 h in the culture medium. To verify whether Me_2SO alone could affect GABAergic transmission, some electrophysiological exper-

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[1] The abbreviations used are: $GABA_A$, γ-aminobutyric acid type A; FZP, flurazepam; TRITC, tetramethylrhodamine isothiocyanate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; TTX, tetrodotoxin; IPSCs, inhibitory postsynaptic currents; mIPSCs, miniature inhibitory postsynaptic currents; sIPSCs, spontaneous inhibitory postsynaptic currents.

iments ($n = 12$) were also performed on cells incubated for 2 h with Me_2SO alone. Me_2SO (0.1%, v/v) did not produce any change in the kinetic properties of miniature inhibitory synaptic currents (data not shown).

Immunofluorescence Staining and Confocal Microscopy—Immunofluorescence labeling (30) of surface $GABA_A$ receptor subunits δ and γ_2 was performed before fixation by incubation of living neurons for 15 min at 4 °C with affinity-purified rabbit polyclonal antibodies raised against the extracellular N-terminal region of the corresponding subunits (*i.e.* region 1–44 of the δ subunit and region 1–33 of the γ_2 subunit) and dilution in the external solution (also used in electrophysiological experiments; 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES (pH 7.4) with NaOH) supplemented with 0.1% bovine serum albumin (Sigma). After washing with the same medium, hippocampal neurons were fixed in 4% paraformaldehyde and 4% (w/v) sucrose in phosphate-buffered saline, blocked with 10% normal serum, and sequentially incubated with biotinylated goat anti-rabbit IgG and fluorescein isothiocyanate-labeled streptavidin. To evaluate the synaptic and extrasynaptic distribution of $GABA_A$ receptors, immunocytochemistry for synaptophysin was performed on the same hippocampal neurons. Cells were therefore permeabilized with 0.1% Nonidet P-40, washed with phosphate-buffered saline, blocked with 10% normal serum, and incubated with an anti-synaptophysin monoclonal antibody (Chemicon International, Inc.). The resulting immune complexes were visualized with TRITC-labeled rabbit anti-mouse IgG (Sigma). All secondary antibodies were from Sigma. These experiments were performed on both untreated and nocodazole-treated neurons.

Because it is known that the microtubular network is temperature-sensitive (31), some control experiments were performed in parallel to our main immunocytochemical analysis to verify the cytoskeleton integrity after *in vivo* labeling. Briefly, the same protocol described above was followed with the exception that, after permeabilization, a rat anti-tubulin monoclonal antibody was used instead of synaptophysin. Confocal analysis performed on these neurons demonstrated that the typical branched microtubular bundles were intact after *in vivo* labeling and comparable with those in similar experiments performed at room temperature (data not shown). Moreover, the same type of control experiments were also performed on nocodazole-treated neurons to verify the expected (27) drug-induced depolymerization of the microtubules (data not shown).

Speciments were observed with a ×63 oil immersion objective, followed by ×2 and ×4.5 digital zoom magnification. Images were acquired on a Olympus BX51WI confocal system using sequential dual channel recording of double-labeled cells.

Electrophysiological Recordings—Currents were recorded in the whole cell and nucleated patch configurations of the patch-clamp technique using an EPC-7 amplifier (List Medical, Darmstadt, Germany). The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiments. Cells exhibiting >15% changes during the experiment were excluded from the analysis. The series resistance was 5–7 megaohms, and it was compensated by 70–80%. All the experiments were performed at room temperature (22–24 °C). The intrapipette solution contained 137 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM BAPTA, 2 mM ATP, and 10 mM HEPES (pH 7.2) with CsOH. The composition of the external solution was 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES (pH 7.4) with NaOH. During whole cell recordings, the holding potential was −70 mV. The external solution was supplemented with tetrodotoxin (TTX; 1 μM), kynurenic acid (1 mM), and CGP 55845 (1 μM) to block voltage-activated Na^+ channels, ionotropic glutamate receptors, and GABA type B receptors, respectively. In some cases, TTX was omitted from the solution to allow action potentials to occur and thus promote a larger release of GABA. Currents were sampled at 50–100 kHz, digitized, low pass-filtered at 3.15 kHz with a Butterworth filter, and stored on the computer hard disk. The acquisition softwares used were Clampex 9 (Axon Instruments, Inc., Foster City, CA) and WCP, kindly given by Dr. J. Dempster (Strathclyde University, Glasgow, Scotland, United Kingdom). Control and drug-containing solutions were delivered to the recorded neurons with a perfusion system consisting of glass barrels positioned close to the soma of the recorded cell (multibarrel RSC-200 perfusion system, Bio-Logic, Grenoble, France). Judging from the onset of the liquid junction potentials, a complete exchange of the solution around the open tip electrode occurred within 10–20 ms.

Because it has been observed previously that the amplitude of tonic GABAergic currents in the hippocampus can be reduced when the cells are perfused with a stream of saline (6, 32), our experiments were performed at a constant low perfusion rate. However, in the experiments with NO-711, the perfusion was turned off to allow a larger accumulation of GABA. The drug was first injected into the recording chamber through the glass barrels described above. After the recording solution was completely replaced with the NO-711-containing solution (~4 min), the perfusion system was turned off to prevent ambient GABA from being partially diffused away by the stream of fluids.

Picrotoxin and CGP 55845 were purchased from Tocris (Bristol, United Kingdom). Flurazepam was a kind gift of Dr. S. Vicini. All other drugs were purchased from Sigma.

In the nucleated patch configuration, the holding voltage was −30 mV. GABA-containing solutions were applied to nucleated patches using an ultrafast perfusion system based on a piezoelectric-driven theta glass application pipette (33). The piezoelectric translator was from Physik Instrumente (Waldbronn, Germany), and the theta glass tubing was from Hilgenberg (Malsfeld, Germany). The open tip recordings of the liquid junction potentials revealed that the 10–90% exchange of the solution occurred within 40–80 μs. The speed of the solution exchange was also estimated around the excised patch by the 10–90% onset of the membrane depolarization induced by application of high potassium saline (25 mM). In this case, the 10–90% rise time value (60–90 μs) was very close to that found for the open tip recordings.

Data Analysis—The amplitude of the tonic current was estimated by the outward shift of the base-line current after the application of the $GABA_A$ receptor antagonists bicuculline (100 μM) and picrotoxin (100 μM) (1). Four epochs of 500 ms each were pooled together to calculate the base-line current amplitude and its S.D. The resulting all-point histogram was fitted with a gaussian function. Only current recordings that exhibited a stable base line were included in the analysis. During the experiments in the whole cell configuration, spontaneous inhibitory postsynaptic currents (IPSCs) were recorded. In particular, depending on the presence or absence of TTX, spontaneous miniature (mIPSCs) or action potential-dependent synaptic (sIPSCs) GABAergic currents were identified. Synaptic currents were analyzed with the AxoGraph 4.9 program (Axon Instruments, Inc.). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events because it was moved along the data trace one point at a time and was optimally scaled to fit the data at each position. The detection criterion was calculated from the template-scaling factor and from how closely the scaled template fit the data. The threshold for detection was set at 3.5 times the S.D. of the base-line noise. Using the same program, the decay time constant of averaged mIPSCs was taken from the biexponential fit of the decay time.

The decaying phase of the IPSCs was fitted with exponential functions in the form shown in Equation 1,

$$y(t) = \sum_{i=1}^{n} A_i \cdot \exp(-t/\tau_i) \qquad \text{(Eq. 1)}$$

where τ_i and A_i are the time constants and relative fractions of the respective components. In the case of analysis of normalized currents, the fractions of kinetic components fulfilled the normalization condition (Equation 2).

$$\sum_{i=1}^{n} A_i = 1 \qquad \text{(Eq. 2)}$$

The deactivation time courses of IPSCs were fitted with the sum of two exponentials ($n = 2$). The mean time constant, calculated as in Equation 3,

$$\tau_{\text{mean}} = \sum_{i=1}^{n} A_i \cdot \tau_i \qquad \text{(Eq. 3)}$$

was used to estimate the speed of the decaying process. The goodness of fit was assessed by minimizing the sum of the squared differences. Analysis of variance of the base-line current (σ^2) allowed estimation of the single channel current (i) and the single channel conductance (γ) of the receptors mediating tonic currents (I) (6, 32, 34, 35). Plotting σ^2 *versus* I gives rise to a parabolic function (Equation 4),

$$\sigma^2 = i(1 - P_o)\, I \qquad \text{(Eq. 4)}$$

where P_o is the channel open probability, which varies from 0 to 1. Assuming that, at extremely low concentrations of GABA (0.1–1 μM), the open probability of the channels mediating tonic currents is extremely small, a simplified Equation 4 suggests the following (Equation 5).

