Original Research *Feature Article*

Selective modulation of tonically active $GABA_A$ receptor functional subgroups by G-proteins and protein kinase C

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Impact statement

Here we study intracellular mechanisms which regulate inhibitory signaling delivered through continuously (tonically) open ionotropic receptors of γ-aminobutyric acid (GABA) of dentate gyrus granule cells (DGCs). We found that, apart of classical GABA-A receptors ($GABA_A$Rs) which can be activated by GABA binding, a significant part of tonic inhibitory current is delivered by newly discovered spontaneously opening $GABA_A$Rs (s-$GABA_A$Rs), which enter active state without binding of GABA. We have also found that conventional $GABA_A$Rs and s-$GABA_A$Rs are regulated by different intracellular mechanisms, which may overlap and thus induce various signaling repercussions. Our results demonstrate that s-$GABA_A$Rs play a key role in the mechanism that implements DGCs functional role in the brain. On top of that, since regulatory mechanisms under study are affected in a number of pathological states, our results may have broad implications for treatment of neurological disorders.

Abstract

Ionotropic receptors of γ-aminobutyric acid ($GABA_A$Rs) produce two forms of inhibitory signaling: phasic inhibition triggered by activation of synaptic $GABA_A$Rs at GABAergic synapses, and tonic inhibition generated in large part through persistent activation of extrasynaptic $GABA_A$Rs. It has recently been demonstrated that tonic inhibition may also involve spontaneously opening $GABA_A$Rs (s-$GABA_A$Rs) whose activation does not require binding of γ-aminobutyric acid (GABA). Here, we examine intracellular mechanisms modulating $GABA_A$Rs' tonic effects in rat dentate gyrus granule cells (DGCs). Cellular control of s-$GABA_A$Rs-delivered tonic current appears to involve signaling inputs from G-protein-dependent and -independent molecular cascades, whereas tonic GABA-dependent current in DGCs is regulated by protein kinase C. The intracellular agents that modulate s-$GABA_A$R-generated inhibition could thus represent a generic mechanism controlling signal integration in central neural circuits.

Keywords: Spontaneously opening GABAA receptors, tonic inhibitory current, action potential generation, G-proteins, protein kinase C

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Introduction

Inhibitory signaling delivered through ionotropic receptors of γ-aminobutyric acid ($GABA_A$Rs) is a key element of brain functioning due to control over neuronal excitability. $GABA_A$R-mediated signaling consists of two main components: phasic and tonic. Phasic inhibition consists of individual inhibitory postsynaptic events, whereas tonic inhibition is generated by continuous receptor activity. It has long been accepted that tonic inhibition is generated by $GABA_A$Rs binding to γ-aminobutyric acid (GABA) which diffused to the extrasynaptic space.[1] In our recent study, however, we found that in hippocampal dentate gyrus granule cells (DGCs) spontaneously opening $GABA_A$Rs (s-$GABA_A$Rs) are the main contributors to the tonic current: these receptors can switch to the active state both after GABA binding or in the absence of GABA.[2]

Due to significant input of tonic inhibitory conductance to neural excitability and neural network oscillations,[3,4] studies of tonic $GABA_A$Rs signaling is a rapidly developing area in neuroscience research. In our previous study, we demonstrated the input of s-$GABA_A$Rs in neuronal signal generation and integration[2]; however, the underlying contributing mechanisms are still unclear.

Protein kinase C (PKC) is able to phosphorylate $GABA_A$Rs and thus plays an important role in the development of a number of neural diseases such as epilepsy, alcoholism and anxiety.[5]

Nevertheless, to date, our knowledge of PKC-triggered modulation of $GABA_A$Rs effects is far from excellence. There are several studies which suggested post-translational modifications of PKC to be inhibitory factor for extrasynaptic $GABA_A$Rs response.[6–8] However, other reports have suggested that activation of PKC increases

$GABA_A$Rs' response[9] or does not alter their function.[10] In turn, kinases that regulate tonically active $GABA_A$Rs are modulated by G-proteins,[11–13] thus providing a number of other molecular candidates for control over s-$GABA_A$R.[14–16]

Intriguingly, in our previous study, spontaneous $GABA_A$R openings were absent in recordings performed on outside-out cell membrane patches (OOPs), thus pointing to cytoplasmic factor as a critical link of s-$GABA_A$Rs' functioning.[2] However, spontaneous openings of s-$GABA_A$Rs were registered earlier in numerous works on OOPs.[14–18] Further, an earlier study on OOPs excised from hippocampal primary neurons demonstrated the presence of s-$GABA_A$Rs insensitive to GABA competitive antagonist SR-95531 (SR-95),[19] questioning the idea of cytoplasmic actors as being the only necessary and sufficient factors enabling s-$GABA_A$Rs' functioning.

Thus, here we focused on the connection between PKC and G-proteins' activity and s-$GABA_A$Rs' effects in DGCs to highlight cytoplasmic mechanisms that regulate s-$GABA_A$Rs' functioning. To achieve this, we set out to proceed from s-$GABA_A$Rs' single-channel properties (conductance, opening probability, average open time) to s-$GABA_A$Rs' input into interneuronal crosstalk, i.e. action potential (AP) generation. With these approaches, we tested which cytoplasmic factors (PKC and/or G-proteins), and to what extent, are responsible for s-$GABA_A$Rs' activation/deactivation and, in a more general context, for s-$GABA_A$Rs input into inhibitory conductance.

