GABA$_B$-mediated inhibition of multiple modes of glutamate release in the nucleus of the solitary tract

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Increasing evidence suggests that a single neurotransmitter may be released through multiple modes sourced from distinct vesicle pools and can interact with different postsynaptic effectors (Atasoy et al. 2008; Sara et al. 2005, 2011; Wasser and Kavalali 2009). Different modes of release may depend on unique calcium-sensing proteins (Kochubey et al. 2011) and colocalization within microdomains with particular voltage-activated calcium channels (VACCs) (Chapman 2008; Sun et al. 2007) and/or with different vesicle populations (Atluri and Yang 1995; Wang et al. 2010), GABA$_B$-mediated inhibition of multiple modes of glutamate release. In the solitary tract (STS) nucleus, primary sensory afferents fall into two broad classes based on the expression of transient receptor potential vanilloid type 1 (TRPV1) receptors. Both afferent classes (TRPV1+/−) have indistinguishable glutamate release mechanisms for ST-evoked excitatory postsynaptic currents (EPSCs). However, TRPV1+ terminals release additional glutamate from a unique, TRPV1-operated vesicle pool that is temperature sensitive and facilitated by ST activity to generate asynchronous EPSCs. This study tested whether presynaptic γ-aminobutyric acid (GABA$_B$) receptors inhibit both the evoked and TRPV1-operated release mechanisms on second-order ST nucleus neurons. In horizontal slices, shocks activated single ST axons and evoked the time-invariant (latency jitter <200 μs), glutamatergic EPSCs, which identified second-order neurons. Gabazine eliminated GABA$_B$ responses in all recordings. The GABA$_B$ agonist baclofen inhibited the amplitude of ST-EPSCs from both TRPV1+ and TRPV1− afferents with a similar EC$_{50}$ (~1.2 μM). In TTX, GABA$_B$ activation decreased miniature EPSC (mEPSC) rates but not amplitudes, suggesting presynaptic actions downstream from terminal excitability. With calcium entry through voltage-activated calcium channels blocked by cadmium, baclofen reduced mEPSC frequency, indicating that GABA$_B$ reduced vesicle release by activated calcium channels (VACCs) (Chapman 2008; Sun et al. 2007; Atluri and Yang 1995; Wang et al. 2010). Recently, an endogenous, TRPV1-operated mode of glutamate release was identified in the ST nucleus (NTS) (Peters et al. 2010). Action potentials evoked tightly synchronized excitatory postsynaptic currents (EPSCs) that depended on a readily releasable pool, which was quite similar for both TRPV1+ and TRPV1− ST afferents (Andresen and Peters 2008; Bailey et al. 2006b; Peters et al. 2008, 2010). However, TRPV1+ ST afferents generated glutamatergic EPSCs from an additional, unique vesicle pool controlled by TRPV1 receptors. This TRPV1-operated pool contributes to an elevated basal frequency of spontaneous EPSCs (sEPSCs), is highly sensitive to temperature, and generates asynchronous volleys of EPSCs following ST activation (Peters et al. 2010; Shoudai et al. 2010). These multiple modes of glutamate release raise the fundamental question of whether they may be modulated independently.

Neurotransmitter release strongly depends on a cascade of events linking action potential invasion of the synaptic terminal to calcium influx, which triggers vesicle fusion (Meir et al. 1999; Neher and Sakaba 2008; Schneggenburger and Neher 2005). Neurmodulators often target the terminal depolarization process, including potassium and calcium channels, and influence presynaptic plasticity in the NTS, as well as other brain regions. G protein-coupled receptors (GPCRs) represent a broad and important class of signal transduction molecules that can transform synaptic transmission. γ-Aminobutyric acid (GABA$_B$) is a prototypical inhibitory GPCR with respect to targeting both terminal potassium and calcium channels, as well as vesicular release proteins (Brown and Sihra 2008; Maximov and Sudhof 2005; Yoon et al. 2007). GABA, the major inhibitory transmitter in the NTS (Andresen and Yang 1995), is synthesized in neurons distributed throughout NTS (Bailey et al. 2008). While ST afferent activation commonly triggers later, likely disynaptic, GABA$_B$-mediated inhibitory postsynaptic currents (IPSCs) in caudal NTS (Andresen and Yang 1995; Wang et al. 2010), GABA$_B$ metabotropic receptor-mediated responses are more varied (Brooks and Glaum 1995; Ruggeri et al. 1996; Zhang and Mifflin 1998). Heterogeneity in responses to GABA$_B$ activation may arise from differences in receptor expression or coupling to its various targets (Bowery 2010; Brown and Sihra 2008; Fernandes et al. 2011).