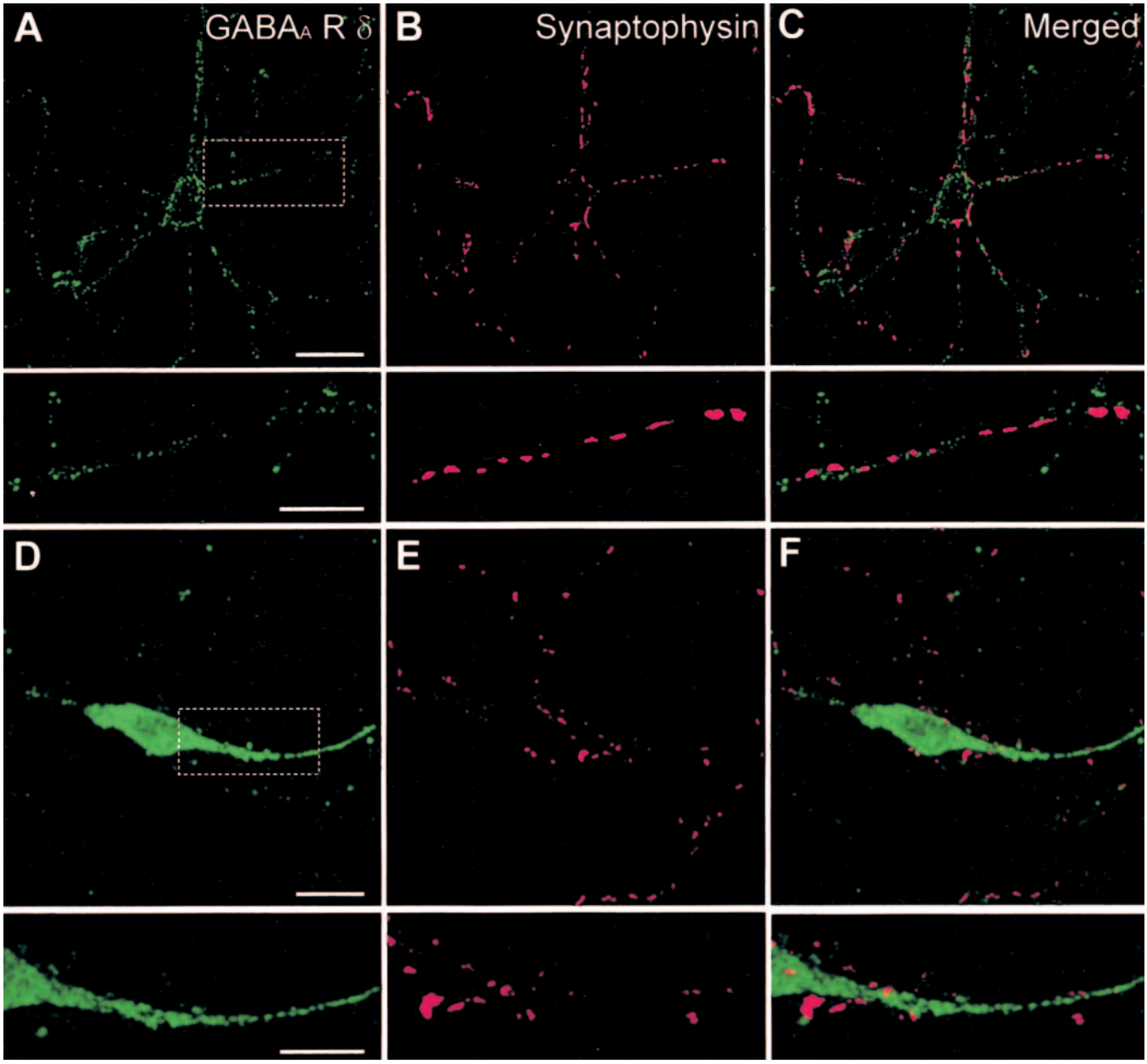

FIG. 1. **Extrasynaptic clusters of δ subunit-containing GABA$_A$ receptors are lost after nocodazole treatment.** Neurons were labeled with a polyclonal antibody recognizing the GABA$_A$ receptor (*R*) δ subunit (*green*) in combination with a mouse anti-synaptophysin antibody (*red*). In untreated neurons, δ subunit-containing GABA$_A$ receptors were arranged in clusters (*A*) localized exclusively at extrasynaptic sites since they were never associated with synaptophysin immunoreactivity (*B* and *C*). After nocodazole treatment, δ subunit-containing GABA$_A$ receptors were uniformly distributed (*D*), and they still did not overlap with synaptophysin staining (*E* and *F*). *Lower panels* are magnifications of the *boxed areas*. *Scale bars* = 10 μm (*upper panels*) and 20 μm (*lower panels*).

$$i = \sigma^2/I \quad \text{(Eq. 5)}$$

However, in the presence of GABA$_A$ receptor antagonist, there is still a variance in the base-line current (σ^2_{bic}, where "bic" is bicuculline). Therefore, Equation 5 must be adjusted for the intrinsic variability of tonic currents (Equation 6).

$$i = (\sigma^2 - \sigma^2_{bic})/I \quad \text{(Eq. 6)}$$

The single channel conductance of the receptors mediating tonic currents was estimated with Equation 7,

$$\gamma = I/(|V_m| - |E_{Cl^-}|) \quad \text{(Eq. 7)}$$

where V_m is the holding potential and E_{Cl^-} is the reversal potential of Cl^- (under our experimental conditions, these values were approximately −70 and 0 mV).

Data are expressed as the means ± S.E., and all values included in the statistics represent recordings from separate cells. Statistical comparisons were made with the use of paired and unpaired *t* tests, the Wilcoxon signed rank test, and the Kolmogorov-Smirnov test ($p < 0.05$ was taken as significant).

Model Simulations—Simulation experiments were performed using the ChanneLab2 software (Synaptosoft). The ChanneLab2 software converted the kinetic model (see Fig. 8*A*) into a set of differential equations and solved them numerically. Since, in the absence of agonist, receptors can spontaneously open at very low probability (36–38), for simulation convenience, it was assumed as an initial condition, *i.e.* at $t = 0$, that no bound or open receptors were present. The solution of such equations yielded the time courses of probabilities of all the states assumed in the model. The fit of the experimental data was performed by optimizing the values of rate constants. The procedure for the rate constant optimization was based on the comparison of the time course of recorded currents and that of simulated responses.

RESULTS

δ and γ$_2$ Subunit-containing GABA$_A$ Receptors Are Clustered at Extrasynaptic Sites and Become Uniformly Distributed after