Methods and materials

Hippocampal slice preparation

For electrophysiological recordings, we used experimental approaches which were tested and validated in our previous study on DGCs.[20]

Transverse hippocampal slices of 300 μm thickness were obtained from four-to-five-week old Sprague Dawley rats. Rats were bred in the institutional animal house, grown on a Rat and Mouse Breeding Diet (Special Diet Services, Witham, UK) and water *ad libitum*, and maintained at 12–12-h light-dark cycle. Animals were sacrificed for slices in the first half of light period of the L/D cycle. To kill rats, we used an overdose of isoflurane according to the United Kingdom Animals (Scientific Procedures) Act of 1986. After decapitation, brains were rapidly removed and dissected, and hippocampi were sliced with a Leica VT1200S vibratome in ice-cold sucrose-based solution composed of the following (in mM): 70 sucrose, 80 NaCl, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 25$NaHCO_3$, 1.25 NaH_2PO_4, 22 glucose, equilibrated with 95% O_2 plus 5% CO_2, pH 7.4, 315–330 mOsm. Slices were maintained in continuously oxygenated sucrose-free storage solution at 33°C for 15 min, equilibrated to a room temperature for 15 min and then placed to recover in continuously oxygenated humid interface holding chamber at room temperature for at least 1 h before recording. After recovering, slices were transferred into recording chamber. The perfusion and storage medium contained (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 22 glucose and was continuously gassed with 95%O_2 and 5%CO_2, pH 7.4; osmolarity was held at 290–298 mOsm.

Electrophysiology

Whole-cell recordings. Visualized patch-clamp recordings from mature dentate granule cells were performed using an infrared differential interference contrast imaging system. To measure tonic $GABA_A$R-mediated currents, we held cells in voltage-clamp mode at holding potential $V_{hold} = -70$ mV in the presence of DL-APV (50 μM), NBQX (20 μM), MCPG (250 μM), strychnine (1 μM) and CGP55845 (1 μM). The intracellular pipette solution for voltage-clamp experiments was composed of the following (mM): 120.5 CsCl, 10 KOH-HEPES, 2 EGTA, 8 NaCl, 5 QX-314 Br^- salt, 2 Mg-ATP, 0.3 Na-GTP; for current-clamp experiments it contained 126 K-gluconate, 4 NaCl, 5 HEPES, 15 glucose, 1 $MgSO_4$·7H2O, 2 BAPTA, 3 Mg-ATP; pH adjusted to 7.2 and osmolarity adjusted to 295 mOsm. Competitive antagonist SR-95531 (SR-95) and allosteric antagonist picrotoxin (Picr) were used to apply full or partial block on $GABA_A$Rs. The tonic current delivered through $GABA_A$Rs was measured as an outward shift in holding current following application of corresponding ligand(s). Changes in root mean square (RMS) noise were also used to study changes in tonic $GABA_A$R-mediated conductance since they are unaffected by current drifts. However, RMS noise can paradoxically decrease when tonic current increases.[21] We thus only used RMS noise as a measure in experiments where the tonic GABA-ergic current was fully or partially blocked. PKC blockers bisindolylmaleimide-II (BII, 50 nM) and GF-109203X (GF-109, 50 nM), PKA blocker PKI 5–24 (PKI, 100 nM), and wide-spectrum blocker of G-proteins pertussis toxin (PeTX, 1 μg/mL) were added to intracellular solution. CGP-55845, BII, GF-109 and picrotoxin were pre-diluted in DMSO; APV and MCPG were pre-diluted in 1 eq. NaOH; all other chemicals were pre-diluted in distilled water.

Recordings were performed at 32–34°C with patch pipettes of 6–7 MΩ electrical impedance.

To monitor series resistance, we used a + 5mV step command. Cells with unstable series resistance or unstable holding current were rejected. Electrical stimuli generated by constant voltage DS2A stimulus isolators (Digitimer LTD) were delivered through bipolar stimulating electrode placed in perforant path.

Outside-out and nucleated patch recordings. Membrane patches were excised from DGCs, recordings were performed in voltage-clamp mode (V_{hold} −70 mV). For solution exchange experiments, we used our earlier published protocol.[22] Briefly, a θ-glass application pipette with ~200 μm tip diameter attached to the micromanipulator was controlled by piezoelectric actuator with a 50–100 μs speed of switch between positions. One pipette channel was filled with the bath solution; another channel had aCSF with GABA, or GABA plus antagonist (SR-95 or Picr). Liquid pressure in pipette channels was regulated

by a PDES-02DX pneumatic micro ejector (npi) using compressed nitrogen, separately for each channel. Solutions with GABA, GABA+SR-95 and GABA+Picr were exchanged in a pipette channel during 7–12 s, while membrane patch was exposed to the bath solution channel.

Acquisition and analysis. Recordings were performed with a Multi-Clamp 700B amplifier (Molecular Devices), on-line filtered at 4 kHz, digitized at 10 kHz, and stored on a PC. pClamp/Clampfit 10× software (Molecular Devices) was used for data storage and further analysis. Wolfram Mathematica 10 software was used for nonlinear fitting of Hill dependencies and statistical calculations. For whole-cell recordings of tonic current, "n" means number of recorded neurons; for single-channel recordings, "n" means number of recorded outside-out patches and nucleated patches; in experiments on GABA dose-response dependencies and on action potential generation, "n" means number of animals.

Analysis of tonic currents. To quantify drifts of tonic currents, we used mean values of holding current during 200 ms epochs free of synaptic currents, taken every 30 s. The amplitude of the tonic current was calculated as the difference between the holding current (ΔI_{hold}) measured at stable baseline intervals before and after the application of an antagonist. To calculate change of RMS noise (ΔRMS), noise values were probed within 200 ms time intervals free of synaptic events.

