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FULL-LENGTH ORIGINAL RESEARCH

Epilepsia®

Loss of constitutive functional γ-aminobutyric acid type A-B receptor crosstalk in layer 5 pyramidal neurons of human epileptic temporal cortex

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Summary

Objective: γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in adult central nervous system, and profound alterations of GABA receptor functions are linked to temporal lobe epilepsy (TLE). Here we describe the functional relationships between GABA receptors type B ($GABA_BR$) and type A ($GABA_AR$) in human temporal cortex and how TLE affects this aspect of GABAergic signaling.

Methods: Miniature inhibitory postsynaptic currents (mIPSCs) were recorded by patch-clamp techniques from human L5 pyramidal neurons in slices from temporal cortex tissue obtained from surgery.

Results: We describe a constitutive functional crosstalk between $GABA_BRs$ and $GABA_ARs$ in human temporal layer 5 pyramidal neurons, which is lost in epileptic tissues. The activation of $GABA_BRs$ by baclofen, in addition to the expected reduction of mIPSC frequency, produced, in cortex of nonepileptic patients, the prolongation of mIPSC rise and decay times, thus increasing the inhibitory net charge associated with a single synaptic event. Block of K^+ channels did not prevent the increase of decay time and charge. Protein kinase A (PKA) blocker KT5720 and pertussis toxin inhibited the action of baclofen, whereas 8Br-cAMP mimicked the $GABA_BR$ action. The same $GABA_BR$-mediated modulation of $GABA_ARs$ was observed in pyramidal neurons of rat temporal cortex, with both PKA and PKC involved in the process. In cortices from TLE patients and epileptic rats, baclofen lost its ability to modulate mIPSCs.

Significance: Our results highlight the association of TLE with functional changes of GABAergic signaling that may be related to seizure propagation, and suggest that the selective activation of a definite subset of nonpresynaptic $GABA_BRs$ may be therapeutically useful in TLE.

KEYWORDS

human neocortex, miniature inhibitory postsynaptic currents, patch-clamp, synaptic current kinetics, temporal lobe epilepsy

1 | INTRODUCTION

Temporal lobe epilepsy (TLE) is the most frequent form of refractory focal epilepsy, and has been associated with dysfunctions in chloride homeostasis and in γ-aminobutyric acidergic (GABAergic) neurotransmission.[1,2] GABA mediates both fast and slow inhibitory transmission. GABA type A receptors ($GABA_A$Rs) are pentameric Cl^--selective ion channels mediating phasic and tonic inhibition, undergoing profound structural and functional alterations in the epileptic brain.[3,4] Nineteen distinct genes encode for $GABA_A$R subunits: α1-6, β1-3, γ1-3, δ, ε, π, θ, and ρ1-3.[5] Different subunit expression confers distinct kinetic and pharmacological properties to $GABA_A$Rs, in part due to different sensitivity to phosphorylation.[6–8] GABA type B receptors ($GABA_B$Rs) are high-molecular-mass complexes of $GABA_{B1}$, $GABA_{B2}$ subunits along with members of a subfamily of the KCTD (K^+ channel tetramerization domain-containing) proteins.[9,10] These receptors act presynaptically by inhibiting release of neurotransmitters, impairing Ca^{2+} conductances in neocortex and hippocampus,[11] and postsynaptically decreasing neuronal excitability by activation of inwardly rectifying K^+ (GIRK) channels and causing a slow inhibitory postsynaptic potential.[12,13] Other $GABA_B$R-mediated postsynaptic mechanisms have been reported, involving the Gi-mediated modulation of $[cAMP]_i$. Although many studies show a reduction of cAMP formation following $GABA_B$R activation,[14] other results point to an increase of $[cAMP]_i$ due to the action of the $GABA_B$R-associated Gi-βγ subunit on adenylyl cyclase types 2 and 4, with the consequent activation of protein kinase A (PKA).[15] Recently, a PKA-dependent modulation of extrasynaptic $GABA_A$Rs by $GABA_B$Rs has been reported in rodents.[16,17] In TLE, the $GABA_B$R-mediated signaling exhibits heterogeneous behaviors, such as a loss of function in layer 2/3 pyramidal neurons from human TLE tissues,[18] or an increase in the expression of $GABA_B$R subunits in human hippocampi of epileptic patients.[19] In any case, to date no functional data are available regarding the TLE-related changes in the interaction between $GABA_A$Rs and $GABA_B$Rs in human temporal cortex, highly relevant for seizure propagation.

To highlight the alteration of the inhibition control of temporal cortex output associated with epilepsy, we have analyzed the changes observed upon $GABA_B$R activation in $GABA_A$R-mediated synaptic currents recorded in temporal L5 pyramidal neurons from both epileptic and nonepileptic (NE) human and rat tissues.

Key Points

- Temporal lobe epilepsy is associated with impaired GABAergic inhibitory neurotransmission
- We show that in human neurons, the activation of $GABA_B$Rs modulates the $GABA_A$Rs, and this crosstalk is lost in epileptic tissues
- In nonepileptic cortex, $GABA_B$R activation prolongs mIPSCs, increasing the inhibition due to a single synaptic event
- This prolongation, absent in neurons from epileptic patients, is phosphorylation-dependent and not related to K^+ channels
- Our results confirm the association of epilepsy with severe changes of GABAergic signaling facilitating seizure propagation

2 | MATERIALS AND METHODS

2.1 | Patients

Surgical specimens were obtained from the temporal neocortex of 31 drug-resistant TLE (Patients 1-15 in Table S1) and NE oncologic patients (Patients 16-31) operated on at the Neuromed Neurosurgery Center (Pozzilli-Isernia, Italy). Oncologic patients referring 1 or more epileptic episodes were excluded from this study. Informed consent was obtained from each patient to use part of the bioptic material for experiments, and the ethics committees of Neuromed approved the selection processes and procedures.

2.2 | Pilocarpine model

Male Sprague-Dawley rats (280-300 g; Charles River Laboratories, Wilmington, MA) were used for the experiments. Animal were housed under standard conditions: constant temperature (22-24°C) and humidity (55-65%), 12-hour dark/light cycle, and free access to food and water. Procedures involving animals and their care were carried out in accordance with European Community and national laws and policies. Pilocarpine was administered intraperitoneally (i.p.; 300 mg/kg; Sigma, St Louis, MO, USA) 30 minutes after scopolamine injection (1 mg/kg i.p.). Within the first hour after injection, all rats developed seizures evolving into recurrent generalized convulsions (status epilepticus [SE]). SE was interrupted 3 hours after onset by administration of diazepam (10 mg/kg i.p.). After a latent period of 2-3 weeks, spontaneous seizures were observed and classified following the Racine scale. Rats were culled after 30-45 days.

2.3 | Whole-cell recordings from cortical slices

Neocortical slices were prepared from human temporal cortex of patients with TLE or with NE temporal tumors, and

from control or epileptic rat brains. Transversal slices (350 μm) were cut in glycerol-based artificial cerebrospinal fluid (ACSF) with a vibratome (VT 1000S; Leica, Wetzlar, Germany). Slices were placed in a slice incubation chamber at room temperature with oxygenated ACSF and transferred to a recording chamber within 1-24 hours after slice preparation. Whole-cell patch-clamp recordings were performed on pyramidal neurons at 22-25°C, as in previous studies on $GABA_BR$-mediated modulation of $GABA_AR$ function.[16]

Miniature inhibitory postsynaptic currents (mIPSCs) were recorded in temporal pyramidal neurons using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA, USA), −70-mV holding potential, in the presence of 1 μmol/L tetrodotoxin, 20 μmol/L 6-cyano-7-nitroquinoxaline-2,3-dione, and 40 μmol/L (2R)-amino-5-phosphonovaleric acid. Input and series resistances were monitored during the experiments every 5 minutes, and >10% changes excluded the cell from further analysis. Baclofen was administrated to the cells by perfusion in the bath solution for 15 minutes.