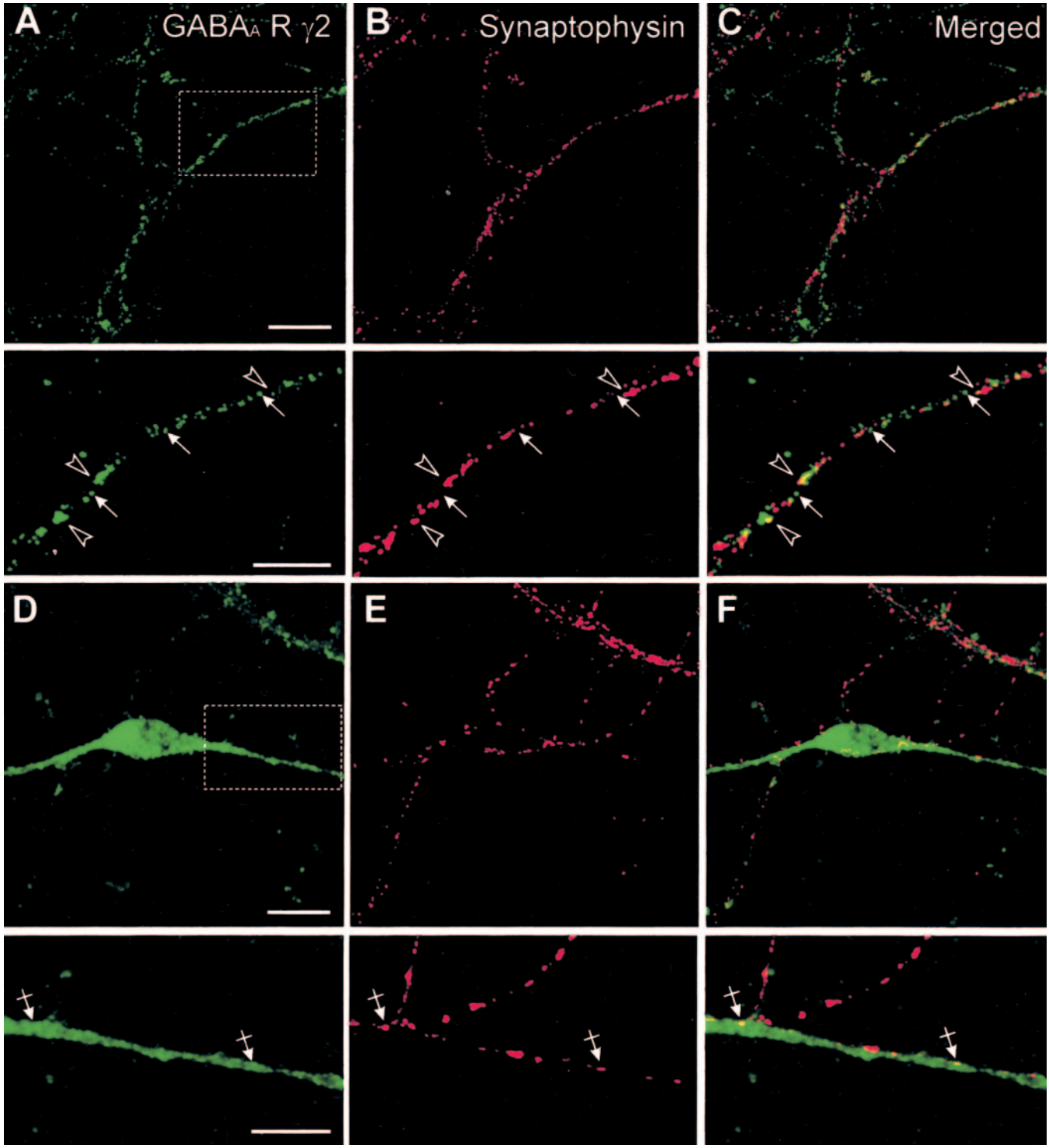

FIG. 2. **Nocodazole treatment promotes the disassembly of γ_2 subunit-containing GABA$_A$ receptor clusters located at both synaptic and extrasynaptic sites.** Neurons were labeled with a rabbit polyclonal antibody specifically recognizing the GABA$_A$ receptor (*R*) γ_2 subunit (*green*) in combination with a mouse anti-synaptophysin antibody (*red*). *Lower panels* are magnifications of the *boxed areas*. In untreated neurons (*A–C*), γ_2 subunit-containing GABA$_A$ receptors formed clusters that either co-localized with synaptophysin immunoreactivity (synaptic clusters) (*lower panels*, *arrowheads*) or not (extrasynaptic receptors) (*arrows*). Nocodazole-treated neurons (*D–F*) displayed a diffuse γ_2 subunit immunoreactivity throughout the cell surface. However, some residual puncta could still be detected. Note that some diffuse γ_2 subunit-containing GABA$_A$ receptors still co-localized with synaptophysin, indicating synaptic receptors (*lower panels*, *crossed arrows*). *Scale bars* = 20 μm (*upper panels*) and 10 μm (*lower panels*).

Nocodazole Treatment—In this work, we analyzed the influence of the clustering of extrasynaptic GABA$_A$ receptors on tonic inhibition. Taking advantage of a previous demonstration that GABA$_A$ receptor clustering can be impaired by inducing the disassembly of the cytoskeleton with the microtubule-disrupting drug nocodazole (27), we first analyzed the distribution pattern and nocodazole sensitivity of δ subunit-containing receptors. In fact, there is much experimental evidence demonstrating that the δ subunit is exclusively expressed at extrasynaptic locations, even though, to our knowledge, its clustering has not yet been addressed.

Immunocytochemical experiments were therefore performed

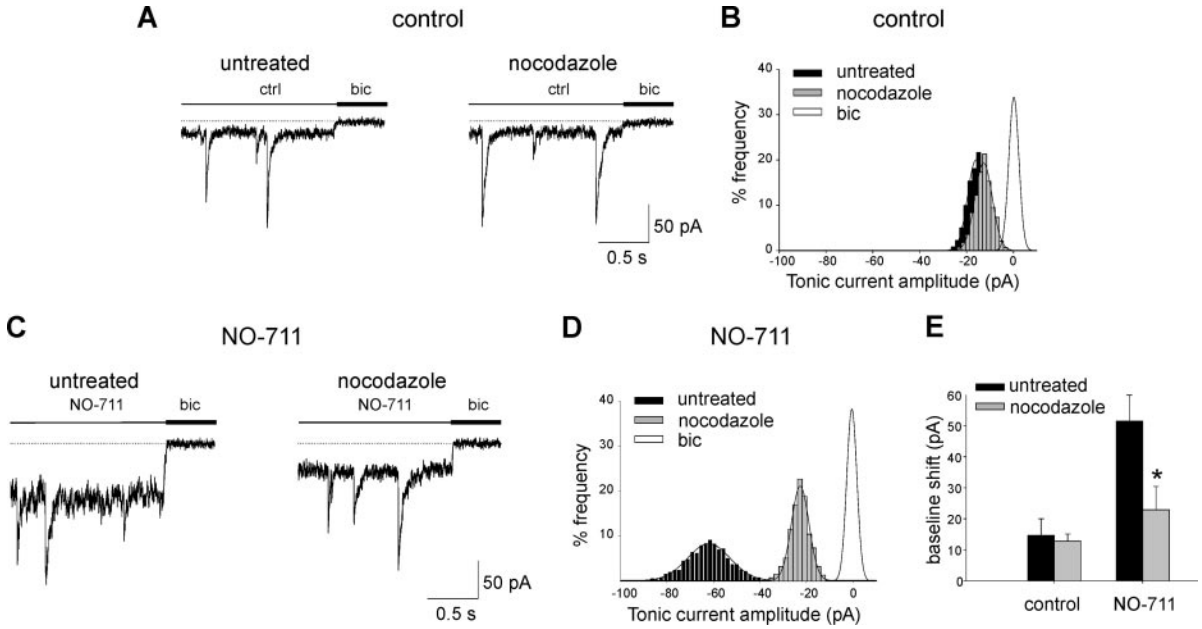

FIG. 3. **Nocodazole attenuates the effect of NO-711 on tonic current amplitudes.** *A*, currents recorded under control conditions (*ctrl*) and in the presence of bicuculline (*bic*) (*solid lines*) from untreated (*left panel*) and nocodazole-treated (*right panel*) neurons. Some sIPSCs were present, but they were excluded from the analysis of tonic current amplitudes. The *dotted lines* represent the holding current in the presence of bicuculline. *B*, all-point histogram of a 500-ms trace recorded under control conditions from untreated and nocodazole-treated neurons (note that the two distributions overlap) and in the presence of bicuculline. The *thin black lines* represent the gaussian fit of the distributions. *C*, tonic currents recorded in the presence of NO-711 and in the presence of bicuculline (*solid lines*) from untreated (*left panel*) and nocodazole-treated (*right panel*) neurons. *D*, all-point histogram of a 500-ms trace recorded in the presence of NO-711 from untreated and nocodazole-treated neurons (note that the two distributions are significantly shifted) and in the presence of bicuculline. *E*, summary of the mean tonic current amplitude (base-line shift) under control conditions and in the presence of NO-711 for untreated and nocodazole-treated neurons ($n = 6$). *, $p < 0.05$.

to detect the surface distribution of δ subunit-containing receptors in untreated primary cultured hippocampal neurons. Interestingly, we found a clearly dotted staining of δ subunits (Fig. 1*A*). δ subunits never co-localized with the presynaptic marker synaptophysin (Fig. 1, *B* and *C*), thus suggesting a clustered arrangement of δ subunit-containing GABA$_A$ receptors at extrasynaptic sites. This punctate staining was significantly affected by nocodazole treatment. In fact, in nocodazole-treated neurons, δ subunits were almost uniformly distributed along the cell surface, although some residual puncta were still detectable (Fig. 1*D*, *lower panel*). The double staining of δ subunit/synaptophysin in nocodazole-treated neurons never showed a co-localization of the two proteins, suggesting that also declustered δ-containing receptors maintained their extrasynaptic localization (Fig. 1*F*, *lower panel*).