Analysis of the single-channel recordings. $GABA_AR$ opening frequency was calculated as $N/\Delta t$, where N is the number of openings and Δt is the time of recording. N was obtained using a detection threshold of 1.5 pA more negative than mean baseline and a minimum opening time of 0.2 ms. Average single channel open time was obtained with threshold-detection algorithm of Clampfit 10X software. Open time fraction was calculated as $t_o/t_f \times 100\%$, where t_o is a time when ion channel(s) were observed in an open state, t_f is an overall time of recording.

Concentration–response curve fitting for GABA effect on $GABA_ARs$ open time per second (T_{GABA}) was performed with Hill equation

$$T_{GABA} = \frac{C_{GABA}^{n_H}}{K_d + C_{GABA}^{n_H}}$$

where C_{GABA} is concentration of GABA, K_d – apparent dissociation constant, n_H – Hill's coefficient.

GABA-receptor antagonists, MCPG, NBQX, BII, GF-109, PKI and PeTX, were purchased from Tocris Bioscience. All other chemicals were purchased form Sigma-Aldrich. All data are given as Mean ± Standard Error of Mean. If other not stated, statistical comparisons were made with Student's unpaired *t*-test. One-way analysis of variance (ANOVA) with Tukey *post hoc* test was used for multiple comparisons.

Results

Modulation of tonic $GABA_ARs$ conductance by cytoplasmic factors

To clarify the input of cytoplasmic signalling pathways into regulation of whole-cell tonic inhibitory current, we first performed continuous recordings where BII (50 nM) and PeTX (1 μg/mL) in intracellular solution were used to modulate s-$GABA_ARs$' conductance. To account for possible side effects of BII on protein kinase A (PKA),[23] we performed the same experiment with PKA antagonist PKI (100 nM) in intracellular solution (Figure 1).

To separate conventional (GABA-dependent) inhibitory response of $GABA_ARs$ and GABA-independent s-$GABA_ARs$ effects, we used different molecular pharmacology of SR-95 and Picr. SR-95 competes with GABA for the binding site at the receptor, i.e. blocks conventional GABA-dependent effects. In turn, Picr binding site is localized inside the $GABA_AR$'s ion channel; thus, Picr binding occurs when $GABA_AR$ channel opens, independently of GABA binding to the receptor. Therefore, Picr blocks both GABA-dependent and GABA-independent effects of $GABA_ARs$. We thus applied a universal pharmacological approach in all our experiments: conventional (GABA-triggered) $GABA_AR$ activity was assessed as the difference between values of given effect obtained in control vs. after application of SR-95. In turn, s-$GABA_AR$ input was assessed as the difference between the effect after SR-95 application vs. that after application of SR-95+Picr. We used a 25 μM SR-95 concentration as it was shown earlier to block in full GABA-ergic conductance in acute tissue.[2]

Application of both SR-95 and SR-95+Picr downregulated the root mean square (RMS) noise in all experiments, indicating the lowering of the number of active $GABA_ARs$; however, SR-95 alone produced a significant decrease only when BII was added to the intracellular solution, suggesting that the block of PKC upregulates conventional (GABA-dependent) tonic conductance. One-way ANOVA demonstrated significant impact of cytoplasmic factors on RMS noise: $F_{3,25}=3.82$, $P=0.022$, Tukey test: $P<0.05$ for Control vs. PeTX, BII vs. PeTX, PKI vs. PeTX (Figure 1(b)).

In line with the results for RMS noise, amount of tonic current (I_{tonic}) through s-GABARs displayed a significant dependence from cytoplasmic regulatory factors. One-way ANOVA on ΔI_{tonic} data: $F_{3,25}=7.26$, $P=0.0012$, Tukey test: $P<0.05$ for Control vs. PeTX, BII vs. PeTX, BII vs. PKI (Figure 1(c)).

Regulation of single-channel properties of tonically active $GABA_ARs$

To further dissect regulatory mechanisms of s-$GABA_ARs$ functioning, we asked which parameters of their single-channel openings are affected by cytoplasmic factors. As an additional control on (possible) side effects of BII in this experiment, we monitored effects of an alternative PKC blocker GF-109.

To minimize inter-sample difference, $GABA_ARs$ openings were recorded first from the OOP where all cytoplasmic signalling chains are destroyed, and then from the