2.4 | Chemicals and solutions

ACSF composition was: 125 mmol/L NaCl, 2.5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1.25 mmol/L NaH_2PO_4, 1 mmol/L $MgCl_2$, 26 mmol/L $NaHCO_3$, 10 mmol/L glucose (pH 7.35). Glycerol-based ACSF solution contained 250 mmol/L glycerol, 2.5 mmol/L KCl, 2.4 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 1.2 mmol/L NaH_2PO_4, 26 mmol/L $NaHCO_3$, 11 mmol/L glucose (pH 7.35). Patch pipettes were filled with the intracellular solution, which contained: 140 mmol/L KCl, 10 mmol/L hydroxyethylpiperazine ethane sulfonic acid, 5 mmol/L BAPTA, 2 mmol/L Mg-ATP (pH 7.35, with KOH). CsCl-based internal solution was used only in the experiments aiming to investigate the role of K^+ conductances. Ten millimoles per liter tetraethylammonium, 2 mmol/L $BaCl_2$, 1 mmol/L 4-AP, and 2 mmol/L CsCl were added to external solution to block $K_v1.1$ and $K_v1.2$, KCNQ, GIRK, K_{Ca}, and K_{ATP} potassium channels.[20] Pertussis toxin (PTX; 1 μg/mL) was applied to slices overnight before the experiment and added to the pipette solution during recordings. All drugs were purchased from Sigma or Tocris Bioscience (Bristol, UK) and freshly prepared before the experiments.

2.5 | Data analysis and statistics

Data throughout the text represent mean ± standard error of the mean. The analysis of mIPSCs was performed with Clampfit 10 software (Axon Instruments). This program uses a detection algorithm based on a sliding template that did not induce any bias in the sampling of events, because it was moved along the data trace 1 point at a time and was 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 4 times the standard deviation of the baseline noise. The rise time was estimated as the time needed for 10%-90% increase of the peak current response and the decay time as the time needed for 90%-10% decrease of peak current. The mean inhibitory charge of a single synaptic event (Q) was measured as the time integral of the $GABA_A$-mediated synaptic currents. Statistical comparisons between groups were made with 1-way analysis of variance (ANOVA) test (Shapiro-Wilk normality test; equal variance test) and all pairwise multiple comparisons with the Holm-Sidak method. The power of all performed tests was >0.8 ($\alpha = .05$). $P < .05$ was taken as significant. In the case of failure of normality test or equal variance test, Kruskal-Wallis 1-way ANOVA on ranks was used, with Dunn method for pairwise comparisons. Paired *t* test on the same data is also performed (Table S2) to highlight the effect of drug application in the same statistical group. Statistical significance of cumulative distribution curves was assessed using the Kolmogorov-Smirnov test.

3 | RESULTS

The spontaneous activity of L5 pyramidal neurons from TLE or NE patients did not exhibit significant differences in overall excitability. However, spontaneous paroxysmal firing was recorded in 3 of 42 cells from TLE patients, whereas it was never observed in 36 neurons from NE patients, indicating that an epileptic phenotype is only detectable in slices from patients with an epileptic history.

3.1 | The activation of $GABA_BRs$ decreases the frequency of mIPSCs recorded from human L5 pyramidal neurons

To study the effects of $GABA_BR$ activation on the postsynaptic $GABA_AR$ function in human temporal cortex, we investigated the properties of mIPSCs recorded from L5 pyramidal neurons in slices obtained from NE or TLE tissue, before and during activation of $GABA_BRs$ with baclofen (100 μmol/L), applied for 15 minutes (Figure 1A,B).

In 11 pyramidal cells from NE tissue, the mIPSC frequency decreased in the presence of baclofen (from 1.2 ± 0.2 Hz to 0.07 ± 0.02 Hz, $P = .005$; Figure 1C), whereas mIPSC amplitude was not modulated (from 46 ± 6 pA to 58 ± 10 pA, $P = .484$; Figure 1E). In TLE neurons, the frequency of events was very similar to control cells and again decreased upon baclofen administration (from 1.3 ± 0.2 Hz to 0.17 ± 0.06 Hz, $P = .003$, n = 8; Figure 1D) without affecting the mean amplitude of events

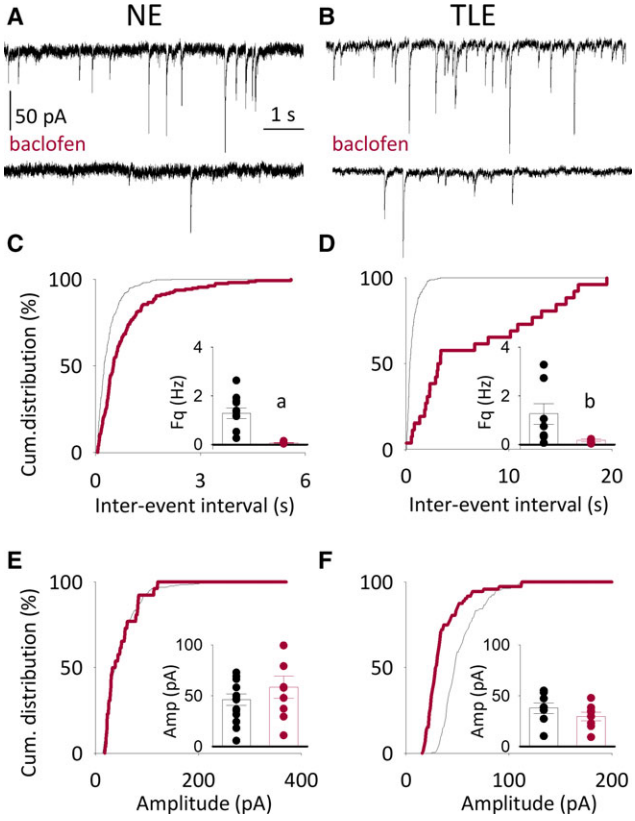

FIGURE 1 Activation of γ-aminobutyric acid type B receptors ($GABA_BRs$) reduces miniature inhibitory postsynaptic current (mIPSC) frequency in both nonepileptic (NE) and temporal lobe epilepsy (TLE) tissues. A, Typical traces recorded in whole-cell configuration from a L5 pyramidal neuron in a slice obtained from the surgically resected temporal pole of an NE patient (#22), showing mIPSCs before (top) and during (bottom) application of baclofen 100 μmol/L. Membrane potential = −70 mV. B, Typical traces recorded as in A from a neuron in a slice obtained from the surgically resected temporal pole of a TLE patient (#5). C, Cumulative distribution of the interevent intervals of mIPSCs recorded from NE tissues before (black line) and during (red line) baclofen application, showing a significant increase of interevent intervals upon $GABA_BR$ activation. The corresponding decrease in mean mIPSC frequency is shown in the inset (circles represent mean mIPSC frequency of individual cells before [left] and during [right] baclofen acquisition; vertical bars indicate overall mean values; 11 cells from 7 patients). D, Cumulative distribution of the interevent intervals of mIPSCs recorded from TLE tissues, as in C (8 cells, 6 patients). E, Cumulative distribution of the amplitudes of mIPSCs recorded from NE tissues (same cells and events as in C). The corresponding mean mIPSC amplitudes are reported in the inset. F, Cumulative distribution of the amplitudes of mIPSCs recorded from TLE tissues (same cells and events as in D). Statistical significance obtained by 1-way analysis of variance: a, $P = .005$; b, $P = .003$