Clusters of γ_2 subunit-containing GABA$_A$ receptors have been recently identified at extrasynaptic sites (26, 39), although their functional role has been poorly investigated. An antibody specific for the extracellular N-terminal domain of the γ_2 subunit was used to decorate γ_2 subunit-containing GABA$_A$ receptors on the cell surface. In untreated neurons, γ_2 subunits displayed a punctate staining pattern over the neuronal somata and dendrites (Fig. 2*A*). Only some of the puncta were co-localized with synaptophysin, thus indicating that γ_2 subunits were present at both synaptic sites (Fig. 2*C*, *lower panel*, *arrowheads*) and extrasynaptic sites (*arrows*). As expected from our previous work (27), nocodazole treatment was associated with the declusterization of γ_2 subunit-containing GABA$_A$ receptors. Most interestingly, we found that, under these conditions, co-localization between declustered γ_2 subunits and synaptophysin was still detectable, thus indicating that nocodazole treatment affected both synaptic and extrasynaptic receptors (Fig. 2*F*, *lower panel*, *crossed arrows*).

Tonic Current Amplitudes in Untreated and Nocodazole-treated Neurons—Currents were recorded from cultured hippocampal neurons in the whole cell configuration of the patch-clamp technique at a holding potential of −70 mV in the presence of kynurenic acid (1 mM) and CGP 55845 (1 mM). Under these conditions, it was possible to record both sIPSCs and a persistent tonic current that was identified upon the application of GABA$_A$ receptor antagonists (see "Experimental Procedures"). The application of bicuculline (100 μM) produced the complete disappearance of sIPSCs and an outward shift of the base-line current (Fig. 3*A*).

In an attempt to test whether nocodazole treatment could affect the amplitude of the tonic current, the shift of the base-line current after the application of bicuculline (100 μM) was measured in untreated and nocodazole-treated neurons. Under these two conditions, tonic currents were not significantly different. In untreated neurons, the base-line shift was 14.7 ± 5.3 pA, whereas in nocodazole-treated neurons, it was 12.7 ± 2.3 pA ($n = 6$; $p > 0.05$) (Fig. 3, *A* and *E*). The all-point histograms of the base-line currents in untreated and nocodazole-treated neurons exhibit overlapping distributions (Fig. 3*B*). Similar results were obtained upon the application of picrotoxin (100 μM). The base-line shifts were 15.4 ± 5.7 and 11.9 ± 3.5 pA in untreated and nocodazole-treated neurons, respectively ($n = 4$; $p > 0.05$) (data not shown). The similarity of the tonic current amplitudes in untreated and nocodazole-treated neurons suggests that the depolymerization of microtubules and the associated declusterization of GABA$_A$ receptors did not reduce the number of the receptors expressed on the cell surface. This hypothesis is further supported by our previous demonstration that also the amplitude of synaptic currents and GABA-evoked currents is not significantly reduced after nocodazole treatment (27).

Nocodazole Treatment Attenuates NO-711-induced Increases in Tonic Current Amplitude—The lack of significant differences between the tonic current amplitudes recorded in untreated and nocodazole-treated neurons could be attributed to the small concentration of ambient GABA derived mainly from spillover of the neurotransmitter from neighboring synapses.

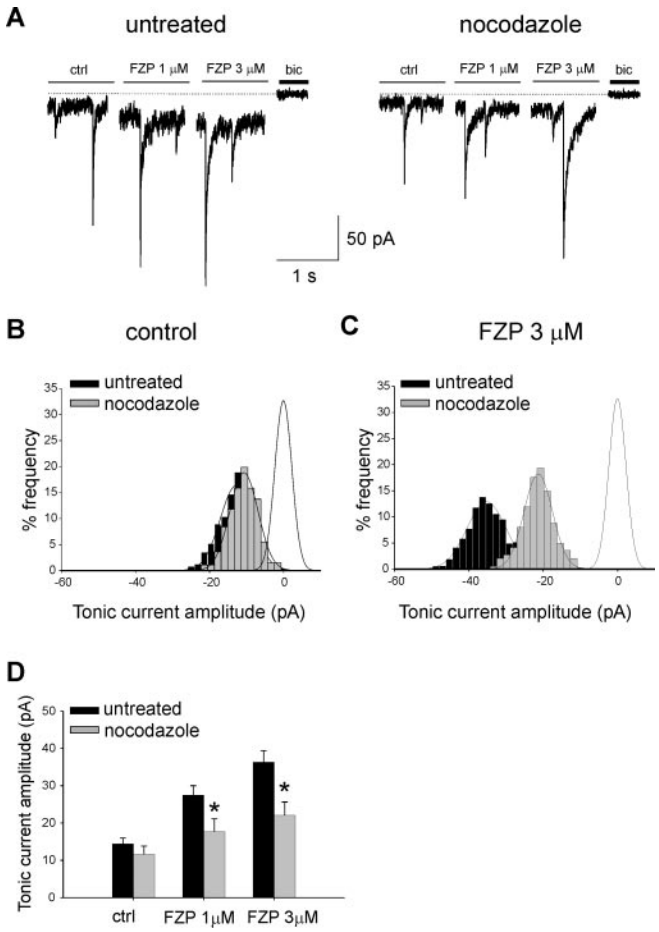

FIG. 4. **Nocodazole treatment reduces the enhancing effect of FZP on tonic currents.** *A*, example currents recorded from untreated (*left panel*) and nocodazole-treated (*right panel*) neurons under control conditions (*ctrl*) and in the presence of FZP (1 and 3 μM) or bicuculline (*bic*) (*solid lines*). The *dotted lines* represent the holding current in the presence of bicuculline. sIPSCs were removed during the analysis of tonic currents. *B*, all-point histogram of a 500-ms trace recorded under control conditions from untreated and nocodazole-treated neurons (note that the two distributions overlap) and in the presence of bicuculline. The *thin black lines* represent the gaussian fit of the distributions. *C*, similar all-point histogram in the presence of 3 μM FZP. Note the different distributions between untreated and nocodazole-treated neurons. *D*, mean tonic current amplitude recorded from untreated and nocodazole-treated neurons under control conditions and in the presence of 1 and 3 μM FZP ($n = 6$). *, $p < 0.05$.

To test this hypothesis, we sought to increase the concentration of ambient GABA by blocking GABA uptake with the GAT-1 antagonist NO-711 (8, 34, 40, 41). In agreement with previous reports (34, 41), NO-711 (100 μM) slowed down the deactivation kinetics of sIPSCs in both untreated and nocodazole-treated neurons. In untreated neurons, the application of NO-711 increased the values of τ_{mean} from 24.1 ± 2.5 to 39.4 ± 4.8 ms ($n = 6$; $p < 0.05$) (data not shown). Consistent with a larger concentration of ambient GABA, in the presence of NO-711, both untreated and nocodazole-treated neurons exhibited larger tonic currents (Fig. 3, *C–E*). In untreated neurons, after the application of NO-711, the tonic current was ~3.6-fold larger than in control neurons. In fact, with NO-711 (10 μM), the base-line shift induced by bicuculline was 51.5 ± 8.5 pA, whereas under control conditions, it was 14.7 ± 5.3 pA ($n = 6$; $p < 0.001$) (Fig. 3, *A*, *C*, and *E*). In contrast, in nocodazole-treated neurons, NO-711 induced a non-significant increase in the amplitude of the tonic current, from 12.7 ± 2.3 to 22.9 ± 7.5 pA ($n = 6$; $p > 0.05$) (Fig. 3, *A*, *C*, and *E*). The all-point histograms of tonic currents recorded in the presence of NO-711 from untreated and nocodazole-treated neurons clearly showed a significant difference in their distributions ($p < 0.001$) (Fig. 3*D*). In conclusion, in the presence of NO-711, the difference between the amplitudes of the tonic currents in untreated and in nocodazole-treated neurons was statistically significant ($p < 0.05$) (Fig. 3*E*).