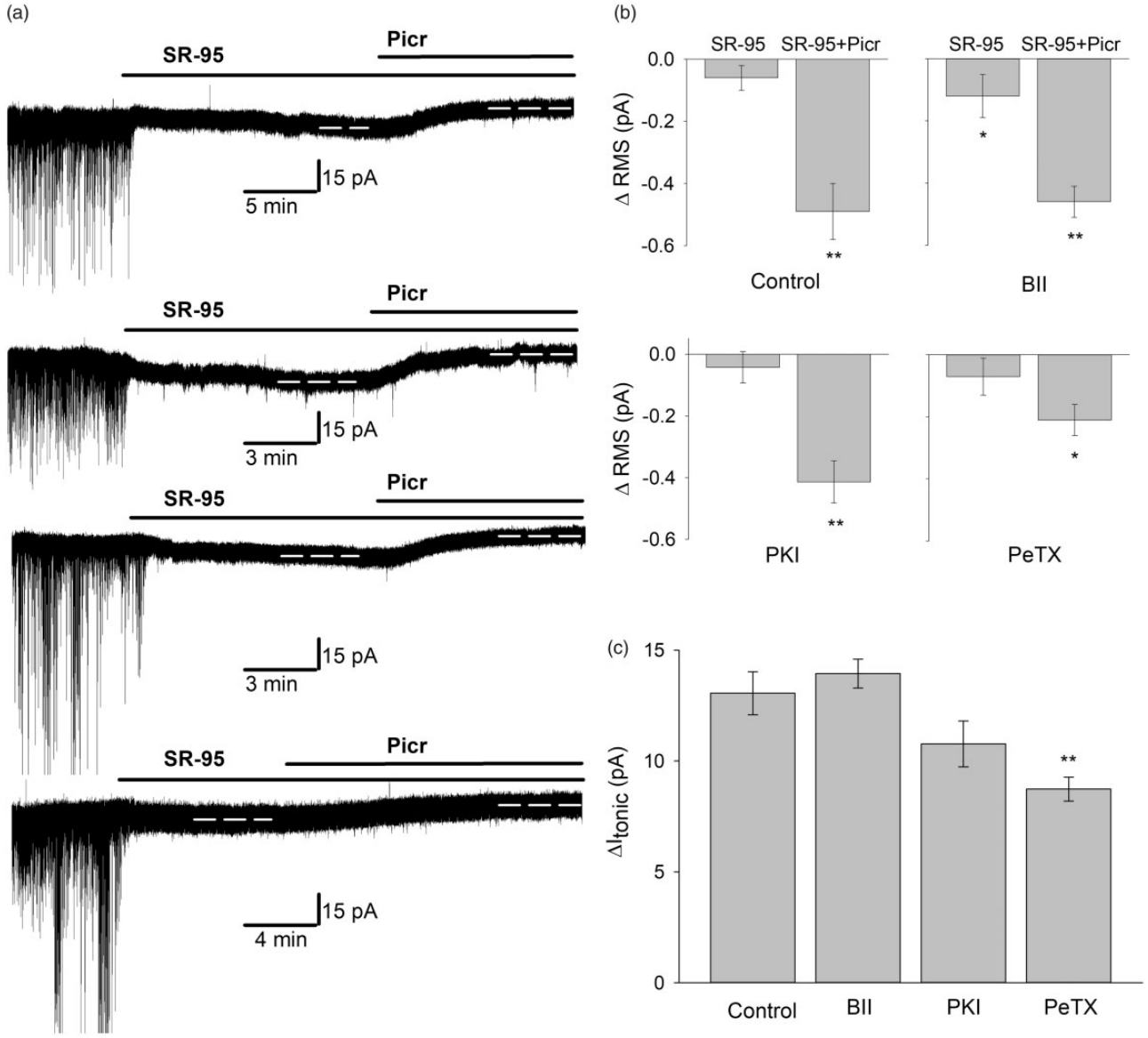

Figure 1. Pharmacology of GABA-mediated tonic currents. (a) Example traces of whole-cell continuous recordings from DGCs. From top to bottom: Control, BII added to internal solution, PeTX added to internal solution, PKI added to internal solution. SR-95 (25 μM) abolished spontaneous synaptic activity, whereas Picr (50 μM) induced an outward shift of tonic current. Dashed lines mark time intervals over which I_{tonic} was averaged for SR-95 (last three minutes before Picr added) and for SR-95+Picr. (b) SR-95 (left columns) and SR-95+Picr (right columns) decrease RMS noise; bars represent change from previous drug application, asterisks indicate significance of difference from zero; $n = 5$–8, Student's *t*-test. Vertical axis signs and bar marks apply to all bar charts. (c) Shifts of I_{tonic} induced by application of SR-95+Picr, asterisks indicate significance of difference from Control; $n = 11$–12, Student's *t*-test. $^*P < 0.05$, $^{**}P < 0.01$.

nucleated patch (NP) pulled from the same neuron, which preserves intracellular milieu (Figure 2). Application of 10 μM GABA generated clear single-channel activity in both patch types. In OOPs, spontaneous activity was still displayed when no GABA was added to the perfusion solution, thus confirming the presence (though of relatively low quantity) of s-$GABA_A$Rs with preserved functionality, when cytoplasmic regulatory chains are destroyed. In both OOPs and NPs, certain level of single-channel activity was still observed after application of SR-95 (25 μM), but application of Picr (50 μM) removed it in full (Figure 2(a)).

$GABA_A$R-generated electrical conductance remained similar under all combinations of patch-clamp modes (OOPs or NPs) and applied ligand cocktails (38.2 ± 4.3 pS, $n = 42$, $P > 0.2$ for all comparisons), thus making it inapplicable for characterization of s-$GABA_A$Rs modulation.

One-way ANOVA on data from OOPs did not display a significant effect of BII, GF-109 and PeTX ($P > 0.25$ for all data sets, refer to Figure 2(d) to (f)), thus ruling out possible involvement of membrane-anchored proteins into modulation of s-$GABA_A$Rs spontaneous activity. When GABA was applied, BII and GF-109, but not PeTX, significantly decreased average open time in NPs compared to control; one way ANOVA results: $F_{3,27} = 4.02$, $P = 0.017$, Tukey test: $P < 0.05$ for Control vs. BII and Control vs. GF-109 (Figure 2 (d)). The opening frequency was upregulated significantly by BII and GF-109, and downregulated by PeTX in the presence of GABA; one-way ANOVA results: $F_{3,27} = 4.82$,