(from 38 ± 5 pA to 30 ± 4 pA, $P = .677$; Figure 1F). These results indicate that $GABA_BRs$ downregulate the mIPSC frequency in human cortical neurons, similarly to observations in rodents.[21]

3.2 | The activation of $GABA_BRs$ prolongs mIPSC kinetics in human NE L5 pyramidal neurons, but not in TLE ones

The application of baclofen affected the kinetics of mIPSCs recorded from neurons in NE tissue, with significant increase of both rise $time_{10\text{-}90}$ and decay $time_{90\text{-}10}$ (from 2.1 ± 0.2 milliseconds to 3.5 ± 0.4 milliseconds, $P = .005$, and from 17 ± 1 milliseconds to 28 ± 2 milliseconds, $P < .001$; Figure 2A-C). As a result, the mean negative charge carried by a single mIPSC (Q) was significantly enhanced (from 0.58 ± 0.04 pC to 1.2 ± 0.2 pC, $P < .001$; Figure 2A,D). In neurons from TLE tissues, rise $time_{10\text{-}90}$ was higher than in control neurons (3.9 ± 0.3 milliseconds, $P = .038$) and baclofen did not affect it (3.6 ± 0.2 milliseconds, $P = .053$; Figure 2A,B). Decay $time_{90\text{-}10}$ values were also not affected by baclofen in TLE neurons (from 16 ± 1 milliseconds to 17 ± 2 milliseconds, $P = .574$; Figure 2C), and the resulting Q was not significantly altered (from 0.53 ± 0.07 pC to 0.43 ± 0.07 pC, $P = .344$; Figure 2D).

The altered mIPSC kinetics may arise from $GABA_BR$-mediated changes in K^+ conductance.[13] Therefore, we repeated the same protocol using a CsCl-based internal solution and adding a mix of K^+ channel blockers. In these conditions, application of baclofen to 5 pyramidal neurons from NE human tissue enhanced the mean decay time of mIPSCs from 17 ± 2 milliseconds to 28 ± 3 milliseconds ($P = .007$; Figure 2A,C). The rise time was significantly higher than control ($P < .001$; Figure 2B), and baclofen application had no effect (from 3.9 ± 0.4 milliseconds to 3.9 ± 0.2 milliseconds, $P = .841$). The baclofen-induced prolongation of mIPSC kinetics enhanced Q (from 0.4 ± 0.1 pC to 0.7 ± 0.1 pC, $P = .043$; Figure 2A,D). In addition to the presynaptic effects on frequency, our data indicate that the activation of $GABA_BRs$ acts at the postsynaptic level, slowing the kinetics of mIPSCs recorded in human temporal pyramidal neurons, with mechanisms that are lost in TLE tissue. The observed $GABA_BR$-mediated kinetic changes are likely due to multiple factors, as the increased duration of current recovery is independent of variations of K^+ conductance, whereas the opposite is true for mIPSC rise time.

3.3 | $GABA_BR$-mediated effects on mIPSC kinetics is due to PKA activation

To understand the molecular mechanisms of $GABA_BR$ modulation of $GABA_AR$-mediated synaptic currents, we analyzed mIPSCS in human NE temporal pyramidal neurons in the presence of selective kinase inhibitors or activators. Adding the selective PKC inhibitor, GF109203X (100 nM), in the patch pipette, the basal values of mIPSC

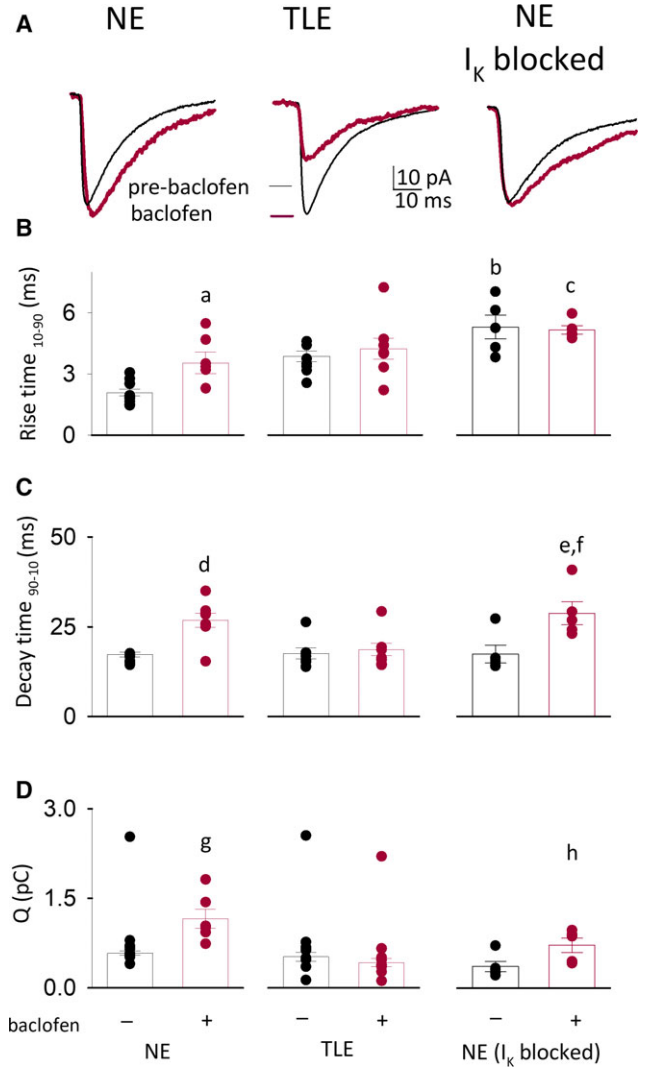

FIGURE 2 Activation of γ-aminobutyric acid type B receptors prolongs miniature inhibitory postsynaptic current (mIPSC) kinetics in temporal L5 pyramidal neurons from nonepileptic (NE) patients, but not from temporal lobe epilepsy (TLE) ones. A, Typical average traces of mIPSCs, recorded before (black line) and during (red line) baclofen application from a single neuron in: NE tissue (left; #13; before baclofen [−], 247 events; during baclofen [+], 72 events); TLE tissue (center; #11; −351 events; +67 events); NE tissue in presence of a cocktail of potassium channel blockers (right; #23; −311 events; +87 events). B, Bar graph representing mean mIPSC rise $time_{10\text{-}90}$ in NE (11 cells from 7 patients), TLE (8 cells from 6 patients), or NE tissues with potassium channels blocked (5 cells, 3 patients). ● Circles represent mean values of individual cells before (left) and during (right) baclofen acquisition; vertical bars indicate overall mean values. C, Bar graph representing mean mIPSC decay $time_{90\text{-}10}$ in the same cells as in B. D, Bar graph representing mean negative charge entering the cell during a single mIPSC (Q) recorded in NE tissues (left panel), TLE tissues (center panel), and NE tissues in presence of K^+ channel blockers. Statistical significance obtained with 1-way analysis of variance: a, $P = .005$; b, $P < .001$; c, $P < .001$; d, $P < .001$; e (I_K blocked + baclofen vs control), $P = .007$; f (I_K blocked + baclofen vs I_K blocked), $P = .006$; g, $P < .001$; h, $P = .043$