Tonic Currents from Untreated and Nocodazole-treated Neurons Are Sensitive to Flurazepam—To compare the pharmacology of tonic currents in untreated and nocodazole-treated neurons and to infer the subunit composition of extrasynaptic GABA$_A$ receptors, we studied the effect of benzodiazepines, known to be effective on γ_2 subunit-containing receptors (42, 43). The efficiency of FZP was assessed by analyzing its effect on the peak amplitude and kinetics of sIPSCs. In agreement with previous studies (6, 44–46), in untreated neurons, FZP (1 μM) significantly ($p < 0.05$) prolonged the decay kinetics of sIPSCs (τ_{mean} = 27.7 ± 2.1 ms under control conditions and 33.9 ± 0.9 ms with 1 μM FZP; $n = 4$) and increased their peak amplitude from 126.9 ± 26.2 to 166.5 ± 28.9 pA ($p < 0.05$) (data not shown). The amplitude of the tonic current was measured under control conditions and during the application of FZP (1 and 3 μM). In untreated neurons, FZP induced a significant dose-dependent increase in the amplitude of the tonic current (from 14.4 ± 1.5 pA under control conditions to 27.5 ± 2.5 pA with 1 μM FZP and 36.3 ± 3.1 pA with 3 μM FZP; $n = 6$; $p < 0.001$) (Fig. 4). After nocodazole treatment, the effect of FZP was attenuated. The mean amplitude of tonic currents increased from 11.6 ± 2.1 pA under control conditions to 17.6 ± 3.4 and 22.9 ± 3.5 pA with 1 and 3 μM FZP, respectively; $n = 6$; $p < 0.02$) (Fig. 4). It is clear from Fig. 4 that the all-point histograms of tonic current amplitudes recorded under control conditions from untreated and nocodazole-treated neurons show two overlapping distributions ($p > 0.05$) (Fig. 4*B*), whereas those recorded in the presence of 3 μM FZP are significantly separated ($p < 0.01$) (Fig. 4*C*). The susceptibility of tonic currents to benzodiazepines suggests the involvement of γ_2 subunit-containing receptors.

Nocodazole Treatment Reduces the Amplitude of Tonic Currents Induced by Low Concentrations of Exogenous GABA—A widely accepted hypothesis is that the spillover of GABA from neighboring synapses contributes to the accumulation of the neurotransmitter in the extracellular space (1, 3, 32), thus influencing tonic inhibition. Direct proof of an activity-dependent modulation of tonic currents came from experiments performed in the presence of TTX, where reduced synaptic activity was accompanied by a tonic current of smaller amplitude (45, 47). In a set of experiments, TTX (1 μM) was added to the external solution in addition to kynurenic acid (1 mM) and CGP 55845 (1 mM). Under these conditions, the amplitudes of the tonic current were 7.5 ± 1.2 and 7.8 ± 3.3 pA ($n = 12–14$) in untreated and nocodazole-treated neurons, respectively (Fig. 5*A*), *i.e.* ~2-fold smaller than in the absence of TTX (see Fig. 3*A*). Similar results were obtained when picrotoxin (8) was used instead of bicuculline. In the presence of TTX, the amplitudes of the tonic current were 7.7 ± 3.2 and 7.2 ± 3.7 pA in untreated and nocodazole-treated neurons, respectively ($n = 4$) (data not shown). These results clearly indicate a direct correlation between synaptic activity and tonic current amplitude.

To quantitatively estimate the correlation between the concentration of GABA in the extracellular space and the amplitudes of the tonic currents in untreated and nocodazole-treated neurons, low concentrations of GABA (0.1–1 μM) were applied. In this set of experiments, TTX (1 μM) was added to the external solution to minimize the amount of endogenously released GABA. In both untreated and nocodazole-treated neurons, GABA produced a dose-dependent downward shift of the base-

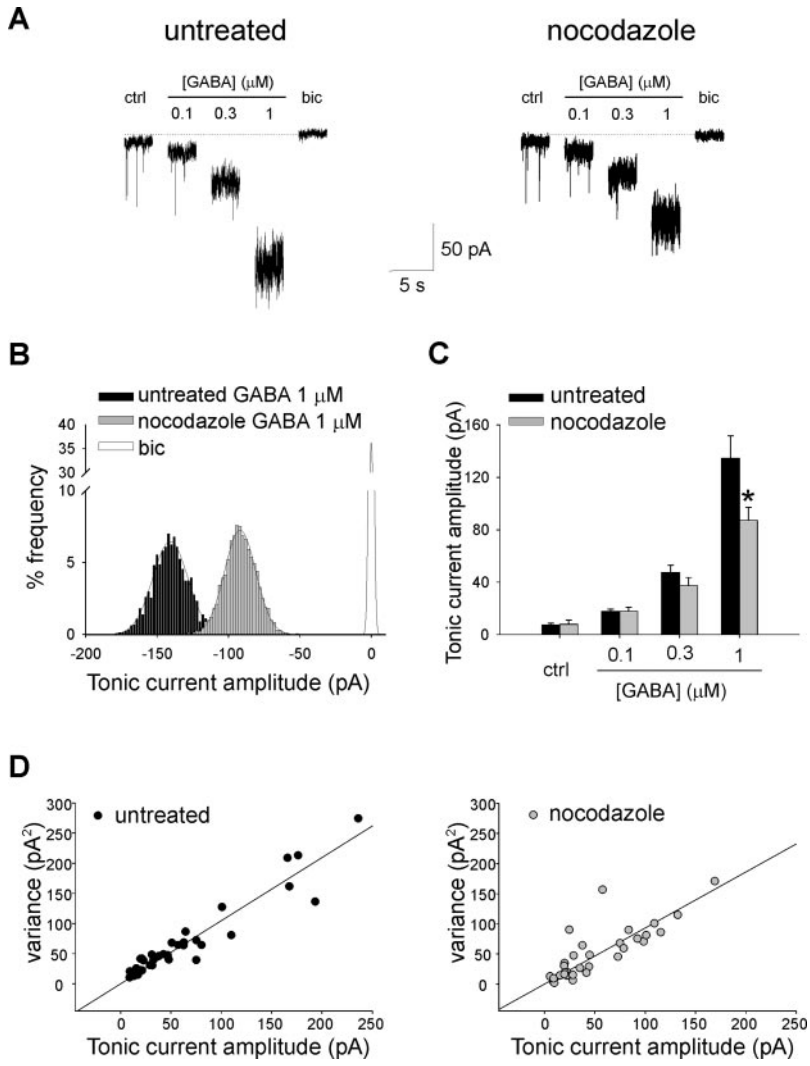

FIG. 5. **Tonic currents evoked by exogenous applications of low [GABA] are smaller in nocodazole-treated neurons.** *A*, currents recorded under control conditions (*ctrl*) and in the presence of different concentrations of exogenous GABA from untreated (*left panel*) and nocodazole-treated (*right panel*) neurons. The holding current in the presence of bicuculline (*bic*) sets the zero level and is represented by the *dotted lines*. *B*, all-point histogram of a 500-ms trace recorded in the presence of 1 μM GABA and bicuculline from untreated and nocodazole-treated neurons. mIPSCs were excluded from the analysis. *C*, mean amplitude of tonic currents recorded under control conditions and in the presence of different concentrations of GABA from untreated and nocodazole-treated neurons (n = 12–14). *, $p < 0.05$. *D*, plots of the variance *versus* the amplitude of the tonic currents recorded from untreated (*left panel*) and nocodazole-treated (*right panel*) neurons (n = 12–14). The *solid lines* are the linear regression fits of all data points. The estimated mean single channel conductance was ~16 pS for untreated and nocodazole-treated neurons.

