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Nerve Terminal $GABA_A$ Receptors Activate Ca^{2+}/Calmodulin-dependent Signaling to Inhibit Voltage-gated Ca^{2+} Influx and Glutamate Release*

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γ-Aminobutyric acid type A ($GABA_A$) receptors, a family of Cl^--permeable ion channels, mediate fast synaptic inhibition as postsynaptically enriched receptors for γ-aminobutyric acid at GABAergic synapses. Here we describe an alternative type of inhibition mediated by $GABA_A$ receptors present on neocortical glutamatergic nerve terminals and examine the underlying signaling mechanism(s). By monitoring the activity of the presynaptic CaM kinase II/synapsin I signaling pathway in isolated nerve terminals, we demonstrate that $GABA_A$ receptor activation correlated with an increase in basal intraterminal $[Ca^{2+}]_i$. Interestingly, this activation of $GABA_A$ receptors resulted in a reduction of subsequent depolarization-evoked Ca^{2+} influx, which thereby led to an inhibition of glutamate release. To investigate how the observed $GABA_A$ receptor-mediated modulation operates, we determined the sensitivity of this process to the Na-K-2Cl cotransporter 1 antagonist bumetanide, as well as substitution of Ca^{2+} with Ba^{2+}, or Ca^{2+}/calmodulin inhibition by W7. All of these treatments abolished the modulation by $GABA_A$ receptors. Application of selective antagonists of voltage-gated Ca^{2+} channels (VGCCs) revealed that the $GABA_A$ receptor-mediated modulation of glutamate release required the specific activity of L- and R-type VGCCs. Crucially, the inhibition of release by these receptors was abolished in terminals isolated from R-type VGCC knock-out mice. Together, our results indicate that a functional coupling between nerve terminal $GABA_A$ receptors and L- or R-type VGCCs is mediated by Ca^{2+}/calmodulin-dependent signaling. This mechanism provides a GABA-mediated control of glutamatergic synaptic activity by a direct inhibition of glutamate release.

$GABA_A$[2] receptors, a large and diverse family of Cl^--permeable ion channels, mediate fast transmission at inhibitory GABAergic synapses and are critical for the development and coordination of neuronal activity underlying the majority of physiological and behavioral processes in the brain (1–3). $GABA_A$ receptors are assembled from seven classes of homologous subunits, α(1–6), β(1–3), γ(1–3), δ, ϵ, π, and θ (4), into heteropentamers that display extensive structural and functional heterogeneity at different subcellular localizations (5). Thus, $GABA_A$ receptors also operate as tonically active extrasynaptic receptors (6–8), and as modulatory auto- or heteroreceptors present on axons and, in some instances, presynaptic nerve terminals in specific areas of the central nervous system (9, 10).

Presynaptic inhibition mediated by $GABA_A$ receptors was first described in the spinal cord (11–15). Recent electrophysiological studies have established the presence of presynaptic $GABA_A$ receptors in a number of brain regions, including cerebellum (16–19), hippocampus (20–26), auditory brainstem (27), ventral tegmental area (28), and others (9). However, from these studies it is apparent that the presynaptic activity of $GABA_A$ receptors can have diverse effects on properties of neurotransmitter release, ranging from inhibition to stimulation of spontaneous or evoked release of neurotransmitters, depending on a type of nerve terminals, brain region, and/or the age of animals (9). Given that neurotransmitter release results from a complex cascade of interactions between voltage-gated ion channels and protein machinery involved in synaptic vesicle exocytosis, and can be regulated at multiple points in this cascade (29), it is important to establish the exact signaling mechanisms activated downstream of $GABA_A$ receptors to regulate this process.

We demonstrate here that $GABA_A$ receptors, by regulating intraterminal calcium concentration ($[Ca^{2+}]_i$), increase Ca^{2+}/calmodulin-dependent signaling to inhibit subsequent voltage-gated Ca^{2+} entry and thereby suppress neurotransmitter release from glutamatergic terminals isolated from the adult rat neocortex. We demonstrate further that the inhibition of glutamate release requires NKCC1 activity, implicating a Cl^- electrochemical gradient subserving a depolarizing activity of $GABA_A$ receptors in this preparation. Moreover, the modula-

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[2] The abbreviations used are: $GABA_A$, γ-aminobutyric acid, type A; GABA, γ-aminobutyric acid; VGCC, voltage-gated calcium channel; CaMK, calcium/calmodulin-dependent kinase; NKCC, Na-K-2Cl cotransporter; GAD, glutamic acid decarboxylase; VGLUT, vesicular glutamate transporter; NKCC1, Na-K-2Cl cotransporter 1; ANOVA, analysis of variance; CDI, Ca^{2+}/calmodulin-dependent inactivation; ω-Aga IVA, ω-agatoxin IVA; ω-CTx GVIA, ω-conotoxin GVIA; CDI, Ca^{2+}/calmodulin-dependent inactivation; 4AP, 4-aminopyridine.

tion is dependent on the influx of Ca^{2+} via L- or R-type VGCCs, although only R-type channels directly contribute to the release. The modulatory entry of Ca^{2+} leads to the activation of calmodulin. Together, our results demonstrate $GABA_A$ receptor-mediated and Ca^{2+}/calmodulin-dependent down-regulation of VGCCs leading to an inhibition of glutamate release from nerve terminals.

EXPERIMENTAL PROCEDURES

Preparation of Synaptosomes—Percoll gradient-purified synaptosomes were prepared as described previously (30) from the cerebral cortices of 2-month-old male Sprague-Dawley rats or from male wild-type or R-type ($Ca_V2.3$) α1E subunit-deficient ($\alpha1E^{-/-}$) mutant mice characterized previously (31). On incubation at 37 °C, synaptosomes show metabolic competence and ability to release neurotransmitters (32, 33).

Glutamate Release Assay—Synaptosomes (0.1 mg) were resuspended in 1.5 ml of Hepes-buffered incubation medium (HBM: 140 mM NaCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose, and 20 mM Hepes, pH 7.4), and placed in an LS-5 spectrofluorimeter (PerkinElmer Life Sciences) at 37 °C with stirring. Glutamate release was assayed in the presence of glutamate dehydrogenase (50 units/ml, Sigma), $NADP^+$ (2 mM), and $CaCl_2$ (1 mM) by on-line fluorimetry, as described previously (32, 34). Where indicated, synaptosomes were incubated in the presence of picrotoxin (50–100 μM, Tocris), SR 95532 (50 μM, Tocris), strychnine (30 μM, Sigma), bumetanide (10 μM, Sigma), W7 (20 μM, Tocris), or W5 (20 μM, Tocris) for 10 min; ω-agatoxin IVA (100 μM, Alomone Labs), ω-conotoxin GVIA (10 μM, Alomone Labs), $NiCl_2$ (50 μM, Sigma), nifedipine (1 μM, Alomone Labs), or SNX-482 (100 nM, Alomone Labs) for 4 min; and muscimol (10–500 μM, Tocris) or isoguvacine (10–500 μM, Tocris) for 3 min prior to the addition of secretagogue. Other experiments were performed in the presence of EGTA (0.2 mM) for 7 min, followed by the addition of either $BaCl_2$ (1 mM) with/without muscimol (200 μM) or only muscimol (200 μM), and further incubated for 3 min prior to the addition of secretagogue. Release was stimulated as indicated with 4-aminopyridine (4AP, 1 mM), KCl (10 mM), or ionomycin (5 μM), 10 min after the start of incubation and monitored at 2-s intervals. Data were analyzed using Lotus 1-2-3, Microcal Origin, and Microsoft Excel. Each *n* indicates the number of individual synaptosome preparations used; each preparation was derived from a single animal. Mean glutamate release ± S.E. in the absence or presence of drugs is reported in time course traces. Unless otherwise indicated, mean release values ± S.E. (nmol/mg protein/5 min) quoted in the text are levels attained at "steady state" 5 min after stimulation. Additionally, for some comparisons, the values obtained in the presence of drugs are expressed as percent of corresponding control values in the absence of drugs and shown as bar graphs. Statistical analysis was carried out using one-way ANOVA followed by post-hoc LSD test. Dose-response curves were fit using a sigmoidal relationship with variable slope employing GraphPad Prism.

Intraterminal Ca^{2+} Measurements—Intraterminal Ca^{2+} levels were assayed by on-line fluorimetry as described previously (33, 35). Synaptosomes (0.1 mg) were resuspended in 1 ml of HBM containing $CaCl_2$ (0.1 mM) and loaded with Fura 2-AM (5 μM) for 20 min at 37 °C. Synaptosomes were washed with HBM by centrifugation, resuspended in 1.5 ml of HBM, and placed in an LS-5 spectrofluorimeter (PerkinElmer Life Sciences) at 37 °C with stirring in the presence of $CaCl_2$ (1 mM). Synaptosomes were incubated for 3 min in the presence of muscimol (10–500 μM) prior to depolarization with 4AP (1 mM). Calibration procedures were performed as described previously (33). Briefly, samples were alternately excited at $\lambda = 340$ nm and $\lambda = 380$ nm; fluorescence emission was monitored at $\lambda = 505$ nm, and fluorescence ratios (excitation 340/380 nm) were calculated. The maximum fluorescence ratio (*i.e.* Ca^{2+}-saturated Fura-2) was determined in the presence of 0.1% SDS and the minimum fluorescence ratio (*i.e.* Ca^{2+}-free Fura-2) in the presence of 20 mM EGTA, added at the end of each experiment. Calculation of cytosolic $[Ca^{2+}]_i$ was carried out assuming a K_d value for Fura-2 and Ca^{2+} of 224 nM, using equations as described previously (36). Cumulative data were analyzed using Lotus 1-2-3. Changes in intraterminal Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$), 5 min after the addition of 4AP in the presence of muscimol, were calculated and expressed as percentage of $\Delta[Ca^{2+}]_i$ measured under control conditions without muscimol. Statistical analysis was performed by one-way ANOVA followed by post-hoc LSD test.