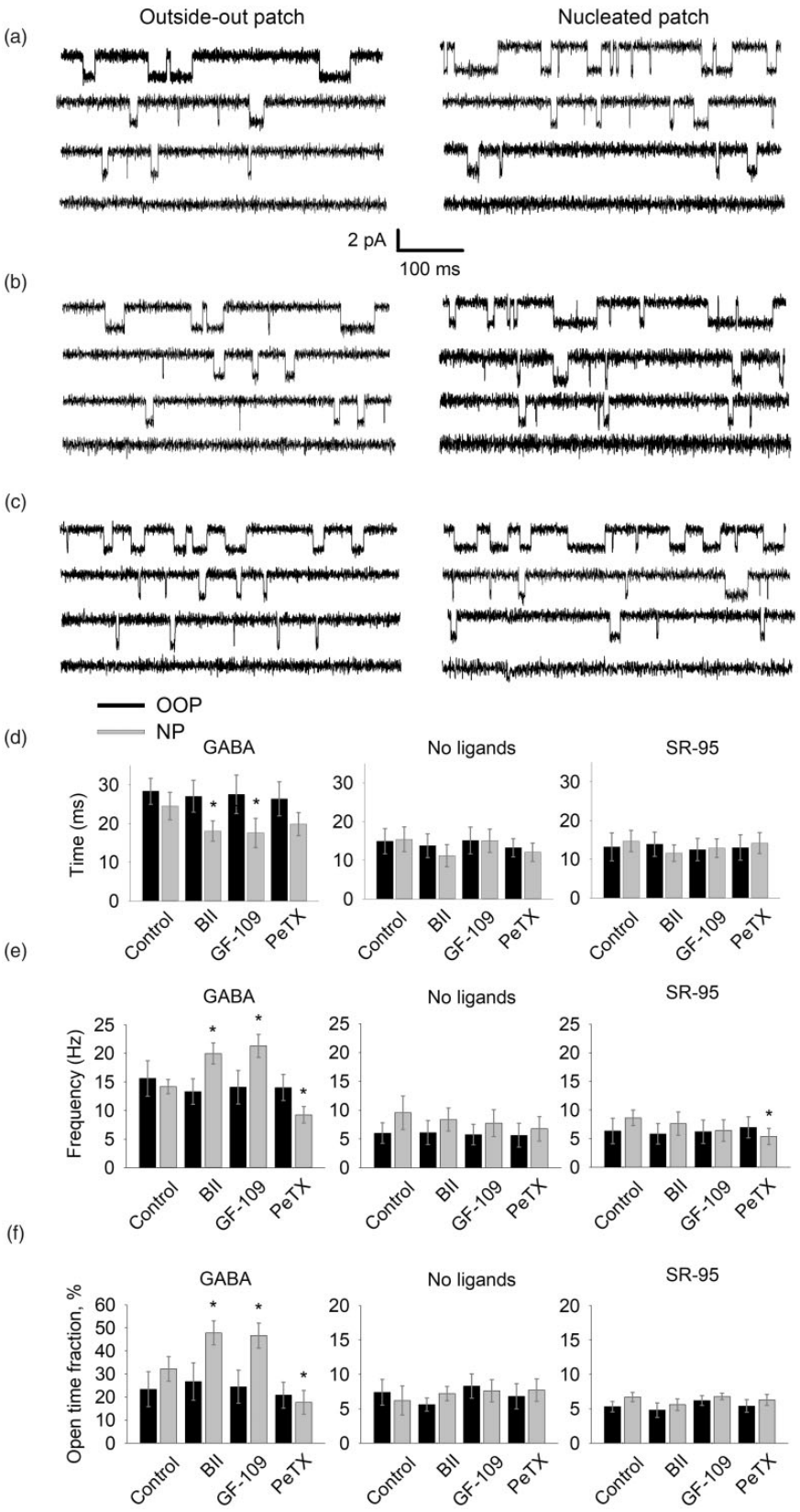

Figure 2. Modulation of $GABA_A$Rs single-channel parameters by cytoplasmic factors. Traces from top to bottom in a–c: GABA, perfusion solution without $GABA_A$R ligands, GABA+SR-95, GABA+Picr. Left panel: outside-out patch (OOP). Right panel: nucleated patch (NP) pulled from the same cell. Panel labels given in a and scale bars apply to a–c. (a) Control. **(b)** Block of PKC by BII (50 nM). (c) Block of G-protein-mediated activity with PeTX (1 μg/mL). (d) Average open time, statistical summary for a–c. (e)Opening frequency, statistical summary for a–c. (f) Open time fraction, statistical summary for a–c. Colour codes apply to d–f. Asterisks denote significance of difference from "Control" NP's column. $^*P < 0.05$; $n = 11$–13, Student's *t*-test.

$P = 0.008$, Tukey test: $P > 0.05$ for BII vs. GF-109 (Figure 2 (e)). Next, we calculated values of an open-time fraction which integrates single-channel average open time and opening frequency. This data set resembled to a large extent the opening frequency readout: in NPs in presence of GABA BII and GF-109 induced a significant increase of the open-time fraction value; in turn, PeTX significantly decreased open-time fraction. One-way ANOVA results: $F_{3,27}$=4.53, $P = 0.01$, Tukey test: $P > 0.05$ for BII vs. GF-109 (Figure 2(f)). In contrast, ANOVA did not display a significant impact of cytoplasmic factors when no ligands were added or in presence of SR-95: $P > 0.1$ for all cases (refer to Figure 2(d) to (f)).

These results suggest that BII and GF-109 upregulate GABA-dependent effects and PeTX downregulates GABA-independent effects of s-$GABA_A$Rs (mainly through the decrease of their opening probability), and thus provide additional support to the observation made in the continuous recording experiment (Figure 1).