kinetics and charge were unaffected and the prolonging effects exerted by baclofen were still observed; rise $time_{10\text{-}90}$ increased from 2.5 ± 0.5 milliseconds to 3.5 ± 0.3 milliseconds ($P = .005$), decay $time_{90\text{-}10}$ from 17 ± 1 milliseconds to 29 ± 1 milliseconds ($P < .001$ vs control and internal control; Figure 3A,C), and Q from 0.5 ± 0.1 pC to 1.1 ± 0.2 pC ($P = .03$, n = 5; Figure 3A,D). By contrast, the PKA inhibitor KT5720 (600 nmol/L, in pipette) blocked the effects of baclofen on rise $time_{10\text{-}90}$ (control 2.1 ± 0.3 milliseconds, baclofen 2.2 ± 0.2 milliseconds, $P = .99$, n = 5; Figure 3A,B), decay $time_{90\text{-}10}$ (14 ± 2 milliseconds vs 17 ± 2 milliseconds, $P = .786$; Figure 3A,C), and Q values (0.6 ± 0.2 pC vs 0.7 ± 0.2 pC, $P = .344$; Figure 3A,D). Furthermore, bath application of the PKA activator 8Br-cAMP (100 μmol/L, 20 minutes) significantly prolonged the mIPSC decay $time_{90\text{-}10}$ value (17.3 ± 0.7 milliseconds in control, 25 ± 2 milliseconds in 8Br-cAMP, $P < .001$, n = 5; Figure 3A,C), occluding the kinetic modulation of subsequent baclofen application (27 ± 2 milliseconds, $P = .578$; Figure 3A,C). Application of 8Br-cAMP did not affect the initial rise $time_{10\text{-}90}$ (2.1 ± 0.2 milliseconds in control, 2.8 ± 0.5 milliseconds in 8Br-cAMP, $P = .882$; Figure 3A,B). The subsequent application of baclofen enhanced the rise time to 3.6 ± 0.2 milliseconds, leaving unchanged the mIPSCs amplitude (38 ± 8 pA in control, 36 ± 6 pA in 8Br-cAMP; not shown). As a consequence, the Q did not change upon 8Br-cAMP and baclofen application (0.72 ± 0.07 pC and 0.7 ± 0.1 pC, respectively, $P = .287$, n = 5; Figure 3A,D). These data indicate that $GABA_BR$ modulation of $GABA_AR$ kinetics observed in human temporal L5 pyramidal neurons depends on PKA activation. Moreover, to deepen the analysis of the involved G proteins, the effect of PTX was studied. This toxin (1 μg/mL) prevented the effects of baclofen on mIPSCs (rise $time_{10\text{-}90}$ from 3 ± 1 milliseconds to 3.4 ± 0.6 milliseconds, $P = .614$; decay $time_{90\text{-}10}$ from 13 ± 2 milliseconds to 15 ± 2 milliseconds, $P = .998$; Q from 0.28 ± 0.04 pC to 0.30 ± 0.04 pC, $P = .765$; Figure 3A-D; n = 5).

3.4 | The $GABA_BR$ activation reduces mIPSC frequency in rat cortical pyramidal neurons

To extend and strengthen the observation in human temporal cortex, we used the pilocarpine-treated rat model, and compared the effects of $GABA_BR$ activation on mIPSCs recorded from pyramidal neurons of temporal cortex of NE and chronically epileptic adult animals (Figure 4A,B). In 16 neurons from NE rats, baclofen significantly reduced the mIPSC frequency (from 0.5 ± 0.1 Hz to 0.10 ± 0.03 Hz, $P = .007$; Figure 4C), without changing the mean amplitude (from 33 ± 5 pA to 25 ± 4 pA,

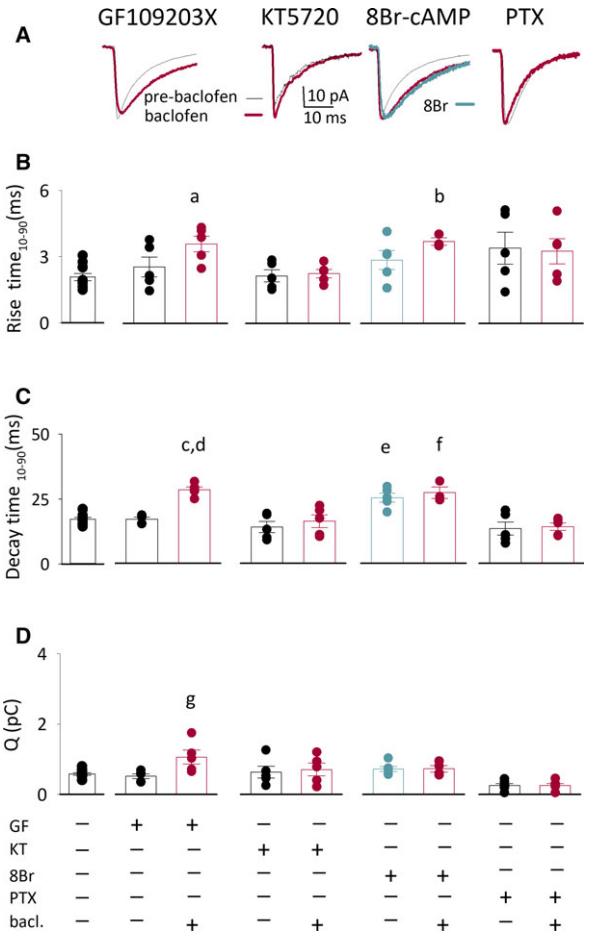

FIGURE 3 The γ-aminobutyric acid type B receptor ($GABA_BR$)-mediated modulation of miniature inhibitory postsynaptic current (mIPSC) kinetics in human nonepileptic (NE) tissue is a protein kinase A (PKA)-dependent process**.** A, Average traces of mIPSCs recorded from temporal L5 pyramidal neurons from human NE tissues before (black line) and after (red line) baclofen application in different experimental conditions, as indicated: from left, in presence of the PKC blocker GF109203X (100 nmol/L in pipette; #22; before baclofen [−], 254 events; during baclofen [+], 57 events); in presence of the PKA blocker KT5720 (600 nmol/L in pipette; #20; −66 events; +16 events); before (black line) and after (light blue line) 8Br-cAMP application (20 minutes, 100 μmol/L), and in the presence of 8Br-cAMP plus baclofen (red line; #27; control, 312 events; 8Br-cAMP, 133 events; 8Br-cAMP plus baclofen, 102 events); overnight exposure to PTX (1 μg/mL; #28, −112 events; +89 events). Holding potential = −70 mV. B, Mean mIPSC rise $time_{10\text{-}90}$ values, in control condition (13 cells, 7 patients), and before and during baclofen application for each other experimental condition, as indicated (5 cells for each panel from 2, 3, 3, and 2 patients, respectively). Circles represent mean values of individual cells before (left) and during (right) baclofen acquisition; vertical bars indicate overall mean values. C, Bar graph showing the effect of $GABA_BR$ activation on the decay $time_{90\text{-}10}$, for the same experiments as in B. D, Bar graph representing mean inhibitory charge of a single synaptic event (Q) values in the same cells as B and C. Statistical significance obtained with 1-way analysis of variance: a, $P = .005$; b, $P = .01$; c (GF + baclofen vs control), $P < .001$; d (GF + baclofen vs GF), $P < .001$; e, $P < .001$; f, $P < .001$; g, $P = .03$