Drug Incubation and Immunoblotting—Synaptosomes were resuspended in HBM (at 1 mg/ml) in the presence of $CaCl_2$ (1 mM) on ice and placed at 37 °C with stirring to start the incubation. As indicated in figure legends, synaptosomes were incubated with GABase (0.02–0.4 unit/ml), picrotoxin (1–100 μM), or bicuculline (10–100 μM) for 10 min or in the presence of muscimol (10–500 μM) or isoguvacine (1–500 μM) for 2 min before the lysis with 2% SDS. Protein concentrations were measured using the BCA assay (Pierce), with bovine serum albumin as standard. Equal amounts of protein were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblotting was carried out with a phosphorylation state-specific antibody that was raised to specifically recognize serine 603 (P-site 3) in synapsin I phosphorylated by Ca^{2+}/calmodulin-dependent kinase (CaMK) II (anti-phospho (P)-site 3 synapsin I antibody, 0.5 μg/ml) or with a total synapsin I antibody (0.5 μg/ml, both kindly provided by Professors P. Greengard and A. C. Nairn, The Rockefeller University, New York). In additional immunoblotting experiments, antibodies specific for α1, α2, β3, γ2, and δ subunits of $GABA_A$ receptors (kindly provided by Professor W. Sieghart, Vienna, Austria) were used as described (37). Primary incubations were followed by incubation with ^{125}I-labeled protein A (0.05 μCi/ml; Amersham Biosciences). Blots were exposed to a PhosphorImager screen, and quantification of immunoblots was carried out using PhosphorImager scanning and ImageQuant software (GE Healthcare). Values stated are mean changes in phosphorylation (% of control) ± S.E.

Immunohistochemistry—Immunofluorescence analysis was performed on neocortical slices as described previously (38, 39). Sections were incubated with primary antibody solutions containing anti-P-site 3 synapsin I antibody (0.5 μg/ml), anti-glutamic acid decarboxylase (GAD) antibody (0.5 μg/ml, Chemicon), and anti-vesicular glutamate transporter 1 (VGLUT1) antibody (0.2 μg/ml, Chemicon), for 14–16 h at

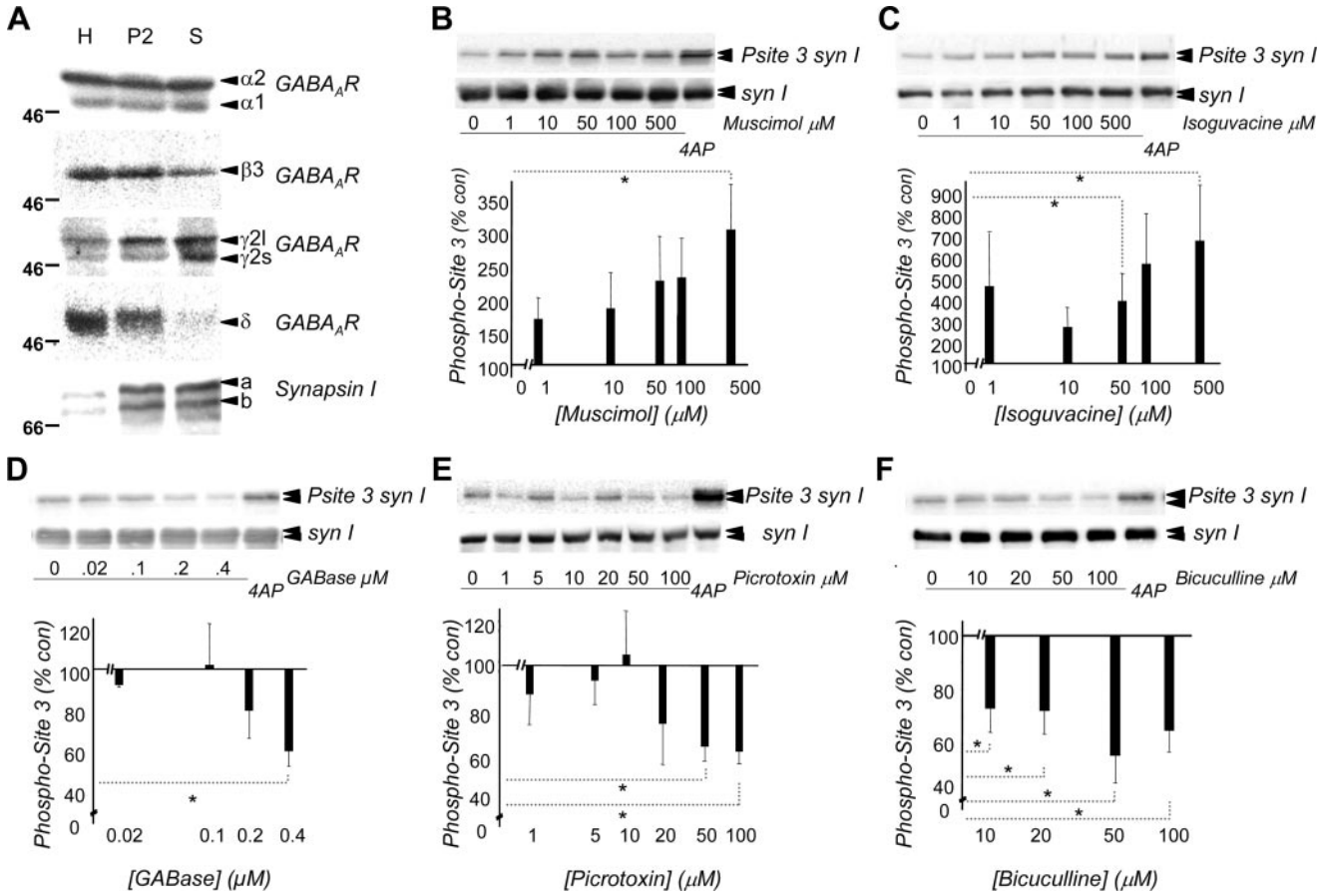


FIGURE 1. **Nerve terminal GABA$_A$ receptor activity regulates Ca^{2+}/calmodulin-dependent phosphorylation of synapsin I.** *A,* immunodetection of GABA$_A$ receptor subunits in purified neocortical synaptosomes (*H,* homogenate; *P2*, crude synaptosomal pellet; *S*, purified synaptosomes). *B* and *C,* dose-dependent increase in CaMKII-dependent phosphorylation of synapsin I at P-site 3 in the presence of increasing concentrations of muscimol ([*Muscimol*], 1–500 μM; $n = 6$) (*B*) and isoguvacine ([*Isoguvacine*], 1–500 μM; $n = 5$) (*C*). *D–F,* dose-dependent decrease in CaMKII-dependent phosphorylation of synapsin I at P-site 3 in the presence of increasing concentrations of GABase ([*GABase*], 0.02–0.4 unit/ml; $n = 5$) (*D*) and picrotoxin ([*Picrotoxin*] 1 μM-100 μM; $n = 5$) (*E*) and bicuculline ([*Bicuculline*] 10 μM-100 μM; $n = 5$) (*F*). Blots (*upper panels, B–F*) show phospho-site 3-synapsin I (P-site 3 syn) and total synapsin I (*syn I*). Histograms (*lower panels, B–F*) show quantification of changes in synapsin I P-site 3 phosphorylation normalized to controls. Total synapsin I levels were not significantly affected under any stimulation condition used. *, $p < 0.05$ compared with 100% 37 °C controls (one-way ANOVA with post-hoc LSD test).

4 °C. Sections were then washed (five times for 5 min) in phosphate-buffered saline and incubated in phosphate-buffered saline with 1% bovine serum albumin (w/v) containing a mixture of fluorescently tagged markers as follows: goat anti-rabbit IgG-Alexa 488, goat anti-mouse IgG-Alexa 555, and goat anti-guinea-pig IgG-Cy5 (all diluted to 1:750; Chemicon). Immunoreactivity was visualized using a Zeiss LSM 510 Meta laser-scanning confocal microscope. The estimation of the percentage of glutamatergic terminals positive for P-site 3 synapsin I was carried out in six randomly selected areas (324 μm^2) in images acquired from neocortical layers I–III, layer IV, and layers V and VI.

RESULTS

Presynaptic GABA$_A$ Receptors Regulate Ca^{2+}-dependent Phosphorylation of Synapsin I in Glutamatergic Nerve Terminals—To detect the activity of GABA$_A$ receptors localized to nerve terminals in the rat neocortex independently from the large postsynaptic pool of these receptors, we isolated presynaptic nerve terminals (synaptosomes) using a well established procedure (30, 40, 41). The presence of GABA$_A$ receptor α1, α2, β3, and γ2 (short, *s* and long, *l*), and the absence of δ subunit, in a highly purified fraction of nerve terminals was detected by immunoblotting with subunit-specific antibodies (Fig. 1*A*, *lane S*). We hypothesized that the activation of these receptors may regulate intraterminal Ca^{2+} levels ($[Ca^{2+}]_i$) in a way similar to changes observed in mossy fiber terminals in the hippocampus (26). To test this hypothesis, we monitored the effects of GABA$_A$ receptor activation or inhibition on the presynaptic CaMKII/synapsin I signaling pathway, using a phosphorylation state-specific antibody that recognizes synapsin I only when phosphorylated by CaMKII at Ser-603 (anti-P-site 3 synapsin I antibody, see Refs. 42, 43). This is a highly sensitive biochemical reporter of changes in intraterminal $[Ca^{2+}]_i$ as synapsin I phosphorylation by CaMKII occurs specifically in response to depolarization-triggered Ca^{2+} influx, as demonstrated previously (44). Accordingly, in all synapsin I phosphorylation experiments, increases in anti-P-site 3 synapsin I in response to depolarization (4AP, 1 mM)-triggered Ca^{2+} influx were monitored as positive controls (Fig. 1, *B–F, lanes 4AP*).