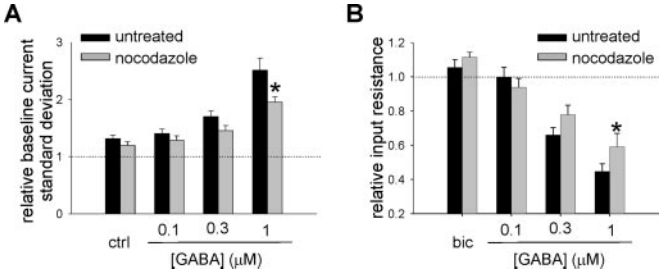

FIG. 6. **GABA$_A$ receptors are less activated in response to low agonist concentrations after nocodazole treatment.** *A*, means ± S.D. of base-line currents recorded under control conditions (*ctrl*) and in the presence of low [GABA], normalized to those recorded in the presence of bicuculline (*bic*) (n = 12–14). *, $p < 0.05$. *B*, mean membrane input resistance recorded from untreated and nocodazole-treated neurons in the presence of bicuculline and low [GABA], normalized to the value recorded under control conditions (n = 6). *, $p < 0.05$.

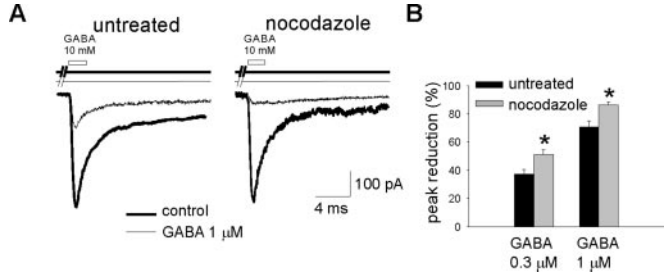

FIG. 7. **Block of GABA-evoked currents after pre-equilibration with low [GABA] is larger in nocodazole-treated neurons.** *A*, current responses to short pulses (2 ms) of saturating [GABA] (10 mM; *open bars*) after a 20-min pre-equilibration with a control solution (*thick lines*) or with 1 μM GABA (*thin lines*) in untreated (*left panel*) and nocodazole-treated (*right panel*) neurons. *B*, mean reduction of control responses after pre-equilibration with low [GABA] in untreated and nocodazole-treated neurons (n = 10–11). *, $p < 0.05$.

line current (Fig. 5, *A* and *C*). However, in nocodazole-treated neurons, this effect was less pronounced, and the amplitude of the tonic current induced by a given concentration of GABA was always smaller than the corresponding value in untreated neurons. In particular, the difference became significant at 1 μM GABA. In untreated and nocodazole-treated neurons, the amplitudes of the currents were 134.7 ± 17.1 and 87.3 ± 9.8 pA, respectively (n = 12–14; $p < 0.05$) (Fig. 5*C*). The all-point histograms of the tonic currents in the presence of 1 μM GABA show distinct distributions with significantly different means ± S.D. (Fig. 5*B*).

To determine whether the nocodazole-induced reduction in the amplitude of tonic currents could be attributed to changes in the single channel conductance of declustered receptors, a simplified form of stationary variance analysis of the base-line currents was performed (6, 32, 34, 35). The single channel conductance of clustered and declustered receptors was calculated assuming that the concentration of GABA responsible for tonic current is so low that the receptor open probability becomes negligible. The relationship between the amplitude (I) and the variance (σ^2) of the tonic current is illustrated in Fig. 5*D*. As shown in the Fig.

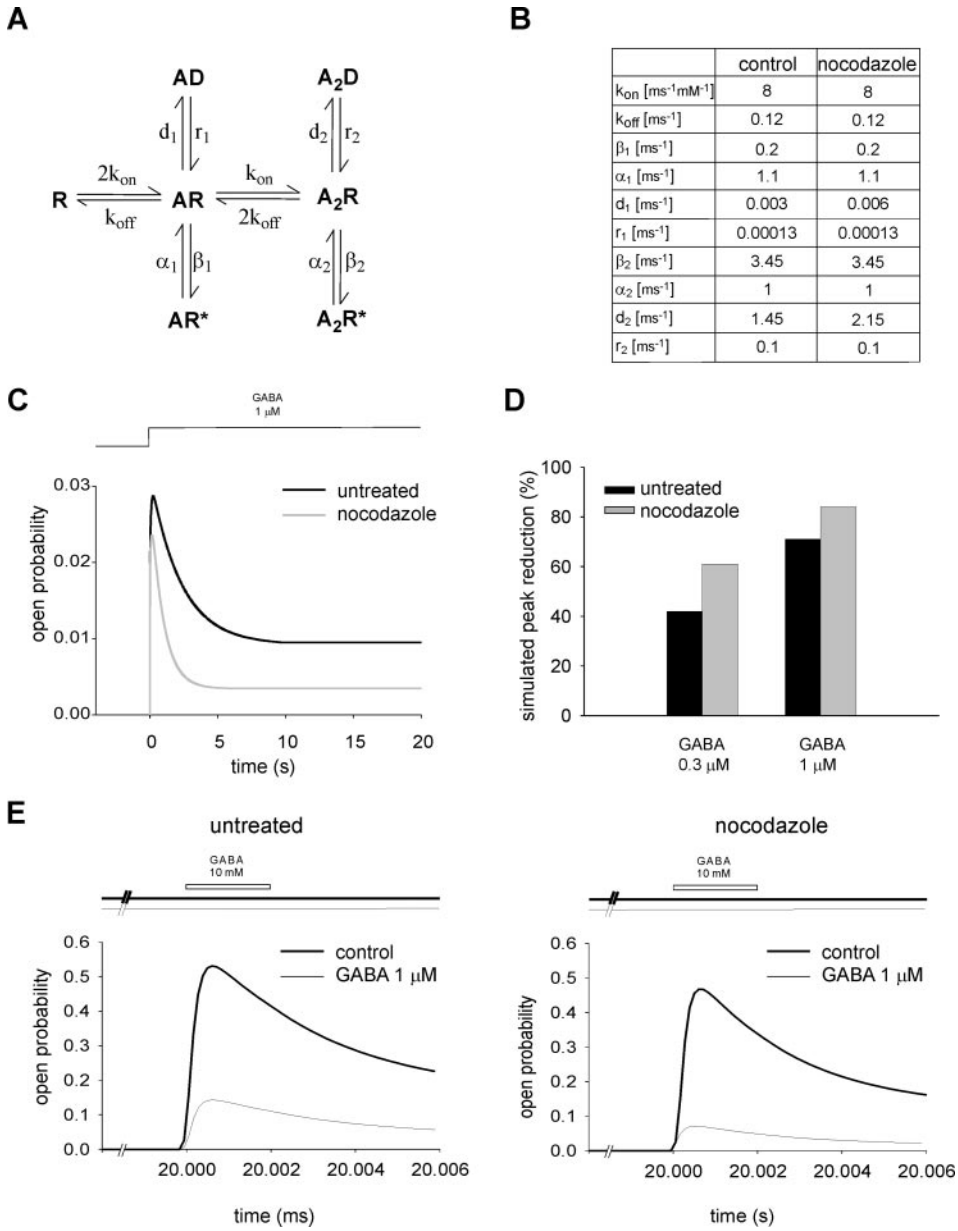

FIG. 8. **Model simulations.** *A*, kinetic model proposed by Jones and Westbrook (48). According to the model, the receptor (*R*) can bind one or two molecules of agonist (*A*), reaching either the singly (*AR*) or doubly (A_2R) bound closed state. From these states, it can open (*AR** and A_2R*) or desensitize (*AD* and A_2D). *B*, values of the rate constants chosen to simulate tonic and GABA-evoked currents under control conditions and in the presence of nocodazole. *C*, simulated tonic current (*lower trace*) evoked by a prolonged exposure to 1 μM GABA (*upper trace*) in untreated and nocodazole-treated neurons. *D*, mean reduction in the amplitude of simulated GABA-evoked currents after pre-equilibration with low [GABA] in untreated and nocodazole-treated neurons. *E*, simulated current responses (*lower traces*) to short pulses (2 ms) of saturating [GABA] (10 mM; *open bars*) after pre-equilibration with a control solution (*thick lines*) or with 1 μM GABA (*thin lines*) in untreated (*left panel*) and nocodazole-treated (*right panel*) neurons.

5*D*, the estimated single channel conductances of $GABA_A$ receptors exposed to 0.1–1 μM GABA in untreated and nocodazole-treated neurons were comparable (17.1 ± 1.0 and 16.6 ± 2.5 pS, respectively; $n = 12–14$; $p > 0.05$). These data allow exclusion of nocodazole-induced changes in single channel conductance or re-assortment of $GABA_A$ receptor subunits exhibiting different conductances.