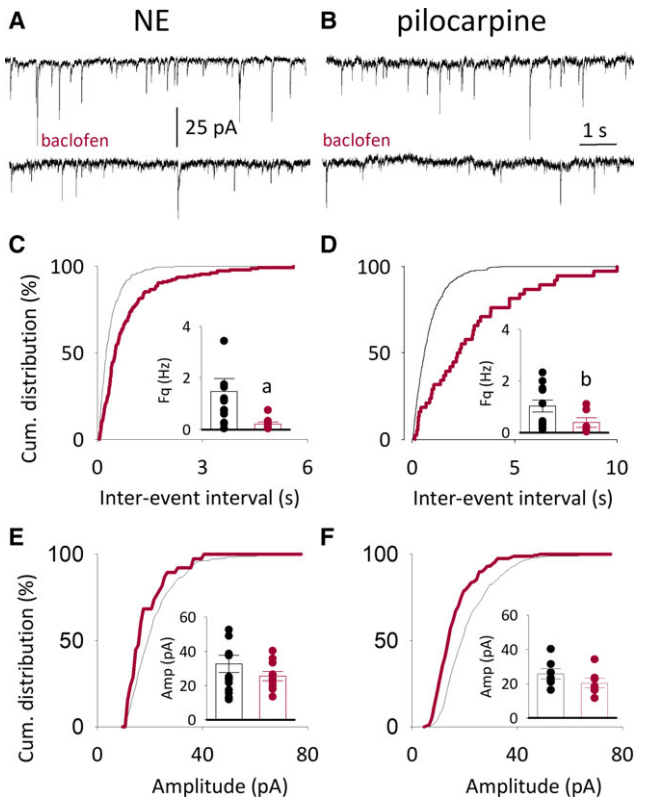

FIGURE 4 Activation of γ-aminobutyric acid type B receptors ($GABA_BRs$) reduces miniature inhibitory postsynaptic current (mIPSC) frequency in both nonepileptic (NE) and epileptic rat tissues. A, Typical traces recorded in whole-cell configuration from a temporal L5 pyramidal neuron from a control rat, showing mIPSCs before (top) and during (bottom) application of baclofen 100 μmol/L. Membrane potential = −70 mV. B, Typical traces recorded as in A from a neuron from a pilocarpine-treated epileptic rat. C, Cumulative distribution of the interevent intervals of mIPSCs recorded from NE tissue before (black line) and during (red line) baclofen application, showing a significant increase of interevent interval duration upon $GABA_BR$ activation. The corresponding decrease in mean mIPSC frequency is shown in the inset. Circles represent mean mIPSC frequency of individual cells before (left) and during (right) baclofen acquisition; vertical bars indicate overall mean values (10 cells, 5 rats). D, Cumulative distribution of the interevent intervals of mIPSCs recorded from epileptic tissues, as in C (7 cells, 5 rats). E, Cumulative distribution of the amplitudes of mIPSCs recorded from NE tissues (same cells and events as in C). The corresponding mean mIPSC amplitudes are reported in the inset. F, Cumulative distribution of the amplitudes of mIPSCs recorded from epileptic tissues (same cells and events as in D). Statistical significance obtained with 1-way analysis of variance: a, $P = .007$; b, $P = .048$

$P = .261$; Figure 4E). The mIPSC frequency did not revert to basal values after 15 minutes of washout (not shown). In 13 neurons from 5 epileptic rats, upon baclofen application the mIPSC frequency fell from 0.6 ± 0.3 Hz to 0.2 ± 0.2 Hz ($P = .048$; Figure 4D) with no change in the mean amplitude (from 26 ± 3 pA to 20 ± 3 pA, $P = .134$; Figure 4F).

3.5 | The $GABA_BR$ activation prolongs mIPSC kinetics in cortical pyramidal neurons from NE rats, but not in epileptic ones

Baclofen slowed mIPSC kinetics only in neurons from NE rats, significantly increasing the decay $time_{90\text{-}10}$ (13 ± 1 milliseconds to 21 ± 2 milliseconds, $P = .002$; Figure 5A,C) but leaving unchanged the rise $time_{10\text{-}90}$ (Figure 5A,B). These prolonged synaptic events were associated with an increased Q, from 0.19 ± 0.01 pC to 0.49 ± 0.04 pC ($P < .001$; Figure 5A,D). The enhanced parameters did not revert to control values after 15 minutes of washout (not shown). In neurons from epileptic rats, baclofen was unable to modulate both rise $time_{10\text{-}90}$ and decay $time_{90\text{-}10}$ (Figure 5A-C). In the same neurons, mIPSC Q value was not significantly altered by the activation of $GABA_BRs$ (from 0.34 ± 0.04 pC to 0.26 ± 0.03 pC, $P = .176$; Figure 5A,D).

To exclude that different mechanisms could be involved in the modulation of mIPSCs kinetics, we pretreated slices with the selective $GABA_BRs$ receptor antagonist, CGP55845 (150 nmol/L, 15 minutes) before baclofen (Figure 5; n = 6, 3 rats). CGP55845 per se did not affect any mIPSC parameter and blocked the baclofen effects, with no change in rise $time_{10\text{-}90}$ (from 2.2 ± 0.3 milliseconds to 2.3 ± 0.4 milliseconds, $P = .225$; Figure 5A,B), decay $time_{90\text{-}10}$ (from 10.5 ± 0.8 milliseconds to 10 ± 1 milliseconds, $P = .688$; Figure 5A,C), and Q (from 0.55 ± 0.05 pC to 0.55 ± 0.07 pC, $P = .225$; Figure 5A, D) of mIPSCs. Moreover, to study the possible involvement of K^+ channels in kinetic changes observed in rat NE neurons, we used a cocktail of K^+ channel blockers. In this condition, baclofen was ineffective in rise $time_{10\text{-}90}$ (Figure 5A,B; n = 7) but prolonged the mean of mIPSCs (from 18 ± 3 milliseconds to 23 ± 2 milliseconds, $P = .02$; Figure 5A,C), resulting in an increase of Q (from 0.62 ± 0.08 pC to 0.9 ± 0.1 pC, $P = .039$; Figure 5D). These data precisely confirm the observations in human tissues.

3.6 | The $GABA_BR$ activation prolongs mIPSC kinetics in NE rat neurons by activating different protein kinases

In the presence of PKC inhibitor GF109203X, baclofen was unable to significantly modulate any of the considered kinetic parameters: rise $time_{10\text{-}90}$ from 1.8 ± 0.5 milliseconds to 2.4 ± 0.5 milliseconds (n = 5, $P = .235$), decay $time_{90\text{-}10}$ from 16.6 ± 0.6 milliseconds to 17 ± 1 milliseconds ($P = .506$), and Q from 0.6 ± 0.1 pC to 0.44 ± 0.04 pC ($P = .552$; Figure 6A-D). Also, the PKA inhibitor KT5720 blocked the effect of baclofen on rise $time_{10\text{-}90}$ (from 1.9 ± 0.2 milliseconds to 2.7 ± 0.4 milliseconds, $P = .144$, n = 5; Figure 6A,B), decay $time_{90\text{-}10}$ (from 15.1 ± 0.6 milliseconds to 15.5 ± 0.9 milliseconds, $P = .306$; Figure 4A,C), and Q (from 0.6 ± 0.1 pC to 0.5 ± 0.1 pC, $P = .620$; Figure 6D).