We first tested whether activation of GABA$_A$ receptors with the agonist muscimol or isoguvacine stimulated CaMKII-dependent signaling in nerve terminals. Immunoblotting with anti-P-site 3 synapsin I antibody revealed a dose-dependent

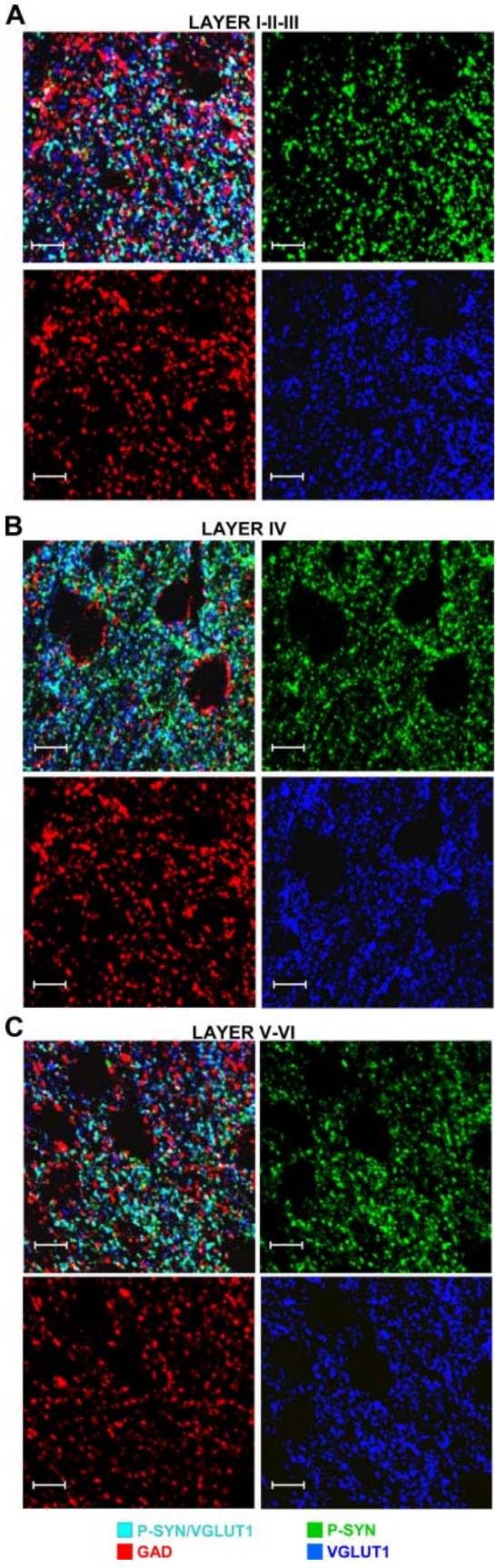


FIGURE 2. **Specific localization of CaMKII-phosphorylated synapsin I to glutamatergic terminals in the rat neocortex.** Immunohistochemical characterization of CaMKII-phosphorylated P-site 3-synapsin I (*P-SYN*, *green, top right*), vesicular glutamate transporter (VGLUT1, *blue, bottom right*) and glutamic acid decarboxylase (GAD, *red, bottom left*) in neocortical layers I–III (*A*); VI (*B*); and V–VI (*C*). Note the colocalization of P-SYN with VGLUT1 (P-SYN/VGLUT1, *turquoise, top left*) but not with GAD, and the mutually exclusive labeling of GAD and VGLUT1. (*Scale bar,* 10 μm).

increase in CaMKII-dependent P-site 3 phosphorylation of synapsin I in the presence of muscimol (1–500 μM), with 500 μM agonist producing a 302.2 ± 68.4% increase compared with controls (n = 6, Fig. 1*B*, *blot P-site 3 syn I* and *bar graph*). Addition of isoguvacine (1–500 μM) caused a similar dose-dependent stimulation of phosphorylation, with 50 and 500 μM, respectively, effecting 385.4 ± 132.5 and 670.7 ± 263.7% increases compared with controls (Fig. 1*C*, *panel P-site 3 syn I* and *bar graph*). Neither agonist affected the total levels of synapsin I in these experiments (Fig. 1, *B* and *C*, *panel syn I*).

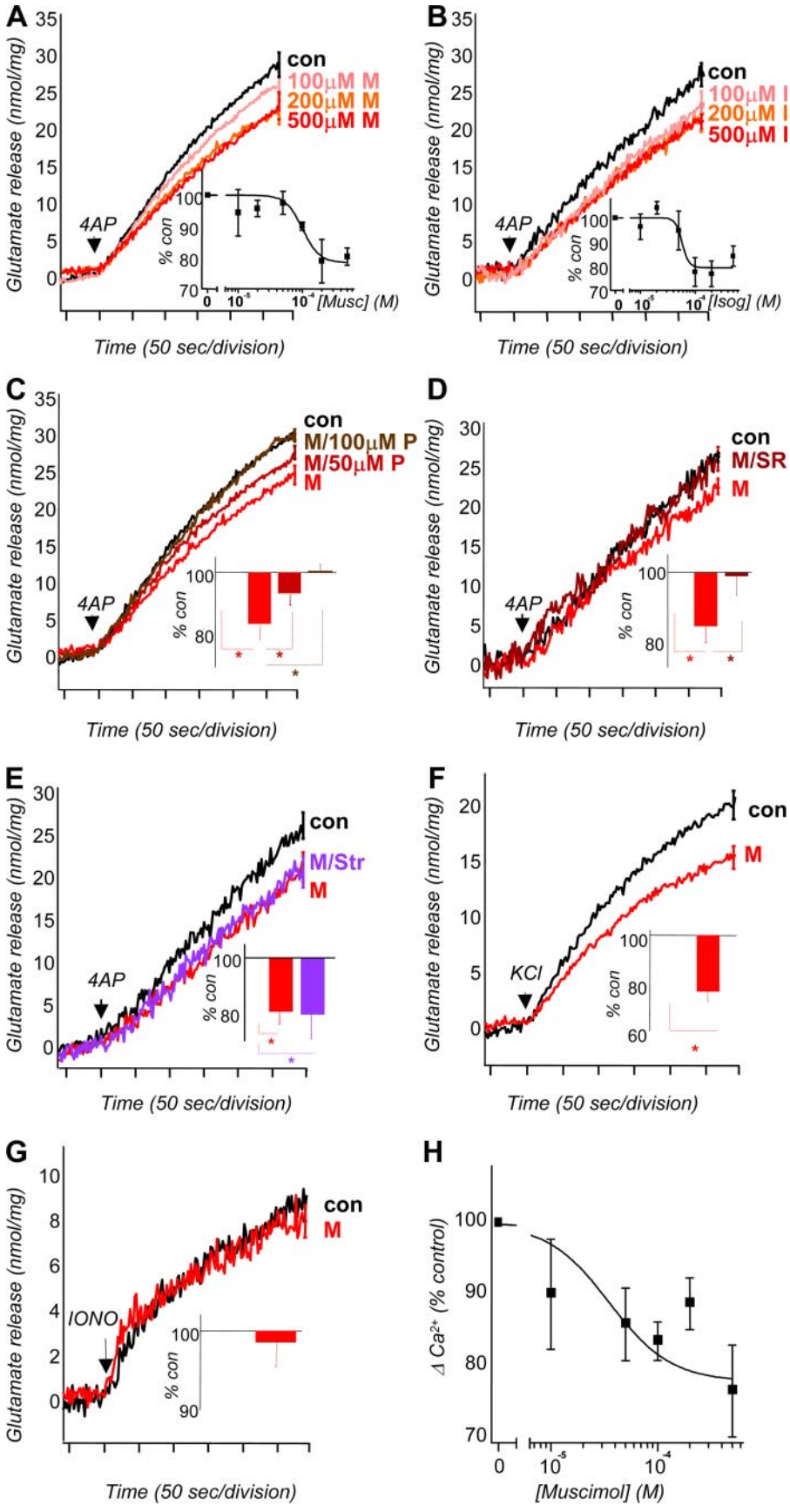
Given the relatively high concentrations of GABA$_A$ receptor agonists required to produce the observed effects, we reasoned that high affinity GABA$_A$ receptors (45) may be tonically active, because the ambient concentration of GABA in our preparation has been estimated to be as high as 1–5 μM (at 1 mg/ml concentration of synaptosomal protein) (46). To reduce the levels of GABA, we incubated synaptosomes with increasing concentrations of GABase (a complex of GABA-aminotransferase and succinic semialdehyde dehydrogenase, 0.02–0.4 unit/ml) (47). Immunoblotting with anti-P-site 3 synapsin I antibody revealed a significant dose-dependent decrease in CaMKII-dependent phosphorylation of synapsin I with increasing concentrations of GABase (0.02–0.4 unit/ml), reaching 59.8 ± 7.7% of control in the presence of 0.4 unit/ml (Fig. 1*D*, *panel P-site 3 syn I* and *bar graph*), without any effect on the total levels of synapsin I (Fig. 1*D*, *panel syn I*).