Interestingly, the dose-dependent increase in the amplitude of the tonic current induced by exogenous GABA was accompanied by a similar increase in the S.D. of the base-line current (Fig. 6*A*). The S.D. has been considered a good parameter for evaluating the variability of the tonic current since it reflects the level of activation of $GABA_A$ receptors (6, 8, 35). The S.D. of the tonic currents under control conditions and in the presence of low [GABA] was normalized to that obtained in the presence of bicuculline. These values were always smaller in nocodazole-treated neurons than in untreated neurons (Fig. 6*A*). In particular, when 1 μM GABA was applied, the normalized S.D. of the tonic current in nocodazole-treated neurons was significantly smaller than that observed in untreated neurons (1.9 ± 0.1 and 2.5 ± 0.2, respectively; $n = 12–16$; $p < 0.05$). This result suggests that, in response to the same GABA concentration, $GABA_A$ receptors are less activated after nocodazole treatment.

A further confirmation of this hypothesis came from the measurement of the membrane input resistance in untreated and nocodazole-treated neurons. The input resistance values obtained in the presence of bicuculline or low [GABA] were normalized to those found under control conditions (Fig. 6*B*). In the presence of bicuculline, the normalized values were similar in untreated and nocodazole-treated neurons (1.05 ± 0.04 and

1.12 ± 0.03, respectively; $n = 6$; $p > 0.05$) and, as expected, were >1. At increasing concentrations of GABA, the normalized input resistance progressively decreased, reflecting a larger flow of ions through the receptors, *i.e.* a larger shunting inhibition. However, in nocodazole-treated neurons, this effect was less pronounced; and in the presence of 1 μM GABA, the normalized input resistance was significantly larger than in untreated neurons (0.45 ± 0.04 and 0.59 ± 0.08 in untreated and nocodazole-treated cells, respectively; $n = 6$; $p < 0.05$).

Block of GABA-evoked Currents Induced by Prolonged Exposure to Low [GABA] Is Smaller after Nocodazole Treatment—The reduction of tonic inhibition observed in nocodazole-treated neurons can be attributed either to a change in the gating properties of GABA$_A$ receptors or to a reduced number of receptor channels. To test for these possibilities, currents evoked by ultrafast applications of saturating [GABA], after a pre-equilibrating protocol with low [GABA] or with a control solution, in both untreated and nocodazole-treated neurons were examined.

If nocodazole treatment *per se* reduced the number of active receptors, all the responses, with and without pre-equilibration with low [GABA], should be smaller than those obtained in untreated neurons. The peak amplitude of the currents evoked by saturating [GABA] after nocodazole treatment was used as an index of the total number of functional declustered GABA$_A$ receptors. Currents were recorded at −30 mV from nucleated patches. GABA pulses (10 mM for 2 ms) were applied every 2 min either under control conditions or after a 20-min pre-equilibration with low [GABA] (0.3–1 μM). Only stable recordings with no signs of rundown were used for the analysis. The responses obtained under control conditions and after pre-equilibration with low [GABA] were averaged separately. The responses evoked by saturating [GABA] in untreated and nocodazole-treated neurons under control conditions (without pre-equilibration with low [GABA]) were very similar (1056.6 ± 144.3 and 1010.5 ± 212.2 pA, respectively; n = 10–11; $p > 0.05$) (Fig. 7*A*). This suggests that nocodazole treatment does not affect the number of active receptors (see Ref. 27). After the pre-equilibrating protocol with 1 μM GABA, the amplitude of the responses elicited by saturating pulses of 10 mM GABA was significantly reduced. However, in comparison with untreated neurons, the responses obtained after nocodazole treatment were significantly smaller (240.2 ± 30.2 and 136.8 ± 47.9 pA in untreated and nocodazole-treated neurons, respectively; n = 10–11; $p < 0.05$) (Fig. 7*A*). This shows that pre-equilibration with GABA produced a block of GABA responses of 70.7 ± 4.3 and 86.1 ± 2.3% in untreated and nocodazole-treated neurons, respectively ($p < 0.05$) (Fig. 7*B*). In the presence of 0.3 μM GABA, the reduction in the peak amplitude of GABA-evoked currents in untreated and nocodazole-treated neurons was significantly different (37.2 ± 3.3 and 51.3 ± 3.4%, respectively; n = 10–11; $p < 0.05$) (Fig. 7*B*). In conclusion, the pre-equilibration of nucleated patches with low [GABA] blocked GABA-evoked currents more extensively in nocodazole-treated neurons than in untreated ones.

Model Simulations—The present findings demonstrate that, in cultured hippocampal neurons, nocodazole-induced microtubule disruption was associated with the declusterization of extrasynaptic GABA$_A$ receptors and with a reduction of tonic inhibition. In particular, after nocodazole treatment, declustered extrasynaptic GABA$_A$ receptors were less activated by ambient (endogenous or exogenous) GABA; thus, the amplitude of the tonic current was smaller. In an attempt to reconstruct the gating properties of declustered extrasynaptic GABA$_A$ receptors, model simulations were used. We referred to the kinetic model (Fig. 8*A*) proposed by Jones and Westbrook (48), which fulfills the minimum requirement to adequately reproduce the gating of GABA$_A$ receptors in different experimental protocols. We adopted and optimized the parameters we proposed previously (27) to reproduce the gating properties of declustered synaptic GABA$_A$ receptors. Because the concentrations of GABA used in that study were >30 μM, the transitions between singly bound open and desensitized states could not be resolved (49, 50). Therefore, the corresponding rate constants were merely adopted from Jones and Westbrook (48). In the present work, the application of extremely low concentrations of GABA (0.1–1 μM) allowed investigation of the singly bound states (50–52). The rate constants were adjusted to reproduce tonic and GABA-evoked currents recorded in the presence of exogenous GABA. The experimental data and our previous study (27) suggested that declustered receptors can be more susceptible to desensitization. For this reason, in this study, we tried to simulate an increased occupancy of the singly bound desensitized state, besides the already assessed larger occupancy of the doubly bound desensitized state (27). It must be pointed out that a larger occupancy of a given conformational state may be due to either a faster entry into or a slower exit from that state. Unfortunately, for the singly bound states, there are no specific protocols to distinguish between these two possibilities. For this reason, we achieved a simulated larger occupancy of the singly bound desensitized state of declustered receptors simply by increasing the value of d_1, the rate constant governing the transition from the singly bound closed state to the singly bound desensitized state (Fig. 8*B*).

With the increased value of d_1, it was possible to reproduce the smaller amplitude of tonic current in nocodazole-treated neurons (Fig. 8*C*). During a simulated prolonged exposure (10 s) to 1 and 0.3 μM GABA, the steady-state open probabilities (tonic current) of declustered GABA$_A$ receptors were 0.0034 and 0.0031, respectively, whereas those of clustered receptors were 0.0094 and 0.0047, respectively. Such small values indicate that when GABA is present in the extracellular space (ambient GABA), the open states of GABA$_A$ receptors are poorly occupied. Nevertheless, after nocodazole treatment, their occupancy is even smaller.

The same sets of rate constants relative to clustered and declustered receptors were used to simulate GABA-evoked currents under control conditions and after pre-equilibration with GABA-containing solutions. The reduction of the simulated open probability induced by pre-equilibration with low [GABA] was very similar to that recorded experimentally. Simulated pre-equilibration with 1 μM GABA induced blocks of 71 and 84% (Fig. 8*E*), whereas that with 0.3 μM GABA induced blocks of 42 and 61% for clustered and declustered GABA$_A$ receptors, respectively (Fig. 8*D*). Model simulations suggest that, after nocodazole treatment, there is a larger occupancy of the singly bound desensitized state of GABA$_A$ receptors and that this is responsible for reduced tonic inhibition.

DISCUSSION

The present results clearly demonstrate that, in cultured hippocampal neurons, nocodazole treatment induced the declusterization of extrasynaptic GABA$_A$ receptors and reduced GABA-mediated tonic inhibition. Analysis of GABA-evoked currents after preincubation with low concentrations of GABA and model simulations suggest that the effects of nocodazole on tonic inhibition are due mainly to changes in the gating properties of GABA$_A$ receptors. In particular, we propose that nocodazole treatment promotes the accumulation of declustered receptors into a singly bound desensitized state during tonic exposure to ambient GABA.