However, if PeTX decreases the number of active s-$GABA_A$Rs, it should modify the relative sensitivity of the overall GABA-receptor pool to neurotransmitter. To test this, we next recorded single-channel openings from NPs in response to increasing concentrations of GABA; average $GABA_A$Rs open time per second was taken as a quantitative indicator of GABA effect (Figure 3). BII, being added to the intracellular solution, increased the maximum effect of GABA (586.4 ± 18.3 ms vs. 496.6 ± 24.4 in control, $P = 0.021$, $n = 11$), thus supporting the conclusion about upregulation of activity of conventional $GABA_A$Rs suggested by changes in RMS noise. PeTX, however, did not induce perceptible changes of maximum GABA effect value (481.3 ± 24.8 vs. 496.6 ± 24.4 ms, $P = 0.62$, $n = 9$; Figure 3(d)). Further, we normalized concentration-effect curves to the effect induced by the highest concentration of GABA and fitted them with Hill's equation. Hill's coefficient obtained for PeTX was significantly higher than that for the control curve (n_H=2.35 ± 0.56 for PeTX vs. 1.05 ± 0.42 for control, $P = 0.028$, $n = 9$), confirming our presumption about increase of receptor pool sensitivity to GABA. In contrast, Hill's coefficient shift induced by BII was not significant (n_H=1.51 ± 0.49 vs. 1.05 ± 0.42 for control, $P = 0.43$, $n = 11$) (Figure 3(e)).

Role of cytoplasmic factors in action potential generation

Previous experiments confirmed a significant input of cytoplasmic factors in regulation of single-channel s-$GABA_A$Rs' characteristics and s-$GABA_A$Rs' effects in the whole cell. Therefore, we next studied the role of cytoplasmic factors controlling tonic $GABA_A$Rs activity in AP generation.

To clarify the input of different groups of $GABA_A$Rs, we performed a current-clamp experiment, where, at the first stage, step current injection was held at the lowest level ample to generate AP(s) (rheobase). Then SR-95 (25 μM) was added to isolate GABA-independent s-$GABA_A$Rs' input, and the current injection was again lowered to the minimum amount which generates AP(s). Next, Picr (50 μM) was added. The ratios of the number of APs generated (i) in control vs. number after SR-95 application and (ii) after adjustment of current injection in the presence of SR-95 vs. number after Picr application were interpreted as an indicator of input into AP generation caused by GABA binding to $GABA_A$Rs and GABA-independent receptor openings, respectively.

When no cytoplasmic signalling pathways were blocked, both SR-95 and SR-95+Picr application caused a significant increase of the AP numbers ratio compared to unity: to 1.52 ± 0.24 and 2.32 ± 0.27, $P = 0.037$ and $P = 0.0085$, respectively, $n = 6$ (Figure 4(a) and (d)). BII added to the internal solution increased the "SR-95 vs. Control" ratio to 2.73 ± 0.42 vs. 1.52 ± 0.24 in the control ($P = 0.019$, $n = 11$, Student's t-test); thus, characterizing PKC-induced modulation of GABA-dependent input into AP-generating machinery (Figure 4(b) and (d)). PeTX significantly reduced the ratio "SR-95+Picr vs. Adjusted SR-95" (1.45 ± 0.22 vs. 2.32 ± 0.27, $P = 0.026$, $n = 11$, Student's t-test), which in turn confirms substantial input of PeTX-sensitive fraction of s-$GABA_A$Rs into modulation of AP generation (Figure 4(c) and (d)). However, the "SR-95+Picr vs. Adjusted SR-95" ratio still remained significantly higher than unity in cells where PeTX was added ($P = 0.034$, $n = 11$, Student's t-test), suggesting the significant role of PeTX-insensitive s-$GABA_A$Rs in AP generation.

Discussion

Here we found that different cytoplasmic factors control separate sets of tonically active $GABA_A$Rs, thus allowing the dissection of tonic inhibitory conductance in DGCs.

First, $GABA_A$Rs-generated tonic current can be clearly divided into GABA-dependent and GABA-independent, with GABA-dependent conductance downregulated by PKC-mediated activity in line with the earlier report for DGCs.[24]

PKC was repeatedly shown to inhibit $GABA_A$R function through two main mechanisms: via activation of phorbol esters – mediated signaling, which disrupts $GABA_A$R internalization and recycling, and via the direct phosphorylation of $GABA_A$R β_{1-3} subunits at residues S408-S410, which lowers $GABA_A$R sensitivity to GABA.[25] This suggests the latter mechanism (as GABA-dependent) to be responsible for PKC-delivered modulation of GABA-dependent $GABA_A$Rs in our preparation. Both PKC inhibitors used in our study (BII and GF-109) are competitive antagonists of ATP at its binding site at PKC[26,27]; in turn, inactivation of ATP binding site abolishes protein phosphorylation by PKC.[28] Therefore, the mechanism of modulation of $GABA_A$R response by BII and GF-109 is likely to be as following. PKC inhibitors occupy ATP binding site, thus blocking direct phosphorylation of $GABA_A$R β subunit by PKC. This, in turn prevents loss of $GABA_A$R sensitivity to GABA (receptor desensitization) and, as a sequence, upregulates inhibitory ion current used as a readout in our experiments.