To confirm that the observed regulation of presynaptic CaMKII/synapsin I signaling by ambient GABA was mediated by GABA$_A$ receptors, we incubated synaptosomes with increasing concentrations of picrotoxin (1–100 μM), a GABA$_A$ receptor Cl^- channel blocker, or bicuculline (10–100 μM), a competitive GABA$_A$ receptor antagonist. Immunoblotting with anti-P-site 3 synapsin I antibody revealed a dose-dependent decrease in basal levels of CaMKII-dependent phosphorylation of synapsin I, with statistically significant effects at 50 μM (63.8 ± 6.8% control) and 100 μM picrotoxin (61.6 ± 5.8% control, Fig. 1*E*, *panel P-site 3 syn I* and *bar graph*). Bicuculline produced a significant dose-dependent decrease in CaMKII-dependent P-site 3 phosphorylation of synapsin I at all the concentrations tested (10 μM, 73.1 ± 8.9% of control; 20 μM, 72.4 ± 8.6% control; 50 μM, 55.8 ± 10.3% of control; and 100 μM, 65.0 ± 7.9% control; Fig. 1*F*, *panel P-site 3 syn I* and *bar graph*). Neither picrotoxin nor bicuculline affected the total levels of synapsin I (Fig. 1, *E* and *F*, *panels syn I*).

Synapsins are presynaptic proteins expressed ubiquitously in all presynaptic nerve terminals in the central nervous system (48). To determine whether the GABA$_A$ receptor-mediated regulation of synapsin I/CaMKII signaling cascade occurs universally throughout the rat neocortex (layers I–VI) or only in certain populations of nerve terminals, we carried out triple labeling immunocytochemistry, followed by confocal microscopy and colocalization analysis, using anti-P-site 3 synapsin I

antibody (Fig. 2, *A–C*, in *green*), together with anti-glutamic acid decarboxylase (GAD) antibody to identify GABAergic nerve terminals (Fig. 2, *A–C*, in *red*), and anti-vesicular glutamate transporter (VGLUT) 1 antibody to identify glutamatergic nerve terminals (Fig. 2, *A–C*, in *blue*). Although terminal-specific staining was detected with all three antibodies, P-site 3 synapsin I was detected exclusively in a population of glutamatergic nerve terminals as reflected by the overlapping (*green/blue*) *turquoise* color and evidently absent from GABAergic terminals (Fig. 2, *A–C*, merged images). The quantitative analysis revealed the presence of P-site 3 synapsin I in 31.1 ± 3.8% (layers I–III), 19.1 ± 1.7% (layer IV), and 19.2 ± 1.5% (layers V and VI) of glutamatergic nerve terminals in the neocortex. Complete lack of colocalization between GAD and VGLUT 1 confirmed the specificity of antibodies used in these experiments. These results, together with our biochemical experiments, are indicative of the presence of functional GABA$_A$ receptors specifically on a subset of glutamatergic nerve terminals in the rat neocortex.

Presynaptic GABA$_A$ Receptors Modulate Glutamate Release through Control of Depolarization-dependent Ca^{2+} Influx—To determine whether presynaptic GABA$_A$ receptor activity regulates glutamate release, synaptosomes (0.1 mg/ml) were incubated in the presence of increasing doses of muscimol (10–500 μM) or isoguvacine (1–500 μM), followed by the addition of 1 mM 4AP to trigger release. Control glutamate release (28.2 ± 1.6 nmol/mg/5 min) was decreased in a dose-dependent manner by 200 μM (21.8 ± 1.6 nmol/mg/5 min, 77.2% of control) and 500 μM (22.6 ± 1.8 nmol/mg/5 min, 80.3% of control) muscimol (Fig. 3*A*). Glutamate release (26.9 ± 1.6 nmol/mg/5 min in control) was also inhibited in a dose-dependent manner by 100 μM (21.0 ± 1.9 nmol/mg/5 min, 80.2% of control), 200 μM (20.5 ± 1.0 nmol/mg/5 min, 76.3% of con-



trol), and 500 μM isoguvacine (23.2 ± 1.5 nmol/mg/5 min, 86.2% of control; Fig. 3*B*). To confirm that the observed inhibition of 4AP-evoked glutamate release was indeed mediated by $GABA_A$ receptors, we incubated synaptosomes with 50 and 100 μM picrotoxin (Fig. 3*C*), or 50 μM SR95531 (Fig. 3*D*), prior to the addition of muscimol. The inhibition of control glutamate release (29.3 ± 0.4 nmol/mg/5 min) by muscimol (200 μM, 24.1 ± 1.3 nmol/mg/5 min, 82.3% of control) was partially reversed by 50 μM picrotoxin (27.1 ± 0.9 nmol/mg/5 min, 92.7% of the control) and abolished by 100 μM picrotoxin (29.4 ± 0.8 nmol/mg/5 min, 100.3% of control; Fig. 3*C*). The inhibition of control release (26.0 ± 1.3 nmol/mg/5 min) by muscimol (200 μM, 22 ± 1 nmol/mg/5 min, 84.5% of control) was also abolished by 50 μM SR95531 (25.6 ± 0.6 nmol/mg/5 min, 98.5% of control; Fig. 3*D*). However, the inhibition of control release (24.9 ± 1.4 nmol/mg/5 min) by muscimol (200 μM, 20.7 ± 1.8 nmol/mg/5 min, 83.1% of control) was unaffected by the addition of 30 μM strychnine, a glycine receptor-specific antagonist (20.1 ± 1.8 nmol/mg/5 min, 80.6% of control; Fig. 3*E*), further demonstrating a specific role of $GABA_A$ receptors in the observed inhibition of glutamate release. Glutamate release evoked by 4AP in the absence of extracellular Ca^{2+} was unaffected by muscimol (200 μM) indicating that $GABA_A$ receptor activity does not influence the release of glutamate occurring by any reversal of plasma membrane glutamate transporter (data not shown).

We confirmed the $GABA_A$ receptor-mediated modulation of glutamate release using an alternative secretagogue, KCl. Control glutamate release evoked by 10 mM KCl (19.7 ± 1.3 nmol/mg/5 min) was potently inhibited by 200 μM muscimol (15.0 ± 1.0 nmol/mg/5 min, 76.1% of control; Fig. 3*F*). Importantly, the release triggered by Ca^{2+}-ionophore ionomycin (5 μM, 8.3 ± 0.5 nmol/mg/5 min) was unaffected by muscimol (200 μM, 7.8 ± 0.7 nmol/mg/5 min; Fig. 3*G*). Thus, in stark contrast to depolarization-dependent release evoked by 4AP or KCl, a direct increase in $[Ca^{2+}]_i$ without any VGCC activation was not modulated by $GABA_A$ receptor activation. This indicates that the molecular mechanisms underlying the observed inhibition of glutamate release by nerve terminal $GABA_A$ receptors involve steps prior to synaptic vesicle recruitment and exocytosis and are likely to operate at the level of voltage-gated ion channels that trigger glutamate release.

To monitor $GABA_A$ receptor-dependent changes in intraterminal $[Ca^{2+}]_i$ directly, we carried out on-line fluorescent assays using a Ca^{2+}-sensitive indicator Fura-2 (36). Although the application of increasing doses of muscimol caused no detectable changes in basal $[Ca^{2+}]_i$ (data not shown), the depolarization-dependent increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) from 155.1 ± 12.2 nM (mean ± S.E., $n = 5$) to 340.2 nM ± 39.1 nM (mean ± S.E., $n = 5$), under control conditions, was significantly attenuated at concentrations of muscimol higher than 50 μM (Fig. 3*H*). These data indicate that the ability of nerve terminals to undergo depolarization-dependent activation of Ca^{2+} channels is significantly attenuated by prior $GABA_A$ receptor activity.

$GABA_A$ Receptor-mediated Inhibition of Glutamate Release Requires NKCC1 Activity, Influx of Ca^{2+}, and Activation of Calmodulin—To dissect the signaling pathway(s) activated downstream of $GABA_A$ receptors in glutamatergic nerve terminals, we tested whether the observed regulation of release was sensitive to the inhibition of the inwardly directed Cl^- transporter, NKCC1, previously reported to maintain high intraterminal $[Cl^-]_i$ in some types of nerve terminals (49, 50). Synaptosomes were incubated with bumetanide (10 μM), a selective NKCC1 inhibitor, and glutamate release was triggered by the addition of 1 mM 4AP (Fig. 4*A*). Glutamate release of 26.1 ± 0.3 nmol/mg/5 min under control conditions was significantly decreased to 21.9 ± 0.3 nmol/mg/5 min (83.9% of control) by muscimol (200 μM). Glutamate release was also significantly reduced by bumetanide (10 μM) to 16.3 ± 1.8 nmol/mg/5 min (62.6% of control release). Crucially, muscimol-dependent inhibition of release was completely abolished in the presence of bumetanide (15.2 ± 1.0 nmol/mg/5 min; Fig. 4*A*), indicating an important role of presynaptic NKCC1 activity, and consequent high intraterminal $[Cl^-]_i$, in glutamate release and modulation thereof by presynaptic $GABA_A$ receptors.