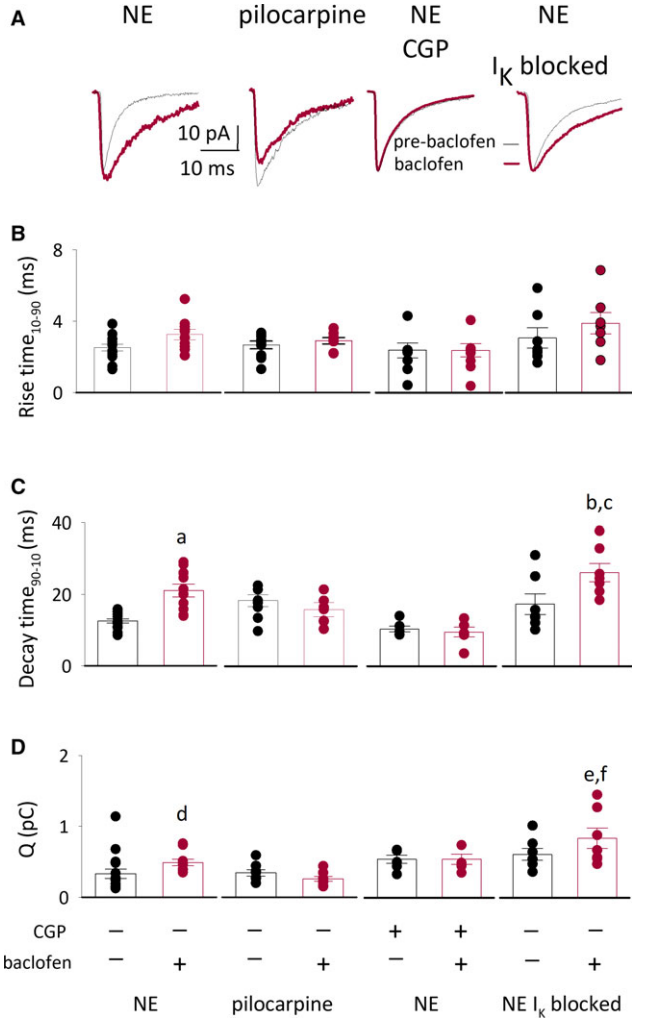

FIGURE 5 Activation of γ-aminobutyric acid type B receptors ($GABA_BRs$) prolong miniature inhibitory postsynaptic current (mIPSC) kinetics in temporal L5 pyramidal neurons from nonepileptic (NE) rats, but not from pilocarpine-treated epileptic ones. A, Typical average traces of mIPSCs, recorded before (black line) and during (red line) baclofen application from a single neuron in (from left) NE tissue, epileptic rat tissue, NE tissue in presence of the $GABA_BRs$ blocker CGP55845 (150 nmol/L, 15 minutes), and NE tissue in presence of a cocktail of K^+ channel blockers. B, Bar graph representing mean mIPSC rise $time_{10\text{-}90}$ in NE tissue (16 cells, 7 rats), epileptic tissue (14 cells, 8 rats), NE tissue in presence of CGP55845 (5 cells, 3 rats), or NE tissue in presence of K^+ channel blockers (6 cells, 3 rats). Circles represent mean values of individual cells before (left) and during (right) baclofen acquisition; vertical bars indicate overall mean values. C, Bar graph representing mean mIPSC decay $time_{90\text{-}10}$, for the same cells as in B. D, Bar graph representing mean inhibitory charge of a single synaptic event (Q) values in the same cell as B and C. Statistical significance obtained with 1-way analysis of variance: a, $P = .012$; b (I_K blocked + baclofen vs control), $P = .003$; c (I_K blocked + baclofen vs I_K blocked), $P = .02$; d, $P < .001$; e, $P = .006$; f, $P = .039$

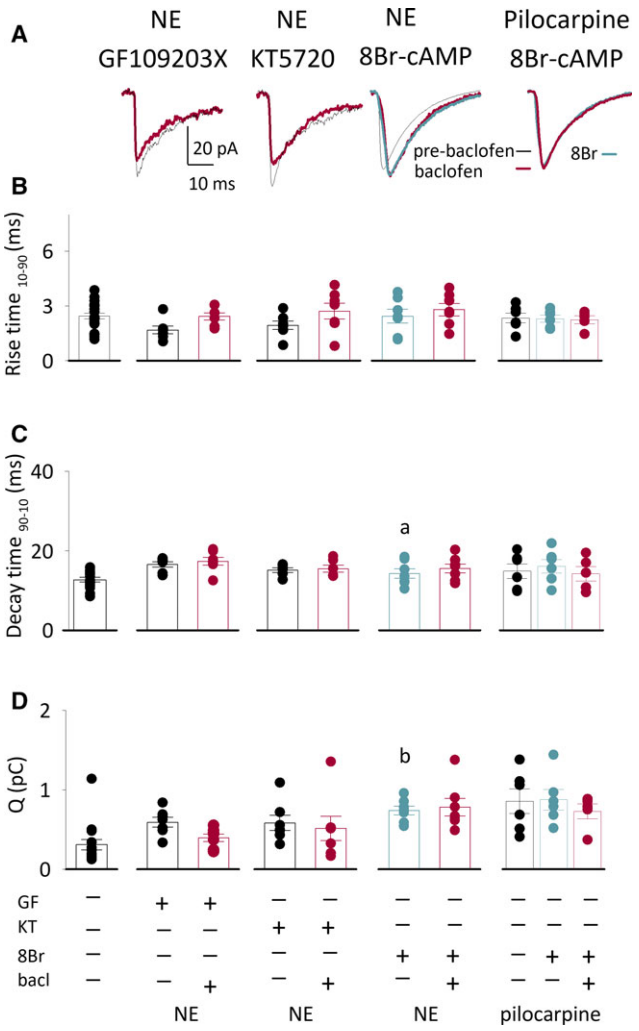

FIGURE 6 The γ-aminobutyric acid type B receptor–mediated modulation of miniature inhibitory postsynaptic current (mIPSC) kinetics in rat nonepileptic (NE) neurons is mediated by both protein kinase A (PKA) and PKC activation. A, Average traces of mIPSCs recorded before (black line) or after (red line) baclofen application from temporal pyramidal neurons in (from left): NE tissue in presence of the PKC blocker GF109203X (100 nmol/L in pipette; before baclofen [−], 65 events; during baclofen [+], 36 events); NE tissue in presence of the PKA blocker KT5720 (600 nmol/L in pipette, −62 events, +38 events); NE tissue in presence of 8Br-cAMP (light blue line, control, 141 events; 8Br-cAMP, 121 events; 8Br-cAMP plus baclofen, 57 events); epileptic tissue in presence of 8Br-cAMP (control, 213 events; 8Br-cAMP, 195 events; 8Br-cAMP plus baclofen, 61 events). B, Bar graph representing rise time$_{10\text{-}90}$ values. Circles represent mean values of individual cells before (left) and during (right) baclofen acquisition; vertical bars indicate overall mean values (the averaged cells are 5, 5, 7, and 6, respectively; animals are 3, 3, 4, and 3). C, Bar graph representing mean decay time$_{90\text{-}10}$ values, for the same cells as B. D, Bar graph representing mean mean inhibitory charge of a single synaptic event (Q) values, for the same cells as B and C. *P* values shown in the graph are: a (NE 8Br-cAMP vs control), $P = .015$; b (NE 8Br-cAMP vs control), $P = .044$

The role of PKA was also investigated by using 8Br-cAMP in both NE and epileptic rats (Figure 6A-D). In NE neurons, 8Br-cAMP (100 μmol/L, 20 minutes) occluded the baclofen effects, prolonging the decay time$_{90\text{-}10}$ (from 11.4 ± 0.6 milliseconds to 14 ± 1 milliseconds, $P = .015$; n = 7, 4 rats; Figure 6A,B) and increasing the Q (from 0.49 ± 0.04 to 0.77 ± 0.05, $P < .001$). Consistently, the subsequent baclofen application had no further effect (Figure 6A-D). In epileptic rats, 8Br-cAMP had no effect on mIPSCs kinetics (Figure 6A-D).

4 | DISCUSSION

Our data show for the first time a crosstalk between $GABA_A$Rs and $GABA_B$Rs in human temporal L5 pyramidal neurons that is lacking in the temporal cortex of TLE patients.

We describe 2 main processes triggered by the activation of $GABA_B$Rs in human temporal cortex and affecting $GABA_A$R signaling: (1) a marked reduction of mIPSC frequency, mediated by presynaptic $GABA_B$ autoreceptors; and (2) a significant prolongation of mIPSC kinetics, leading to the increase of the inhibitory charge carried by a single synaptic event, mediated by nonpresynaptic $GABA_B$Rs. Whereas the reduction of the mIPSC frequency is present both in NE and TLE tissues, the $GABA_B$R-induced kinetic changes of $GABA_A$R-mediated currents are lost in neurons from epileptic tissues.