Establishment of the role of PKC as a modulator of inhibitory tonic conductance is accompanied by uncertainty due to a number of side effects generated by kinase blockers, such as BII. To get rid of this uncertainty, we repeated an

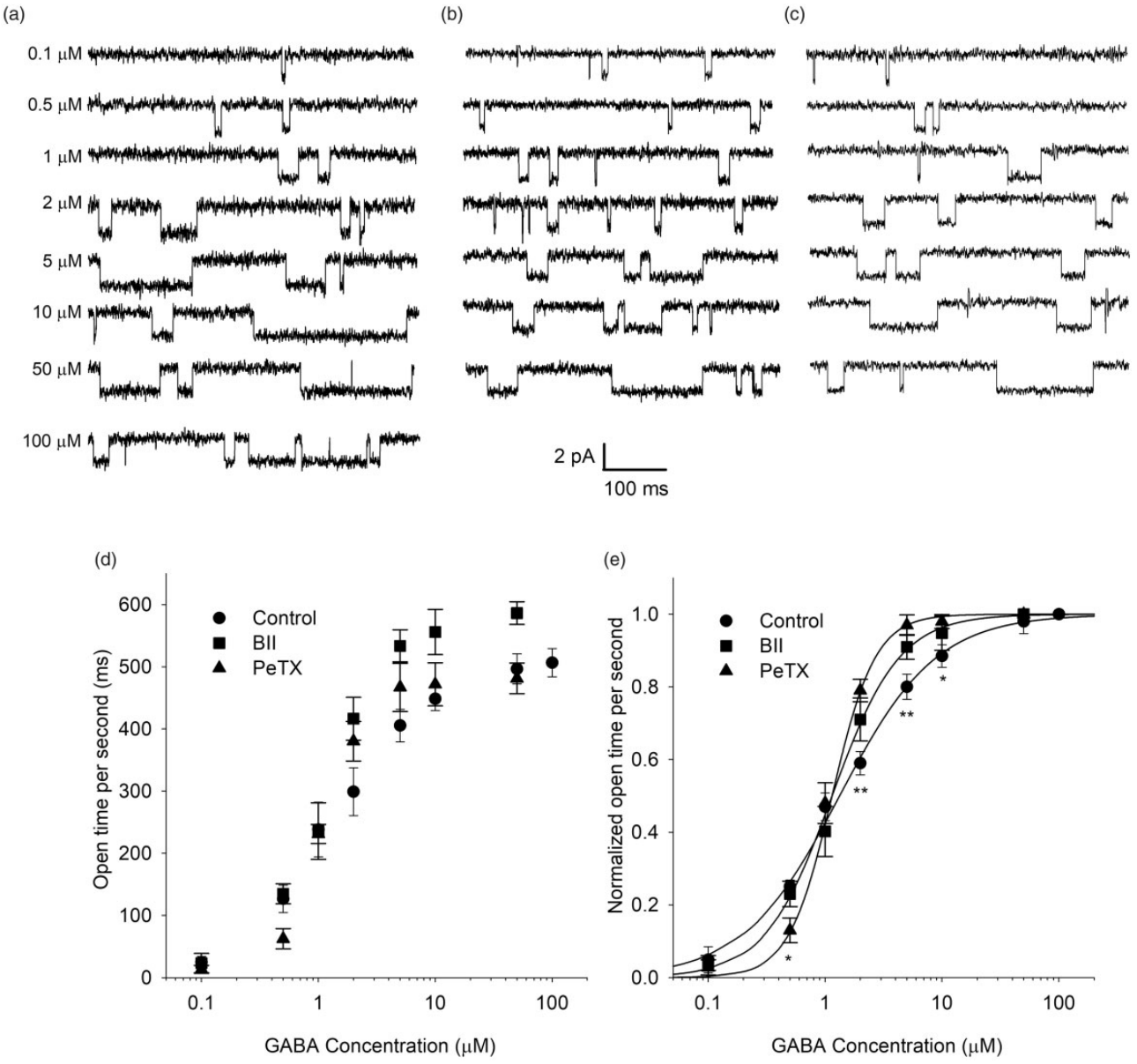

Figure 3. Modulation of $GABA_A$Rs sensitivity to GABA by cytoplasmic factors. a–c: Single-channel openings recorded from nucleated patches. Traces from top to bottom: augmenting concentrations of GABA in perfusion solution. (a) Control. (b) BII (50 nM) in recording pipette. (c) PeTX (1 μg/mL) in recording pipette. Concentrations of GABA given in a and scale bars apply to a–c. (d) $GABA_A$Rs open time per second as a function of GABA concentration. (e)Normalized $GABA_A$Rs open time per second as a function of GABA concentration. Asterisks denote significance of difference between points for PeTX and control. $^*P < 0.05$, $^{**}P < 0.01$, $n = 9$–11, Student's *t*-test.

experiment on single-channel functional characteristics (Figure 2) with another PKC silencer GF-109, which has the same action mechanism as BII,[26,27] but different set of possible side effects, e.g. enzymes which it can modulate.[29] We found no difference between BII and GF-109 effects (Figure 2(d) to (f)). Next, since PKA is an enzyme which can be modulated by both GF-109 and BII,[29] we tested possible impact of PKA on tonic inhibitory current: no significant effect was found (Figure 1). After PKA activation/deactivation is excluded from possible mechanisms of $GABA_A$R modulation, sets of side effects pertinent to BII and GF-109 and having the potential to influence $GABA_A$Rs tonic conductance become substantially different. Thus, it is unlikely that these sets of side effects generate similar response. We, therefore, conclude that BII and GF-109 modulate GABA-dependent tonic current via PKC. Another indirect confirmation of this conclusion comes from our recently published observation that 5-HT_3 receptors, which can be blocked by GF-109,[30,31] do not exert a significant impact on tonic inhibitory current in DGCs.[20]

GABA-independent tonic current subdivides into PeTX sensitive, i.e. modulated via G_i, G_o and G_t subgroups of G-proteins,[32] and PeTX insensitive. Further, at least a part of both PeTX- and GABA-insensitive current detected in whole-cell experiments and experiments on nucleated