With a high intraterminal $[Cl^-]_i$, presynaptic $GABA_A$ receptors would be predicted to depolarize glutamatergic nerve terminals and activate voltage-gated Ca^{2+} entry. Although this increase in Ca^{2+} entry may not be sufficient to trigger vesicle fusion, it may suffice to instigate the observed modulation of release by these $GABA_A$ receptors. We tested this hypothesis by excluding Ca^{2+} during the activation of $GABA_A$ receptors, *i.e.* by incubating synaptosomes with muscimol in the absence of

FIGURE 3. **Nerve terminal $GABA_A$ receptor activity inhibits stimulus (4AP)-evoked glutamate release and Ca^{2+}-influx.** *A* and *B,* 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence of increasing concentrations of $GABA_A$ receptor agonists (in *red*) muscimol (*M*, [*Musc*] (1–500 μM; $n = 6$) (*A*) and isoguvacine (*I*, [*Isog*] 1–500 μM; $n = 7$) (*B*). *Insets* show dose-response curves of decreases in 4AP-evoked glutamate release in the presence of agonists (% control 5 min after 4AP addition). *C,* 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence of muscimol (*M*, 200 μM, *red*) or muscimol and picrotoxin (*M/P*, 50 μM, *dark red*; and 100 μM, *brown*, $n = 4$). *Inset* quantifies occlusion of muscimol (*red*)-induced decrease of 4AP-evoked glutamate release (% control 5 min after 4AP addition) by picrotoxin (50 μM, *dark red*, and 100 μM, *brown*). *D,* 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence of muscimol (*M*, 200 μM, *red*) or muscimol and SR95531 (*M/SR*, 50 μM, dark red, $n = 4$). *Inset,* quantifies occlusion of muscimol (*red*)-induced decrease of 4AP-evoked glutamate release (% control 5 min after 4AP addition) by SR95531 (50 μM, *dark red*). *E,* 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence muscimol (*M*, 200 μM, *red*) or muscimol and strychnine (*M/Str*, 50 μM, *purple*, $n = 4$). *Inset* demonstrates the lack of an effect of strychnine (*purple*) on muscimol-induced decrease of 4AP-evoked glutamate release (% control 5 min after 4AP addition). *F,* muscimol (*M*, 200 μM, *red*)-induced inhibition of glutamate release triggered by 10 mM KCl ($n = 5$). *Inset* quantifies the reduction of KCl (10 mM)-evoked glutamate release by muscimol (*red*, % control 5 min after KCl addition). *G,* muscimol (200 μM, *red*) has no significant effect on ionomycin (5 μM)-induced glutamate release ($n = 4$). *Inset* quantifies the effect of muscimol (*red*) on ionomycin-induced glutamate release (% control 5 min after ionomycin addition). *A–G,* glutamate release (mean ± S.E., nmol/mg) values were calculated for every 2-s time point, with the cumulative release 5 min after secretagogue (4AP/KCl/ionomycin) addition used for statistical analysis. *, $p < 0.05$ (one-way ANOVA followed by post-hoc LSD test). *H,* muscimol-induced decrease in 4AP-evoked change in intraterminal Ca^{2+} concentration (ΔCa^{2+}, increase in 4AP-evoked intraterminal $[Ca^{2+}]$ in the presence of muscimol (10–500 μM) is presented as a percentage of an increase obtained with 4AP in the absence of muscimol). Data points show effects on Ca^{2+} influx obtained 5 min after the addition of 4AP and represent mean ± S.E. of five independent experiments. *, $p < 0.05$ (one-way ANOVA followed by post-hoc LSD test).

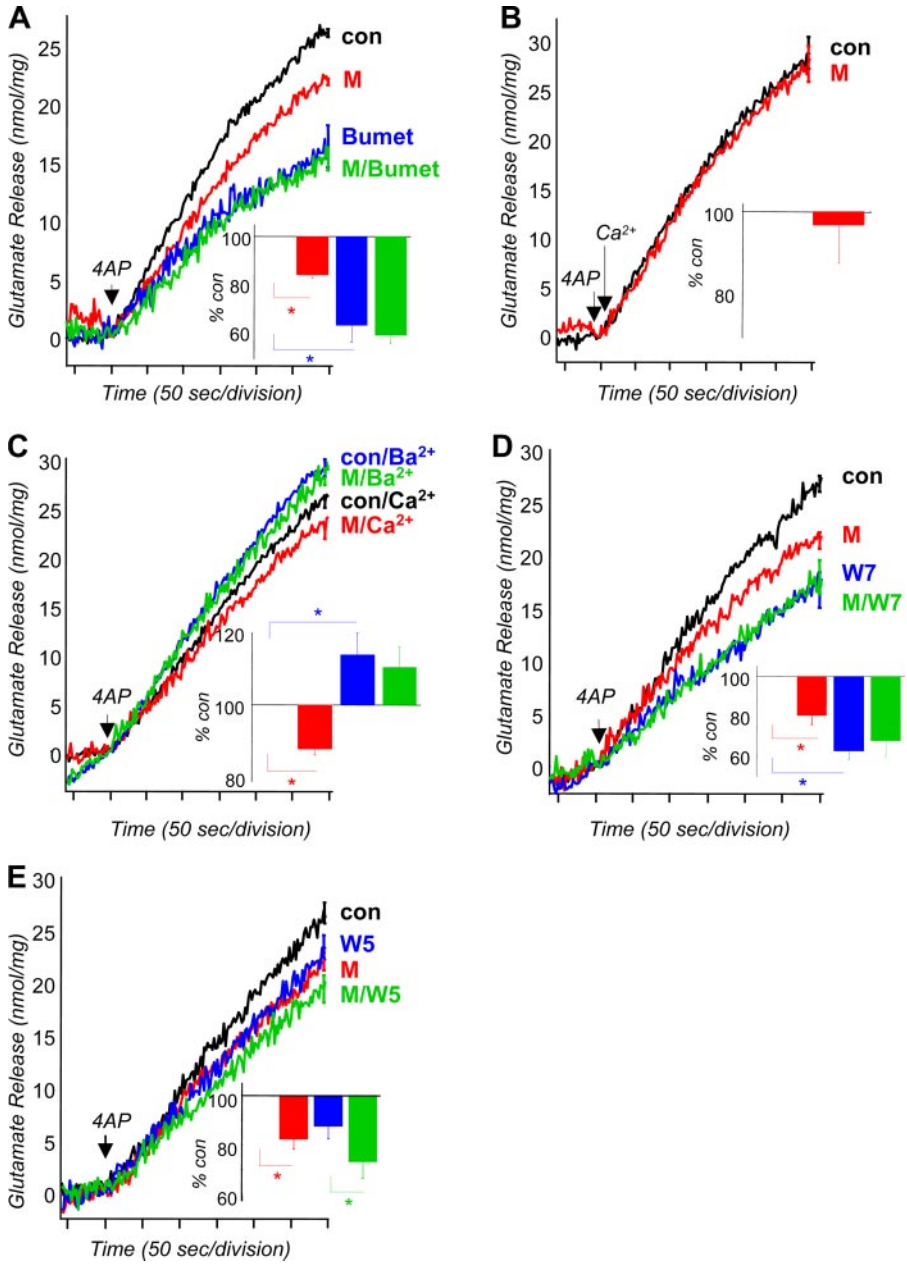


FIGURE 4. **GABA$_A$ receptor-mediated inhibition of glutamate release requires NKCC1 activity, Ca^{2+} influx prior to stimulation of release, and calmodulin activation.** *A,* 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence of 1 mM $CaCl_2$, and in the absence (*con*, *black*) or presence of muscimol (*M*, 200 μM, *red*), bumetanide (Bumet, 10 μM, *blue*), or both (*M/Bumet*, *green*). *Inset* compares decreases in 4AP-evoked glutamate release by muscimol (*red*), bumetanide (*blue*), or muscimol and bumetanide (*green*) as % control release 5 min after 4AP addition ($n = 4$). *B,* synaptosomes were incubated in the absence of Ca^{2+} and in the absence (*con*, *black*) or presence of muscimol (*M*, 200 μM, *red*). The release was triggered by the addition of 4AP (1 mM), immediately followed by the addition of $CaCl_2$ (1 mM). *Inset* compares 4AP-evoked glutamate release in the absence (control) or presence of muscimol (*red*, % control 5 min after the addition of 4AP) when Ca^{2+} is omitted during muscimol treatment ($n = 3$). *C,* 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence of $CaCl_2$ (1 mM) and in the absence (*con/*Ca^{2+}, *black*) or presence of muscimol (*M/*Ca^{2+}, *red*), or in the presence of $BaCl_2$ (1 mM) and in the absence (*con/*Ba^{2+}, *blue*) or presence of muscimol (*M/*Ba^{2+}, *green*). *Inset* compares changes in 4AP-evoked glutamate release (% control 5 min after 4AP addition) under the same conditions. Note the lack of the effect of muscimol in the presence of $BaCl_2$ ($n = 4$). *D,* 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence of 1 mM $CaCl_2$, and in the absence (*con*, *black*) or presence of muscimol (*M*, 200 μM, *red*), calmodulin inhibitor W7 (*W7*, 20 μM, *blue*), or both (*M/W7*, *green*). *Inset* compares decreases in 4AP-evoked glutamate release by muscimol (*red*), W7 (*blue*) or muscimol and W7 (*green*) as % control release 5 min after 4AP addition ($n = 4$). *E,* 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence of 1 mM $CaCl_2$, and in the absence (*con*, *black*) or presence of muscimol (*M*, 200 μM, *red*), W5 (*W5*, 20 μM, *blue*), or both (*M/W5*, *green*). *Inset* compares decreases in 4AP-evoked glutamate release by muscimol (*red*), W5 (*blue*), or muscimol and W5 (*green*) as % control release 5 min after 4AP addition ($n = 6$). Glutamate release (mean ± S.E., nmol/mg) values were calculated for every 2-s time point, with the cumulative release 5 min after secretagogue (4AP) addition being used for statistical analysis. *, $p < 0.05$ (one-way ANOVA with post-hoc LSD test).

extrasynaptic Ca^{2+} prior to triggering glutamate release by the addition of 1 mM 4AP (in the presence of 1 mM Ca^{2+}; Fig. 4*B*) (51). In these experiments, although glutamate release *per se* (29.1 ± 1.6 nmol/mg/5 min) was not altered, muscimol-dependent inhibition of release was completely abolished. The release measured with this protocol was 27.9 ± 1.8 nmol/mg/5 min, similar to levels obtained in the absence of muscimol (Fig. 4*B*). This indicates an obligate requirement for extrasynaptosomal Ca^{2+} during the GABA$_A$ receptor-mediated modulation phase. Evidently the modulatory pathway needs to be activated prior to depolarization with 4AP, given that the presence of muscimol and Ca^{2+} during depolarization as such (Fig. 4*B*) is insufficient to produce an effect. This also implies that the GABA$_A$ receptor-mediated inhibition of glutamate release is contingent upon the activation of a long lasting Ca^{2+}-dependent signaling mechanism.