Consistent with previous reports (20, 25, 26, 39, 53), our immunocytochemical data clearly demonstrate that, in addition to synaptic receptors, also extrasynaptic ones are arranged

in clusters and that they become uniformly distributed throughout the cell surface after nocodazole treatment. In agreement with other groups (39, 54–56), we have found that extrasynaptic receptors in the hippocampus include the δ and γ_2 subunits. However, it seems quite clear that the δ and γ_2 subunits cannot be assembled within the same receptor (57, 58), yet the exact subunit composition of extrasynaptic GABA$_A$ receptors is still unknown, although a substantial effort has been recently made in this direction (58–60). Although the contribution of δ subunits has been well documented, particularly in the dentate gyrus (55, 56), that of γ_2 subunits has often been neglected (20, 61). The lack of complete disappearance of the tonic current in the hippocampus of $\delta^{-/-}$ mice favors the involvement of other receptor subunits (15, 62). Possible candidates are the γ_2 subunits since they are required for the observed facilitatory effect of FZP on tonic inhibition (42, 43, 63–66). However, it must be pointed out that benzodiazepine sensitivity can also be due to α_5 subunit-containing receptors (67–69), which are largely expressed in the hippocampus (68–71). On the basis of recent studies that have demonstrated the co-assembly of α_5 and γ_2 subunits and that have predicted that the native receptors in the hippocampus are $\alpha_5\beta_3\gamma_2$ (68, 71), we can speculate that, in our preparation, γ_2 subunits may belong to $\alpha_5\beta_3\gamma_2$ receptors.

In the present experiments, nocodazole was used as a pharmacological tool to promote the loss of clustered arrangement of GABA$_A$ receptors. The observation that, after nocodazole treatment, the declusterization of receptors that did not co-localize with synaptophysin (*i.e.* extrasynaptic receptors) was associated with changes in tonic inhibition suggests that the two events are correlated. Although nocodazole treatment affected both synaptic and extrasynaptic receptors, it is conceivable that the contribution of synaptic receptors to tonic currents is very small or even negligible since the number of extrasynaptic receptors largely exceeds that of synaptic ones (24, 72).

By comparing the amplitudes of tonic currents evoked by endogenous and exogenous applications of GABA, it was possible to estimate the ambient agonist concentration in our preparation. Consistent with previous reports on cerebellar granule cells (45) and cultured hippocampal neurons (73), we found a value ~0.1 μM. However, these estimations may not exactly reflect the physiological value *in vivo* since they all refer to neurons in culture. In fact, the loss of the anatomical arrangement and the modification of the architecture of the extracellular space in primary cultures may influence the concentration of ambient GABA. From our results, differences between tonic inhibition mediated by clustered and declustered receptors could be revealed only when ambient GABA was increased (*i.e.* in the presence of NO-711 or at the highest concentration of exogenous GABA). This evidence suggests that, under normal conditions, the modulatory effect of receptor clustering on tonic inhibition is not detectable, but it becomes effective when ambient [GABA] exceeds a threshold, as during sustained GABAergic activity.

Noise analysis has demonstrated that, in both untreated and nocodazole-treated neurons, "low conductance channels" mediate tonic inhibition. The value of the single channel conductance estimated here for both clustered and declustered receptors (~16 pS) is consistent with that reported previously (32, 45, 49–51, 74) for receptors mediating tonic inhibition (74, 75). It has been proposed that receptors can be partially or fully activated depending on the concentration of agonist they "see" (76–78). Therefore, it is possible that, at ambient concentrations of GABA, tonic currents are mediated mainly by monoliganded receptors in a lower conductance state (6, 35, 50). However, it cannot be excluded that the conductance level of a given receptor can also be influenced by its subunit composition (45, 69, 74). Therefore, the comparable estimation of the single channel conductance of clustered and declustered receptors suggests that, at low concentrations of agonist, the receptors are similarly activated since nocodazole treatment affects neither the molecular structure/subunit composition nor the proportion of monoliganded GABA$_A$ receptors.

The widespread action of nocodazole raises the possibility that modulation of tonic inhibition can be due to multiple indirect effects. For instance, it is possible that this drug alters the release machinery and therefore influences the concentration of ambient GABA or reduces the number of GABA$_A$ receptors, changes their subunit composition, or affects their gating properties. On the basis of our previous work (27), we can exclude a presynaptic site of action of nocodazole because this drug did not affect the frequency of mIPSCs and because comparable results were obtained when nocodazole was added either to the culture medium or to the intracellular solution. The similar effect of nocodazole on tonic currents induced by endogenous and exogenous applications of low concentrations of GABA argues against a reduction of ambient GABA. As discussed above, we believe that nocodazole did not modify the number of GABA$_A$ receptors on the cell surface since the peak amplitudes of tonic and GABA-evoked currents were comparable in untreated and nocodazole-treated neurons (see also Ref. 27). The similar single channel conductance estimated for clustered and declustered receptors allows exclusion of a re-assorted subunit composition of GABA$_A$ receptors after nocodazole treatment. In conclusion, we believe that the effect of nocodazole on tonic inhibition is due to changes in the gating properties of declustered GABA$_A$ receptors. This idea is supported by the results from the pre-equilibrating protocols combined with kinetic modeling and computer simulations.

Interestingly, it has been demonstrated that nocodazole treatment impairs the clustering of recombinant GABA$_A$ receptors overexpressed in QT6 cells along with the tubulin-binding GABA$_A$ receptor-activated protein (79). Since the GABA$_A$ receptor-activated protein contributes to the trafficking and clustering of GABA$_A$ receptors (80, 81), we cannot exclude the possibility that the effect of nocodazole reported here may involve chaperone proteins such as the GABA$_A$ receptor-activated protein.

Previous studies have demonstrated that pre-equilibration of excised patches with micromolar or submicromolar agonist concentrations traps the receptors in slow and strongly absorbing desensitized states (41, 52, 82, 83). In this work, we found that the reduction of current responses to saturating [GABA] after pre-equilibration with low [GABA] was larger in nocodazole-treated neurons than in untreated ones. This suggests a larger occupancy of slow desensitized states of declustered GABA$_A$ receptors, presumably in the singly bound conformation. For this reason, the kinetic model previously proposed to reproduce the gating of declustered receptors (27) was optimized, taking into account the experimental data obtained with extremely low [GABA]. In fact, it is widely accepted that the probability of occupancy of the receptors in the singly bound conformation is relevant only at very low concentrations of GABA (≤10 μM), whereas it can be neglected at saturating or subsaturating concentrations of agonist (50–52). It is for this reason that the increased occupancy of the singly bound desensitized state of declustered receptors allows mimicking the effect of nocodazole on tonic currents and the results of the pre-equilibration experiments. It is worth noting that the parameters used in this work did not alter the simulations of previous results on mIPSCs and GABA-evoked currents (27). However, the lack of specific protocols to selectively unmask

the kinetics of the singly bound states allows only a qualitative estimation. In fact, we cannot precisely attribute the larger occupancy of the singly bound desensitized conformation to an increased rate of entry or to a decreased rate of exit from that state. Nevertheless, we cannot exclude the possibility that slowly absorbing doubly bound desensitized states (84, 85) neglected in the model of Jones and Westbrook (48) can also be involved.

We conclude that desensitization of $GABA_A$ receptors, in particular of extrasynaptic γ_2 subunit-containing ones, contributes to the modulation of tonic inhibition. This conclusion is supported by data reported previously (85) demonstrating that the significant but still incomplete desensitization of γ_2 subunit-containing receptors allows a current amplitude comparable with that mediated by low desensitizing δ subunit-containing receptors. Moreover, less apparent desensitization is observed in γ_2 subunit currents evoked by low agonist concentrations (82, 84).

This work suggests that receptor clustering is an additional regulating factor for tonic inhibition. Since clustered extrasynaptic $GABA_A$ receptors mediate larger tonic currents, it is possible that, under either physiological or pathological conditions, receptor clustering can modulate tonic inhibition and in turn influence synaptic efficacy and integration (6, 10, 13, 14, 20).

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Clustering of Extrasynaptic $GABA_A$ Receptors Modulates Tonic Inhibition in Cultured Hippocampal Neurons

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