The two ST phenotypes distributed in caudal NTS generally correspond to myelinated A-fiber (TRPV1−) and unmyelinated C-fiber (TRPV1+) afferent sensory neurons (Jin et al. 2004b). Myelinated and unmyelinated (C-fiber) cranial visceral afferents have substantially different physiological discharge patterns and reflex functions (Coleridge and Coleridge 1984;
GABA<sub>B</sub> INHIBITS MULTIPLE MODES OF GLUTAMATE RELEASE

Fan et al. 1999; Schlid et al. 1994). C-fibers dominate the total population of ST afferents (e.g., 90% in baroreceptors) (Andresen et al. 1978; Kubin and Davies 1995), and this is likely preserved at their central synapses. Although peptide receptors are often discretely distributed to subsets of ST terminals (Bailey et al. 2006b; Barnes et al. 2003; Peters et al. 2008), the distribution of GABA<sub>B</sub> receptors is not known. Here, we investigate the role of GABA<sub>B</sub> activation on ST-NTS neurotransmission and test the hypothesis that different modes of afferent glutamate release can be inhibited selectively. Our results demonstrate that GABA<sub>B</sub> depressed all modes of glutamate release across TRPV1+ and TRPV1− afferent types. This included inhibition of TRPV1-operated thermal release under conditions when voltage-dependent coupling and VACC pathways were blocked. Thus GABA<sub>B</sub> coordinately depressed multiple modes of glutamate release across ST afferent subtypes. The findings have broad implications for activity-dependent and -independent afferent transmission in the caudal NTS.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Oregon Health and Science University approved all methods, and it conforms to the guidelines of the National Institutes of Health publication, “Guide for the Care and Use of Laboratory Animals”. Male Sprague-Dawley rats (n = 43, 150–200 g) were purchased from Charles Rivers Laboratory (Wilmington, MA) and housed under a 12-h/12-h light/ dark cycle. Under deep isoflurane anesthesia (5% in air), hindbrain slices were prepared as described previously (Doyle and Andresen 2001). Briefly, the brainstem was tilted so that horizontal slices (250-μm thick) contained 1–3 mm segments of the ST in the same plane as the cell bodies in the caudal NTS, using a vibrating microtome (Leica VT1000 S, Leica Microsystems, Bannockburn, IL) with a sapphire blade (Delaware Diamond Knives, Wilmington, DE). Slices were submerged in a perfusion chamber, and all recordings were performed in an artificial cerebrospinal fluid (ACSF) composed of (mM): 125 NaCl, 3 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 10 glucose, and 2 CaCl<sub>2</sub>, bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The chamber was perfused continuously (1.5–2 ml/min) with ACSF (32–34°C, pH 7.4). Drugs were added to the ACSF and perfused for at least 5 min. Bath temperature was controlled within 1°C using an inline heating system (TC2BIP with HPRE2 and TH-10Km bath probe, Cell Micro Instruments, Union City, CA). Synaptic currents were measured at 1322A analog to digital converter, and pClamp 9.2 software (Axon Instruments, Union City, CA). Synaptic currents were measured at a holding voltage of −60 mV, and all glutamatergic-mediated currents were inward. Liquid junction potentials were not corrected. In all experiments, the GABA<sub>B</sub> receptor antagonist gabazine (SR-95531, 3 μM) was present to isolate EPSCs. The GABA<sub>B</sub> receptor antagonist CGP 52432 (5 μM) was used in select experiments. Spontaneously released glutamate in the absence of action potentials was measured as miniature EPSCs (mEPSCs) using pharmacological isolation in TTX (1 μM). VACCs were nonselectively blocked by adding 100 μM CdCl<sub>2</sub> to the normal bath solutions. Drugs were purchased from Tocris (Ellisville, MO). Thermally evoked increases in mEPSC frequency were tested using small step changes in bath temperature imposed by command steps to the electronic inline heating system.

*ST afferent-activated EPSCs.* A concentric bipolar-stimulating electrode (200 μm outer-tip diameter; Frederick Haer, Bowdoinham, ME) was placed on the ST >2 mm from the recorded neuron, and constant current shocks (thresholds generally between 0.02 and 0.1 mA) were delivered in short bursts (five stimuli at 50 Hz every 6 s, 100 μs duration) using a Master-8 isolated programmable stimulator (A.M.P.I., Jerusalem, Israel). The threshold for activation of ST afferents was tested using selective recruitment and identification protocols for TRPV1+− and ST-activated EPSCs. Initially, the presence of asynchronous EPSCs, following bursts of ST shocks, was considered indicative of TRPV1+ classification, and tests with 100–200 nM capsaicin identified sensitive (TRPV1+) or insensitive (TRPV1−) afferents, as described previously (Bailey et al. 2006a; Doyle and Andresen 2001; Peters et al. 2010). The latency (time from stimulus artifact until onset of EPSC) and jitter (SD of latency) for the first ST-activated EPSC in each burst were measured across ≥30 ST shocks. Low-jitter (<200 μs) responses were classified as monosynaptic (i.e., second-order neurons). Neurons receiving only high-jitter responses were considered polysynaptic to the ST (i.e., higher-order NTS neurons) and not studied further. All monosynaptic ST-activated EPSCs had abrupt, all-or-none threshold profiles to gradual increases in shock intensity, and this identified the input as arising from a single afferent axon. This test was performed at the beginning of every experiment, and only identified second-order neurons were included in this study.