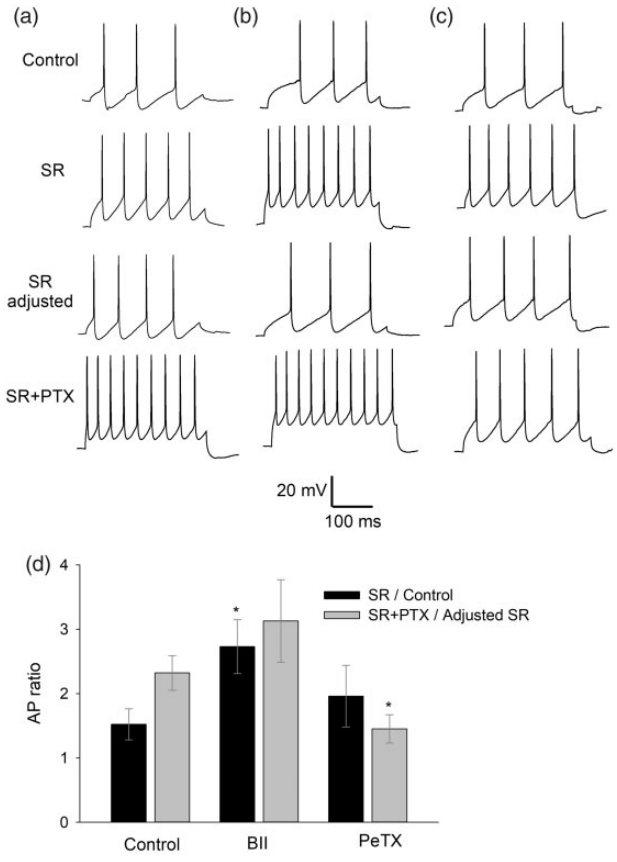

Figure 4. Cytoplasmic factors modulate s-$GABA_A$Rs impact on action potential generation. Minimum current injection which generates APs was used in control; after SR-95 (25 μM) application, the amount of injected current was again adjusted to minimum value generating APs. (a) No ligands added to intracellular solution. (b) BII (50 nM) added to intracellular solution. (c)PeTX (1 μg/mL) added to intracellular solution. Scale bars (bottom) and trace denominations (left) apply to a–c. (d) Bar chart shows "SR-95 to Control" and "Picr to adjusted SR-95" ratios of number of APs (see text for more details). Asterisks display significance of difference of ratios obtained from BII- and PeTX-loaded neurons from ratios generated at the same stage of control experiment. $^*P < 0.05$, $n = 6$–9, Student's *t*-test.

patches corresponds to spontaneous, SR-95-insensitive receptor openings recorded from OOPs, i.e. is independent from any cytoplasmic modulation and/or membrane-anchored PKC[33] (Figure 2).

Earlier works demonstrate that regulation of conventional (GABA-dependent) $GABA_A$Rs by PKC is tightly connected to activation of G-protein signaling chains.[34,35] However, in our study, we found that PKC has a significant impact on GABA-dependent tonic current and single-channel opening characteristics, but not on functioning of s-$GABA_A$Rs in NPs and in a whole-cell experiment (Figures 1(c) and 2(d) to (f)). In turn, PeTX-sensitive G-proteins appeared to exert a significant effect on GABA-independent conductance through s-$GABA_A$Rs. DGCs were shown to express a number of $GABA_A$R subunits: α1, 2 and 4, β1-3, γ1 and 2, δ and ε.[36,37] Therefore, the plausible explanation of observed pharmacological profile of $GABA_A$R-delivered tonic current is that GABA-independent $GABA_A$R effects are generated by receptors of specific subunit composition (such as α4βδ) which are insensitive to PKC, but can be modulated by other G-protein-controlled enzymes.[38] Since α4 extrasynaptic $GABA_A$Rs are affected in a variety of pathological states,[39] our results may thus have broad implications for treatment of neurological disorders. Another potential candidates to be involved into formation of s-$GABA_A$Rs are α1 and β2 subunits which were shown earlier to modulate spontaneous $GABA_A$Rs gating.[40]

Hippocampal network signaling layout includes DGCs as linear integrators of information, transferred via synaptic inputs from entorhinal cortex.[41] To perform this role, DGCs developed a high threshold of AP generation which makes them strong attenuators of incoming excitatory signaling.[41,42] This, in turn requires relatively large number of synchronized synaptic inputs to drive the cell to spike firing. Therefore, performance of the main DGCs functional role requires modulation of AP generation triggered by G-proteins- and PKC-connected signalling chains, and delivered through s-$GABA_A$Rs (Figure 4). On top of that, in our recent paper, we demonstrated a modulatory impact of s-$GABA_A$Rs of DGC on coincidence detection of excitatory synaptic inputs,[20] which implies significant role of DGCs in fine tuning of the local neuronal network signalling pattern. These observations suggest s-$GABA_A$Rs to be a new important actor controlling long-term hippocampal plasticity and, as a consequence, memory formation. However, apart of being necessary and/or important, is hypothetic input of s-$GABA_A$Rs into memory formation critical? It was shown previously that genetic silencing of expression of α1 subunit (a potential candidate for s-$GABA_A$R formation) can lead to the loss of ~60% of functional $GABA_A$Rs in the brain; but, nevertheless, the connected effects on animal phenotype and behaviour can be successfully compensated by alternative neurotransmission mechanisms.[43] This indeed suggests that, to test a hypothesis about critical importance of s-$GABA_A$Rs in memory formation, present data collected in *in vitro* experiments should be supplemented with further studies at a level of whole organism.

AUTHORS' CONTRIBUTIONS

NO performed experimental work, data analysis and provided critical revision of the paper; SS designed the study, performed experiments and data analysis, and wrote the paper.

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DECLARATION OF CONFLICTING INTERESTS

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