We further investigated the signaling role of Ca^{2+} in the observed GABA$_A$ receptor-mediated inhibition of glutamate release by measuring the effects of muscimol in the presence of 1 mM $BaCl_2$ instead of $CaCl_2$. Ba^{2+} is an effective substitute for Ca^{2+} in triggering glutamate release (33) because it can enter nerve terminals through VGCCs (52, 53). However, Ba^{2+} does not bind calmodulin and is therefore ineffective in activating calmodulin-dependent signaling pathways (54). Glutamate release measured in the presence of Ba^{2+} (29.0 ± 0.9 nmol/mg/5 min) was comparable with the release measured in the presence of Ca^{2+} (25.6 ± 0.6 nmol/mg/5 min; Fig. 4*C*), although slightly increased. However, the decrease in glutamate release effected by muscimol (200 μM) seen in the presence of Ca^{2+} (22.5 ± 0.7 nmol/mg/5 min, 87.9% of control release) was abolished in the presence of Ba^{2+}, *i.e.* release in the presence of Ba^{2+}/muscimol (28.1 ± 0.8 nmol/mg/5 min) was indistinguishable from control

release in the presence of Ba^{2+} (Fig. 4*C*). These results point to a key role of Ca^{2+}-dependent activation of calmodulin downstream of presynaptic $GABA_A$ receptors.

To directly confirm that calmodulin is involved in $GABA_A$ receptor-dependent inhibition of glutamate release, we tested the effects of W7, a specific calmodulin inhibitor (55). Under control conditions, glutamate release triggered by 1 mM 4AP (26.8 ± 0.7 nmol/mg/5 min) was decreased to 21.4 ± 0.8 nmol/mg/5 min (79.9% of control) by muscimol (200 μM, Fig. 4*D*). In the presence of W7 (20 μM), glutamate release was potently inhibited to 16.7 ± 1.7 nmol/mg/5 min (62.3% of control release), but crucially, muscimol-induced inhibition of release was abolished (17.9 ± 1.6 nmol/mg/5 min, 66.7% of control; Fig. 4*D*). However, in the presence of W5, an analogue of W7 with ~10-fold lower affinity for calmodulin (56, 57), inhibition of the control glutamate release (26.8 ± 1.0 nmol/mg/5 min) by muscimol (200 μM, 21.9 ± 0.8 nmol/mg/5 min, 81.7% of control) was unaffected (19.3 ± 1.3 nmol/mg/5 min, 82.8% of W5 alone; Fig. 4*E*). W5 caused a small but statistically insignificant inhibition of glutamate release (23.3 ± 1.1 nmol/mg/5 min, 86.9% control; Fig. 4*E*). This comparison of the structurally similar W7 and W5 indicates that it is the more potent anti-calmodulin activity of the former that abrogates the $GABA_A$ receptor-mediated modulation of release. These results emphasize that Ca^{2+} mediates the observed $GABA_A$ receptor-dependent inhibition of glutamate release through the specific activation of calmodulin.

Specificity of VGCCs Involved in the GABA$_A$ Receptor-mediated Inhibition of Glutamate—Given that inhibition of glutamate release by presynaptic $GABA_A$ receptor activity requires an influx of Ca^{2+} from the extracellular milieu, we applied selective VGCC inhibitors to characterize the role of individual Ca^{2+} channel subtypes in this process. We started by investigating the role of N-, P-, and Q-type VGCCs, which are known to directly participate in triggering glutamate release from synaptosomes (58, 59). To assess the role of P/Q-type VGCCs specifically, synaptosomes were preincubated with 100 μM ω-agatoxin IVA (ω-Aga IVA) (60). Glutamate release evoked by 1 mM 4AP (25.6 ± 0.8 nmol/mg/5 min; Fig. 5*A*) was significantly decreased in the presence of ω-Aga IVA alone (17.2 ± 1.5 nmol/mg/5 min, 67.2% of control release, or muscimol alone (19.5 ± 1.5 nmol/mg/5 min, 76.2% of control release). However, in the presence of ω-Aga IVA, glutamate release was further inhibited by muscimol (12.1 ± 1.6 nmol/mg/5 min, 70.3% of release in the presence of ω-Aga IVA alone; Fig. 5*A*). The additive relationship between ω-Aga IVA and muscimol indicates that, although P/Q-type VGCCs contribute significantly to 4AP-evoked glutamate release, they appear not to mediate the modulatory effects of presynaptic $GABA_A$ receptors on glutamate release.

To block N-type VGCCs specifically, synaptosomes were preincubated with 10 μM ω-conotoxin GVIA (ω-CTx GVIA) (61). Glutamate release evoked by 1 mM 4AP (25.6 ± 0.9 nmol/mg/5 min; Fig. 5*B*) under control conditions was significantly decreased in the presence of ω-CTx GVIA alone (21.4 ± 1.3 nmol/mg/5 min, 84% of control) or muscimol alone (21.0 ± 1.9 nmol/mg/5 min, 82.1% of control). Crucially, in the presence of ω-CTx GVIA, addition of muscimol caused a further inhibition

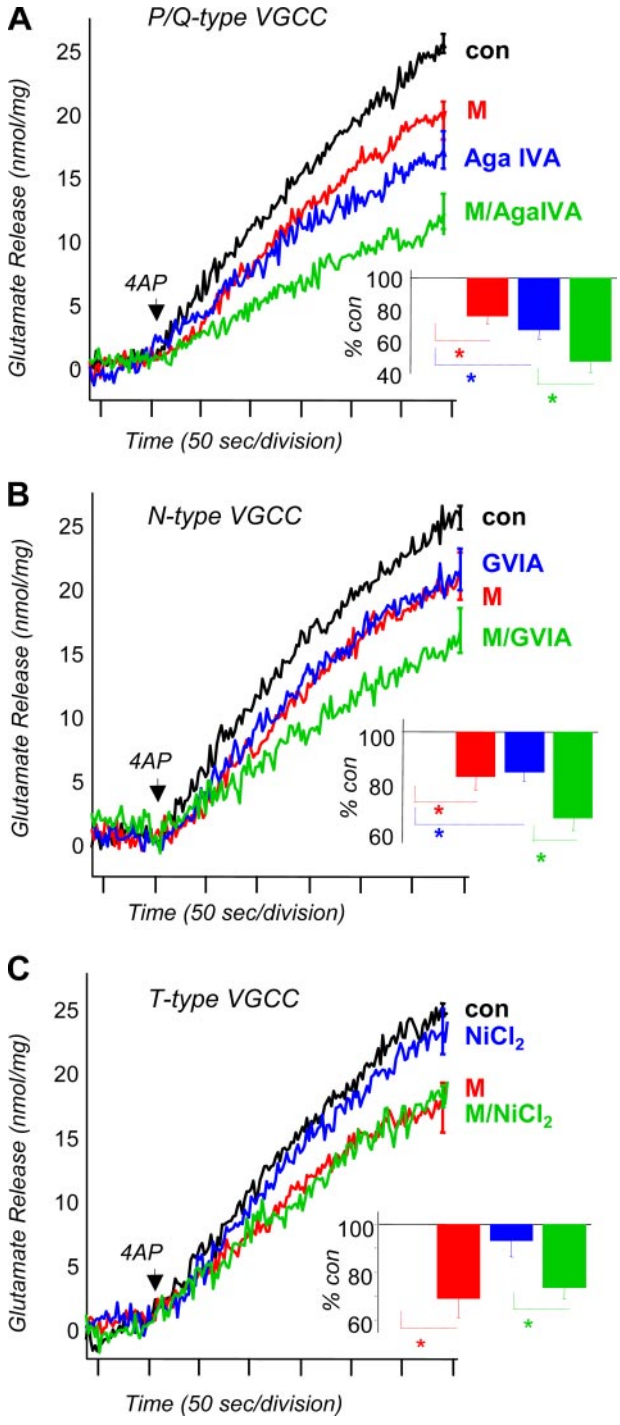


FIGURE 5. **GABA$_A$ receptor-mediated inhibition of 4AP-evoked glutamate release in the presence of P/Q-, N-, or T-type VGCC blockade.** 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence of 1 mM $CaCl_2$, and in the absence (*black*) or presence of ω-agatoxin IVA (100 μM, *blue*), muscimol (200 μM, *red*), or both (*green*, $n = 7$) (*A*); ω-conotoxin GVIA (10 μM, *blue*), muscimol (200 μM, *red*), or both (*green*, $n = 4$) (*B*); or $NiCl_2$ (50 μM, in *blue*), muscimol (200 μM, *red*), or both (*green*, $n = 5$) (*C*). *Insets* compare the effects of P/Q-, N-, or T-type VGCC blockade (*A–C*, respectively) on 4AP-evoked glutamate release (in *blue*), or the inhibition thereof by muscimol (in *green*), as % control release 5 min after the addition of 4AP. Glutamate release (mean ± S.E., nmol/mg) values were calculated for every 2-s time point, with the cumulative release 5 min after secretagogue (4AP) addition being used for statistical analysis. *, $p < 0.05$ (one-way ANOVA with post-hoc LSD test).