4.1 | Modulation of mIPSC frequency

$GABA_A$Rs produce synaptic currents much longer than the lifetime of GABA in the synaptic cleft.[22] Thus, differences in synaptic current kinetics depend on changes in the functional properties of postsynaptic $GABA_A$Rs, whereas modulation of mIPSC frequency depends on presynaptic mechanisms.[23] Upon baclofen application, a strong reduction of the mIPSC frequency was observed both in control and TLE human tissues, as well as in control and epileptic rat tissue. These data are in line with previous results obtained in animal models indicating that presynaptic $GABA_B$ autoreceptors decrease the release probability of GABA, likely impairing presynaptic voltage-gated Ca^{2+} channels.[24] In TLE tissue, this presynaptic action is slightly reduced but not abolished, as described also in epileptic rats.[25]

4.2 | Prolongation of mIPSC kinetics

The second $GABA_B$R-triggered process, consisting of a prolongation of mIPSC kinetics associated with an increase of the net influx of negative charge, was observed only in L5 pyramidal neurons from NE tissues, supporting the notion that in the epileptic cortex a complex rearrangement of

$GABA_A$Rs occurs.[2–4] We show an enhancement of both rise and decay times of GABAergic mIPSCs, observed upon $GABA_B$R activation both in human and rat L5 neurons. The application of a cocktail of K^+ channel blockers induced per se a significant increase of mean rise time, occluding the baclofen effect on this parameter. This prolongation could be due to several K^+-dependent mechanisms, such as variable space-clamp due to changes in the overall K^+ conductance, altered local extracellular K^+ concentrations, modulated GABA reuptake function, and even K^+-dependent astrocyte modulation of the GABAergic synapse. In any case, the block of K^+ channels did not affect the baclofen-induced increase of decay times and inhibitory charge. Thus, the selective activation of nonpresynaptic $GABA_B$Rs in NE human temporal cortex is associated with a higher inhibitory response to GABA release, but the exact location of the $GABA_B$Rs mediating this effect is not known. Given that the $GABA_B$Rs responsible for the prolongation of GABA-mediated mIPSCs functionally affect $GABA_A$Rs in the recorded neuron, the coexpression of both receptors on the same postsynaptic cell could be hypothesized, with the support of the effectiveness of kinase inhibitors in the recording pipette. However, it is not possible to rule out the involvement of astrocytic $GABA_B$Rs, given their role in potentiating the neuronal $GABA_A$R-mediated synaptic currents.[26] The activation of $GABA_B$Rs in astrocytes could in turn induce the action of different mediators on L5 pyramidal neurons via PTX-sensitive G-coupled receptors. In any case, the mechanism linking $GABA_B$R activation and the prolongation of $GABA_A$R-mediated current decays does not involve the modulation of K^+ membrane permeability, because this effect is still present when K^+ channels are blocked. By contrast, our data suggest the involvement of a $GABA_B$R-triggered PKA activity. Kinetic changes of $GABA_A$Rs-mediated currents are strictly dependent on the receptor phosphorylation.[27,28] Our results show that in human NE tissues, $GABA_B$R-mediated modulation of mIPSCs kinetics was abolished by inhibiting PKA and was mimicked by 8Br-cAMP. $G\beta\gamma$ subunit released from Gi-coupled receptors has been shown to conditionally stimulate adenylyl cyclases of group II (types 2, 4, and 7), acting synergistically with activated Gs subunit.[29,30] In particular, in rat pyramidal neurons from CA1 hippocampal region, baclofen is able to liberate $G\beta\gamma$ subunits, which in turn synergize with activated $Gs\alpha$ to stimulate type 2 adenylyl cyclases.[15] Furthermore, the observation that PKA activity affects mIPSC kinetics but not mIPSC frequency clearly indicates that the baclofen-induced prolongation is not due to a presynaptic selection of a particular "long" subset of the heterogeneous GABAergic inputs to L5 pyramidal neurons. In NE human tissue, the block of the baclofen-induced kinetic modulation by PTX confirms the involvement of a Gi protein in the prolongation mechanism.

Interestingly, the animal model used in this study is able to recapitulate the main features of the $GABA_B$R-mediated effects observed in human tissue, but not all; in NE rat neurons, the $GABA_B$R-mediated modulation of mIPSCs kinetics was dependent also on PKC activation. In rat cell lines and native neurons, a crosstalk between PKC and PKA has been described in the context of transduction mechanisms triggered by $GABA_B$R activation.[31,32] In particular, the inhibition of both PKA and PKC has been shown to affect basal GABA release and the response to baclofen in the rat basal forebrain, and the inhibition of PKA was able to occlude the effects of PKC.[31] The absence of a PKC effect on the baclofen-induced kinetic modulation in humans confirms differences in the modulation of $GABA_B$R transduction mechanisms across species.[33]

4.3 | Changes of GABAergic signaling in epileptic tissue

The longer rise time and the smaller amplitude of basal mIPSCs observed in TLE neurons compared to NE ones, along with the inability of $GABA_B$R activation to modulate mIPSC kinetics in TLE tissues, may be ascribed to different mechanisms, such as reorganization of $GABA_B$R expression during epileptogenesis,[14,34] change in the phosphorylation/dephosphorylation balance in pyramidal neurons, and variation in synaptic $GABA_A$R subunit composition, affecting their posttranslational regulation. These processes are probably not mutually exclusive, as evidence for the involvement of all these mechanisms in TLE has been provided. In particular, the expression of $GABA_B$Rs is modulated in human TLE tissue, with specific modulation of $GABA_B$R isoforms in the hilus, in the dentate gyrus, and in other brain regions.[19] However, the presynaptic action of $GABA_B$Rs in epileptic tissue suggests that changes in $GABA_B$R expression pattern may occur with subcellular selectivity, as reported in succinic semialdehyde dehydrogenase–deficient mice.[35] In human TLE, changes in the expression levels of different kinases have been described,[36,37] and PKA activity significantly changes in the cortex and the hippocampus of pilocarpine-treated rats.[38] Furthermore, in the kindling epilepsy model, the phosphorylation status of $GABA_A$Rs is enhanced.[39] In addition to all of these considerations, the rearrangement of the subunit composition of $GABA_A$Rs following epileptogenesis[2–5] may severely change the impact of phosphorylation on receptor function.[40]

4.4 | Implications of the lost GABAergic crosstalk for TLE pathology

Our data extend to human temporal cortex previous studies on the functional crosstalk between $GABA_B$Rs and

$GABA_AR$s. The enhancement of extrasynaptic $GABA_AR$ function by $GABA_BR$s has been described in rodent neurons,[18,19] with phosphorylation-based mechanisms. Our results broaden this functional crosstalk to human synaptic $GABA_AR$s and highlight the association of epilepsy with profound alterations of the GABAergic signaling. In particular, in NE human cortex the $GABA_B$-mediated positive modulation of $GABA_AR$-expressing synapses leads to an extremely significant twofold increase of the negative charge entering the cell during a single synaptic event. Thus, the lack of this mechanism in TLE patients is expected to promote hyperexcitability of the L5 neurons projecting to subcortical areas, thus facilitating seizure propagation. The enhancement of the net inhibitory charge could be considered in contrast with the strong $GABA_BR$-mediated reduction of the mIPSC frequency, but these 2 mechanisms are simultaneously observed in particular experimental conditions, when all $GABA_BR$s are activated by exogenous baclofen application. We hypothesize that in more physiological conditions they could be separately recruited, given that nonpresynaptic $GABA_BR$s may be activated independently from presynaptic ones, usually triggered by excessive GABA release and consequent spillover from synaptic cleft. It is also possible to envisage that during high activity phases the increased concentration of extracellular GABA could activate both presynaptic and nonpresynaptic $GABA_BR$s, leading to concurrent reduction of GABA release probability and postsynaptic increase of inhibitory charge; in this case, in NE tissue, the second mechanism could contribute to limiting the excitatory effects of the first one.