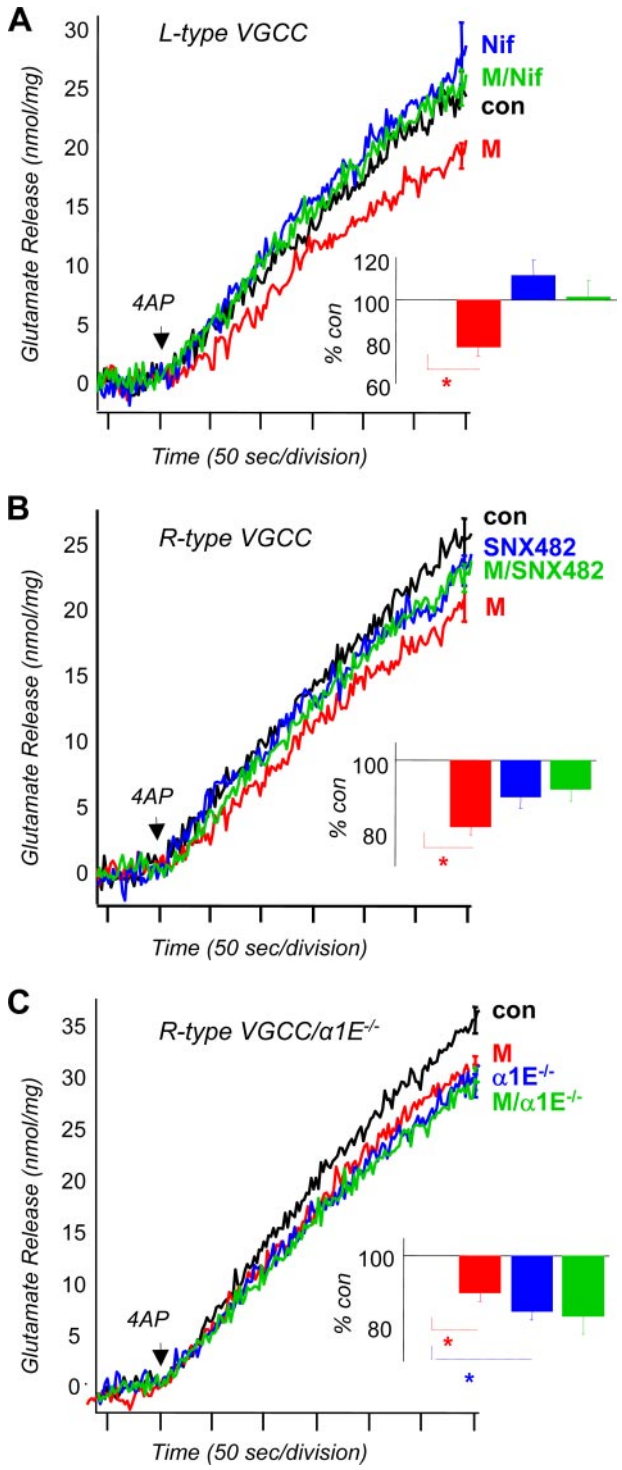


FIGURE 6. **GABA$_A$ receptor-mediated inhibition of 4AP-evoked glutamate release is abolished by L- or R-type VGCCs blockade.** 4AP (1 mM)-evoked glutamate release from synaptosomes incubated in the presence of 1 mM $CaCl_2$ and in the absence (*black*) or presence of nifedipine (*Nif*) (1 μM, *blue*), muscimol (*M*) (200 μM, *red*), or both (*M/Nif*) (*green*, $n = 4$) (*A*); SNX-482 (100 nM, *blue*), muscimol (200 μM, *red*), or both (*green*, $n = 6$) (*B*). *C*, muscimol-induced inhibition of 4AP-evoked glutamate release in R-type VGCC knock-out mice. Synaptosomes from wild-type (control, *con*, *black*, and muscimol, *M*, *red*) and R-type VGCC knock-out mice (control, $\alpha 1E^{-/-}$, *blue*, and muscimol, $M/\alpha 1E^{-/-}$, *green*) were incubated in the presence of 1 mM $CaCl_2$, and in the absence (*con*, $\alpha 1E^{-/-}$) or presence of muscimol (200 μM, *M*, $M/\alpha 1E^{-/-}$) prior to the addition of 1 mM 4AP ($n = 5$). *Insets, A–C,* respectively, compare effects of nifedipine, SNX-482, or genetic ablation of the α1E-subunit supporting R-type VGCC activity, on 4AP-evoked glutamate release (*blue*), or the inhibition thereof by muscimol (*green*), as % control release 5 min after the addition of 4AP. Glutamate release (mean ± S.E., nmol/mg) values were calculated for every 2-s time point, with the cumulative release 5 min after secretagogue (4AP) addition being used for statistical analysis. *, $p < 0.05$ (one-way ANOVA with post-hoc LSD test).

of 4AP-evoked glutamate (17.1 ± 1.4 nmol/mg/5 min, 67.3% of release in the presence of ω-CTx GVIA alone; Fig. 5*B*), indicating that the modulatory influence of GABA$_A$ receptor-mediated inhibition of glutamate release is independent of N-type Ca^{2+} channel activity, even though, similarly to P/Q type VGCCs, these channels are directly involved in mediating neurotransmitter release.

A potential role of T-type VGCCs in GABA$_A$ receptor-mediated modulation was tested in the presence of $NiCl_2$, which selectively blocks these channels at the 50 μM concentration used in our experiments (62, 63). Glutamate release evoked by 1 mM 4AP (24.5 ± 0.5 nmol/mg/5 min) under control conditions was unaffected by $NiCl_2$ (22.3 ± 1.8 nmol/mg/5 min) indicating that T-type VGCCs do not directly support glutamate release from synaptosomes (Fig. 5*C*). Muscimol (200 μM) caused a significant inhibition of glutamate release (18.7 ± 1.9 nmol/mg/5 min, 76.3% of control release) as shown earlier, but notably the modulation of glutamate release by GABA$_A$ receptor activation was unaffected by the presence of $NiCl_2$ (18.1 ± 0.9 nmol/mg/5 min, 81.2% of $NiCl_2$ release; Fig. 5*C*), indicating that T-type VGCCs, similar to P/Q- and N-type VGCCs, are not involved in the observed modulation of glutamate release by presynaptic GABA$_A$ receptors.

Next, the effect of inhibition of L-type VGCCs on the observed muscimol-dependent inhibition of release was investigated in the presence of 1 μM nifedipine. Glutamate release triggered by 1 mM 4AP (24.9 ± 1.5 nmol/mg/5 min) under control conditions was comparable with that obtained in the presence of nifedipine (27.9 ± 2.6 nmol/mg/5 min; Fig. 6*A*). Importantly, although control release was significantly inhibited by muscimol alone (200 μM, 19.2 ± 1.1 nmol/mg/5 min, 77.1% of control), this effect of GABA$_A$ receptor activation was abrogated in the presence of nifedipine, with release in presence of nifedipine and muscimol (25.0 ± 1.5 nmol/mg/5 min; Fig. 6*A*) being comparable with that in the presence of nifedipine alone. Thus, although presynaptic L-type VGCCs are not directly involved in triggering glutamate release (58, 59), their activity appears critical for GABA$_A$ receptor-mediated inhibition of glutamate release.

Finally, the contribution of R-type VGCCs activity to muscimol-dependent inhibition of glutamate release was investigated using SNX-482 (100 nM), a toxin shown to specifically block α1E-subunit containing VGCCs at the concentration used in our assays (64). Control glutamate release evoked by 4AP (25.2 ± 1.7 nmol/mg/5 min) was reduced in the presence of SNX-482 (22.3 ± 1.1 nmol/mg/5 min, 88.5% of control release), indicating that R-type VGCCs directly contribute to release of glutamate from synaptosomes. Although 4AP-evoked glutamate release was significantly reduced (20.4 ± 1.4 nmol/mg/5 min, 81% of control release) in the presence of muscimol alone (200 μM), this modulation was abolished by the addition of SNX-482, with the release measured in the presence

of SNX-482 and muscimol (22.9 ± 1.1 nmol/mg/5 min) being similar to that obtained in the presence of SNX-482 alone.

To authenticate the role of R-type VGCCs in the observed modulation of glutamate release by muscimol, we carried out experiments utilizing synaptosomes prepared from the neocortex of α1E subunit knock-out (α1E$^{-/-}$) mice lacking the R-type VGCC activity (31). In wild-type mice, glutamate release evoked by 1 mM 4AP under control conditions (35.5 ± 1.3 nmol/mg/5 min) was significantly reduced by muscimol (200 μM, 31.6 ± 0.4 nmol/mg/5 min, 89% of control release in wild-type mice; Fig. 6*C*). Glutamate release triggered by 1 mM 4AP was also significantly reduced in R-type knock-out mice (29.8 ± 1.1 nmol/mg/5 min, 83.9% of the release in wild-type mice). However, the inhibition of release by muscimol observed in wild-type synaptosomes was completely abolished in synaptosomes isolated from R-type VGCC knock-out mice, with the release measured in the presence of muscimol (29.1 ± 1.3 nmol/mg/5 min) being similar to that in the absence of muscimol (Fig. 6*C*). Together, these experiments demonstrate a key role of R-type VGCCs as mediators of the presynaptic GABA$_A$ receptor-mediated regulation of glutamate release.

DISCUSSION

The data presented here reveal the presence and examine the function of presynaptic GABA$_A$ receptors in a population of glutamatergic nerve terminals in the adult rat neocortex. We demonstrate that nerve terminal GABA$_A$ receptors activate presynaptic Ca^{2+}/calmodulin-dependent signaling to inhibit depolarization (4AP)-evoked Ca^{2+} influx and glutamate release from isolated nerve terminals. This inhibitory action is dependent on the activity of the NKCC1 transporter, Ca^{2+} and calmodulin, and the activity of L- and/or R-type VGCCs. Although both L- and/or R-type VGCC appear essential for modulation, they evidently play distinctive roles in this process given that, of the two, only R-type channels contribute directly to the release of glutamate.