Further studies will be needed to test the therapeutic hypothesis that the selective activation of a definite subset of nonpresynaptic $GABA_BR$s may increase the net inhibitory input without impairing the GABA release probability.

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DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Supplementary Data

Supplementary Table 1. **Patients included in the study**

T: temporal, F: frontal, L: left, R: right, Lat: lateral, MES: mesial, Ant: anterior, ETL: extensive temporal lobectomy, FCD: focal cortical dysplasia, LES: lesionectomy, ATL: anterior temporal lobectomy, MES: hippocampal mesial sclerosis, AS: astrocytoma; AC: cavernous angioma, G: ganglioglioma, GB: glioblastoma multiforme, OA: oligoastrocytoma, DNET: Dysembryoplastic neuroepithelial tumour, GC: gangliocytoma I° grade WHO.

Patient	Sex	Age (years)	Age at onset of epilepsy (years)	MRI findings	Epileptogenic zone	Surgery	Histopathology
#1	F	27	4	L-T-Mes	L-T-Mes-Ant	ATL	MES
#2	F	27	17	R-T-Mes	R-T-Mes-Ant	ATL	MES
#3	F	41	28	L-T-Ant	L-T-Mes-Ant	ATL	MES
#4	M	26	5	L-T-Mes	L-T-Mes-Lat	ETL	MES
#5	F	43	13	R-T-Mes	R-T-Mes-Ant	ATL	MES
#6	F	41	13	L-T-Mes	L-T-Mes-Ant	ATL	MES
#7	F	17	8	L-T-Mes	L-T-Mes-Ant	ATL	MES
#8	F	45	4	R-T-Mes	R-T-Mes-Ant	ATL	MES
#9	M	40	14	R-T-Mes	R-T-Mes-Ant	ATL	MES
#10	F	46	7	L-T-Mes	L-T- Mes-Lat	ETL	MES
#11	F	46	27	R-T-Ant	R-T-Mes-Ant	ATL	MES
#12	F	36	6	R-T-Ant	R-T-Mes-Ant	ATL	MES
#13	M	54	15	L-T-Mes	L-T- Mes-Lat	ETL	MES
#14	F	52	50	R-T-Mes	R-T-Mes-Ant	ATL	MES
#15	F	61	60	R-T-Mes	R-T-Mes-Ant	ATL	MES
#16	M	63	17	R-T-Mes	R-T-Mes-Ant	LES	AC
#17	M	45	18	L-T-Mes	L-T-Mes-Lat	LES+ETL	DNET

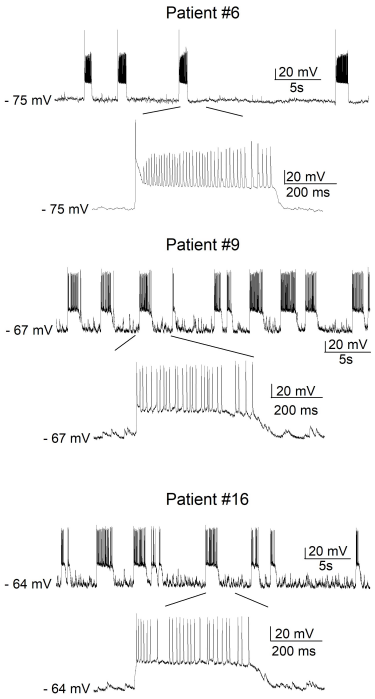
#18	M	55	-	R-T-Mes	R-T-Mes	LES	GB IV WHO
#19	M	30	-	R-T-Mes	R-T-Mes	LES	OA II WHO
#20	F	19	-	L-T-Mes	L-T-Mes	LES	FCD IIA + G
#21	F	39	-	R-T-Lat	-	LES	GB IV WHO
#22	M	72	-	R-T-Mes	-	LES	GB IV WHO
#23	M	74	-	R-T-Lat	-	LES	OA II WHO
#24	F	70	-	R-T-Mes	-	LES	GB IV WHO
#25	M	84	-	R-T-Mes	-	LES	GB IV WHO
#26	M	44	-	L-T-Mes	-	LES	GB IV WHO
#27	M	66	-	L-T-Mes	-	LES	AS
#28	F	68	-	L-T-Mes	-	LES	GC I WHO
#29	F	55	-	R-T-Mes	-	LES	GB IV WHO
#30	M	82	-	R-T-Lat	-	LES	MET
#31	F	21	-	L-T-Mes	-	LES	GC I WHO

Supplementary Table 2. Evaluation of statistical significance (p values) from paired t-test analysis of measurements before and during treatment on the same cells.

Human	Rise time 10-90%	Decay time 90-10%	Charge
NE ctrl vs baclofen (n=8)	*p=0.012	**p<0.001	*p=0.009
TLE ctrl vs baclofen (n=9)	p=0.089	p=0.410	p=0.132
NE I_K-blocked vs NE I_K-blocked + baclofen (n=5)	p=0.786	*p=0.043	*p=0.040
NE GF109203x vs +baclofen (n=5)	*p=0.045	**p<0.001	*p=0.016
NE KT5720 vs +baclofen (n=5)	p=0.683	p=0.921	p=0.396
NE control vs 8Br-cAMP (n=6)	p=0.069	*p=0.026	p=0.121
NE 8Br-cAMP vs +baclofen (n=6)	p=0.120	p=0.694	p=0.518
NE PTX vs +baclofen (n=5)	p=0.738	p=0.751	p=0.973
Rat	Rise time 10-90%	Decay time 90-10%	Charge
NE ctrl vs baclofen (n=10)	p=0.426	*p=0.003	**p<0.001
PILO ctrl vs baclofen (n= 7)	p=0.607	p=0.15	p=0.099
NE ctrl vs CGP (n=6)	p=0.559	p=0.645	p=0.907
NE CGP vs CGP+baclofen (n=6)	p=0.965	p=0.400	p=0.993
NE I_K-blocked vs NE I_K-blocked + baclofen (n=7)	p=0.133	*p= 0.003	p=0.155
NE GF109203x vs +baclofen(n=5)	p=0.070	p=0.426	p=0.315
NE KT5720 vs +baclofen (n=5)	p=0.219	p=0.711	p=0.484
NE control vs NE 8Br-cAMP (n=7)	p=0.096	*p=0.015	*p=0.045
NE 8Br-cAMP vs +baclofen (n=7)	p=0.163	p=0.234	p=0.095
PILO control vs 8Br-cAMP	p=0.811	p=0.417	p=0.819
PILO 8Br-cAMP vs +baclofen	p=0.568	p=0.171	p=0.203

Supplementary Figure 1. Paroxysmal activity recorded in sporadic L5 pyramidal neurons from TLE tissue.

Three examples of spontaneous paroxysmal activity recorded in current clamp configuration (I=0; bottom, enlarged time scale) from 3 L5 neurons used in experiments for this study, from different TLE patients (#6, #9 and #16), before any drug application.


Patient #6
20 mV
5s
- 75 mV
20 mV
200 ms
- 75 mV
Patient #9
- 67 mV
20 mV
5s
20 mV
200 ms
- 67 mV
Patient #16
20 mV
5s
- 64 mV
20 mV
200 ms
- 64 mV