We initially defined the presynaptic effects of GABA$_A$ receptors using Ca^{2+}/calmodulin-dependent phosphorylation of synapsin I, as a direct and sensitive biochemical readout of alterations in intraterminal $[Ca^{2+}]_i$ (43, 65). The synapsin family of small synaptic vesicle-associated proteins is ubiquitous throughout the central nervous system but is exclusively localized to presynaptic nerve terminals (66). With this definitive presynaptic marker, we demonstrate that GABA$_A$ receptor activity increases basal intraterminal $[Ca^{2+}]_i$, as reflected by increased CaMKII-dependent phosphorylation of synapsin I, whereas their inhibition produces a decrease in this parameter. Moreover, our immunohistochemical analysis to delineate this signaling revealed its exclusive localization to a subpopulation of glutamatergic terminals and its notable absence from GABAergic terminals, the latter finding being in agreement with the absence of Ca^{2+}/calmodulin-dependent kinases and phosphatases at GABAergic synapses (67, 68). Crucially, this analysis evinces GABA$_A$ receptors as modulatory heteroreceptors on ~20% of glutamatergic terminals in the rat neocortex.

The observed changes in intraterminal $[Ca^{2+}]_i$ suggest that nerve terminal GABA$_A$ receptors effect presynaptic depolarization rather than the hyperpolarization that is typical of postsynaptically localized receptors (1). The functional outcome of GABA$_A$ receptor activation is predominantly determined by the Cl^- electrochemical gradient established across the plasma membrane. This is in turn regulated by the activity of cation/chloride cotransporters such as KCC2, which extrudes Cl^-, and/or NKCC1, which transports Cl^- into the cytosol (1). Whereas KCC2 is absent from presynaptic nerve terminals (69), NKCC1 has been implicated in the regulation of intraterminal $[Cl^-]$ (49, 50), to achieve the high levels of 20–22 mM (*cf.* somatodendritic compartment), directly measured in the calyx of Held (70), pituitary terminals (71), and retinal bipolar cell terminals (72). In experiments reported herein, the inhibition of glutamate release by GABA$_A$ receptor agonists was highly sensitive to blockade of the cation/chloride cotransporter NKCC1. Together, the data suggest that high intraterminal $[Cl^-]_i$, leading to the depolarization of nerve terminals by GABA$_A$ receptors, is of critical importance for the regulation of glutamate release by these receptors.

Whereas depolarizing effects of presynaptic GABA$_A$ receptors have been supported by electrophysiological evidence (1, 9), the final functional outcome of their activity at the level of neurotransmitter release has been contentious given the conflicting reports indicating both stimulation and inhibition of release being mediated by GABA$_A$ receptors (9). As described here, inhibition of the evoked release by nerve terminal GABA$_A$ receptors has also been observed in the spinal cord (73), frontal cortex (74), hippocampus (20, 75), suprachiasmatic nucleus (76), ventromedial hypothalamus (49), posterior pituitary (71, 77), etc. Conversely, stimulation of spontaneous and evoked release of neurotransmitter by presynaptic GABA$_A$ receptors has been described in the cerebellum (18, 19), hippocampus (21–25), auditory brainstem (27), locus ceruleus (50), ventral tegmental area (28), etc. Typically, facilitation is largely observed in tissue from young animals. In addition to factors such as the age of animals, it is apparent that the type of nerve terminal or the central nervous system region determines the final functional outcome of GABA$_A$ receptor activity. This would be determined by the repertoire of presynaptically expressed GABA$_A$ receptor subtypes, as well as the operative signaling pathways and their targets (including voltage-gated ion channels such as VGCCs modulated to regulate basal as well as evoked intraterminal $[Ca^{2+}]_i$; see our biochemical experiments and Ref. 26).

We demonstrate that an increase in basal intraterminal $[Ca^{2+}]_i$ resulting from GABA$_A$ receptor activity is an obligate requirement for subsequent inhibition of depolarization (4AP)-evoked Ca^{2+} influx and glutamate release, *i.e.* the modulation of release is absent in the absence of extracellular Ca^{2+} or in the presence of extracellular Ba^{2+}. Interestingly, when ionomycin was used as the secretagogue after GABA$_A$ receptor activation, no modulation was observed. These data indicate that GABA$_A$ receptor- and Ca^{2+}-dependent regulation of VGCC activity underpins the subsequent regulation of excitation-secretion coupling.

We elucidated the VGCC subtype(s) involved in the GABA$_A$ receptor invoked regulatory pathway by monitoring its sensitivity to VGCC subtype-specific antagonists added during the activation of GABA$_A$ receptors. Although blockade of P/Q-type

(by ω-Aga IVA) or N-type (by ω-CTxGVIA) VGCCs reduces 4AP-evoked release substantially, conspicuously, the residual release in each case is still inhibited by $GABA_A$ receptor activation with muscimol. T-type VGCC block (by $NiCl_2$) had no effect on evoked release or the inhibition thereof by prior muscimol treatment.

In contrast, L-type VGCC block by nifedipine abolished the inhibition of glutamate release produced by muscimol (note nifedipine has no effect on glutamate release in control conditions). Although L-type VGCCs are mainly located at the soma and dendrites (78), Ca^{2+} influx through these channels during repetitive stimulation has been implicated in regulation of GABA release from cultured GABAergic neurons, presumably through a presynaptic localization and operation (79). More directly, L-type VGCCs also appear to be present in mossy fiber terminals where they mediate large Ca^{2+} currents (80). It is therefore feasible that depolarizing action of nerve terminal $GABA_A$ receptors initiates a long lasting Ca^{2+}-dependent regulatory mechanism following L-type VGCC activation.

Finally, we examined the role of R-type VGCCs in the observed regulation using both pharmacological blockade by SNX-482, and by the genetic ablation of the α1E channel subunit in mice (31). In both these models, the inhibition of glutamate release by muscimol was abolished. Thus, together with L-type VGCCs, R-type VGCCs are implicated in the modulation of release produced by presynaptic $GABA_A$ receptor activation.

A key question arising is: what is the Ca^{2+}-dependent mechanism invoked by presynaptic $GABA_A$ receptors to inhibit glutamate release? Our observations that $GABA_A$ receptor-dependent inhibition of release is ablated by the inhibitor W7, but unaffected by its inactive analogue W5, clearly implicates calmodulin as a modulatory player. The effects of Ca^{2+}/calmodulin on the observed regulation could operate at any number of loci in the release cascade. Judging from the lack of $GABA_A$ receptor-mediated regulation of release when ionomycin is used as the secretagogue, involvement of targets downstream of Ca^{2+} entry is obviated. This leaves potential regulation at the level of Ca^{2+} entry or upstream at loci controlling nerve terminal excitability.

Direct inhibitory action of $GABA_A$ receptor-mediated Ca^{2+} entry on VGCCs themselves is a tenable possibility given that all the high threshold VGCCs have been shown to be subject to Ca^{2+}/calmodulin-dependent inactivation (CDI) (81–85). If VGCCs are indeed direct targets for $GABA_A$ receptor-mediated inhibition, R-type VGCCs surface as the only feasible targets given our data showing that P/Q- and N-type VGCC blockade is of no consequence to the regulation seen, and L-type VGCCs do not support release. These observations not only support R-type VGCCs as key players in the regulation seen, but they obviate the possibility that $GABA_A$ receptor-dependent regulation impinges upstream at the level of channels regulating nerve terminal excitability, as such an action would then be predicted to equally affect all release-supporting VGCCs.

There are increasing examples of R-type VGCCs supporting neurotransmitter release in the calyx of Held (86), glutamatergic transmission in the rat hippocampus (87) and in oxytocin secretion in neurohypophysial terminals (88). Our data with α1E-subunit knock-out mice now show that there is significant support of glutamate release by these VGCCs. The question as to whether direct CDI of R-type VGCCs underlies the $GABA_A$ receptor-mediated inhibition is more open. This is because CDI is generally short lived, yet the regulation of release by muscimol is long lasting because it evidently occurs/persists for some time after initiation of Ca^{2+}/calmodulin-dependent signaling. Moreover, CDI *per se* would also invoke P/Q- and N-type VGCCs in the regulation, which we observe clearly not to be the case. An alternative possibility is that the Ca^{2+}/calmodulin-dependent regulation impinges on R-type VGCC activity indirectly, by mediating activation of a phosphorylation or dephosphorylation cascade. Interestingly in this regard, in the dendritic spines of the CA1 pyramidal cells, L-type VGCC-mediated elevations of Ca^{2+} are suggested to activate CaMKII (and/or Ca^{2+}-dependent adenylyl cyclase), which then leads to a relatively long lived depression of R-type VGCCs (89). Similarly, in GABAergic terminals projecting to Meynert neurones, L- and R-type VGCCs collaborate in mediating spontaneous and evoked neurotransmitter release, respectively (90). A tenable hypothesis arising from the present data would be that $GABA_A$ receptor-mediated depolarization leads to presynaptic L-type VGCC-mediated activation of Ca^{2+}/calmodulin-dependent signaling, which subsequently effects depression of Ca^{2+} entry through R-type VGCCs during the release of glutamate. This hypothesis is consistent with the abolition of $GABA_A$ receptor-mediated inhibition of glutamate release following the pharmacological block of L- or R-type VGCCs, or deletion of the α1E-subunit underpinning R-type Ca^{2+} conductance.

Together, our results demonstrate that nerve terminal $GABA_A$ receptors play an important and specific role in the regulation of glutamate release from a population of glutamatergic nerve terminals in the neocortex. Thus $GABA_A$ receptors may instigate a powerful activity-dependent presynaptic control of excitatory synapses, and as such, they represent a novel functional target for a plethora of GABAergic pharmacological agents.

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