Synchronous, asynchronous, and autonomous synaptic events. For comparisons, five shocks were delivered at 50 Hz every 6 s, with activity recorded during the entire 6-s interval, and then averaged over 20 successive trials (2 min). To measure synchronous release, we analyzed the amplitudes of the first evoked EPSC in the train of five shocks. Since ST-EPSC amplitudes are highly variable across neurons, the drug responses were normalized to the control response value within neurons and expressed as a percent relative change. Aggregate comparisons of the percent of control across neurons were then expressed as mean ± SEM. Basal release rates were measured as the rate of sEPSCs occurring in the 1 s preceding ST activation and collected over a minimum of 20 trials. Asynchronous glutamate release rates were measured as the rate of sEPSCs occurring in the 1 s following the final ST shock in the train of five (over a minimum of 20 trials) and expressed as the net frequency change above the basal rate for that period (i.e., poststimulus rate – the prestimulus rate). Similar to synchronous release, asynchronous and basal sEPSC rates varied greatly across neurons, so that for some aggregate comparisons, individual rates were normalized by dividing by the rate during the control condition. For statistical comparisons, data were tested for normal distributions, and the appropriate parametric or nonparametric statistics were used, including ANOVA and the Student-Newman-Keuls method for post hoc analysis. Statistically significant differences were determined when P < 0.05. The frequency and amplitudes of mEPSCs for individual tests were collected over 2-min periods using MiniAnalysis (Synaptosoft, Decatur, GA) and compared using the Kolmogorov-Smirnov test. Synchronous events >10 pA were detected (threshold and time-course fitting), counted, and analyzed to generate cumulative histograms for comparison. Group data were compared using t-tests (two-group comparisons) or one-way ANOVA with Student-Newman-Keuls post hoc testing when comparing more than two groups.

RESULTS

Two classes of ST-EPSCs differ by asynchronous release. Bursts of five ST shocks activated fixed latency, highly synchronous EPSCs that identified each of these caudal NTS neurons as second order (Figs. 1A and 2A). This report is limited to these second-order neurons in the medial subnu-
**GABA\textsubscript{B} activation inhibits action potential-synchronized and basal glutamate release.** In all second-order NTS neurons, pharmacological activation of GABA\textsubscript{B} receptors, with the high-affinity agonist baclofen, uniformly reduced the amplitudes and increased synaptic failures of ST-EPSCs, regardless of TRPV1 subtype, with aggregate concentration-response relations that overlapped (Fig. 3A). The baclofen concentration-response profiles for TRPV1\textsuperscript{−} neurons were indistinguishable from that for TRPV1\textsuperscript{+} neurons with a combined EC\textsubscript{50} of 1.2 μM (Fig. 3A), a value similar to other pharmacological reports (Dittman and Regehr 1996). In the absence of ST shocks, basal release was substantially greater for TRPV1\textsuperscript{+} than for TRPV1\textsuperscript{−} neurons. Basal spontaneous release onto TRPV1\textsuperscript{+} neurons (11.0 ± 1.8 Hz, n = 18) averaged 10-fold higher (P < 0.001, t-test) than in TRPV1\textsuperscript{−} neurons (1.1 ± 0.2 Hz, n = 9). Baclofen produced substantially steeper concentration-dependent declines in sEPSC rates of TRPV1\textsuperscript{+} neurons than TRPV1\textsuperscript{−} neurons (Fig. 3B). A high concentration of baclofen (10 μM) increased the failure rate similarly (P = 0.8, t-test) from no failures to 52 ± 15% failures in TRPV1\textsuperscript{+} (n = 6) and 44 ± 23% failures in TRPV1\textsuperscript{−} (n = 5). Selective GABA\textsubscript{A\textsubscript{L}} or combined GABA\textsubscript{A\textsubscript{L}}-GABA\textsubscript{B} blockade (data not shown) had no effect on synchronous EPSC amplitude (n = 3, P = 0.6 and 0.3, respectively, one-way repeated measures ANOVA) or basal/asynchronous sEPSC frequency (P = 0.4 for both, one-
way repeated measures ANOVA), indicating that neither class of GABA receptors constitutively controlled glutamate release in our conditions. As expected, the GABA<sub>B</sub> receptor antagonist, CGP 52432, prevented baclofen actions on ST-EPSCs, basal, and asynchronous EPSCs (n = 4, P > 0.1 in all cases). Thus activation of presynaptic GABA<sub>B</sub> receptors powerfully inhibits both evoked and spontaneous modes of glutamate release from ST afferents regardless of TRPV1 subtype.

GABA<sub>B</sub> activation inhibits asynchronous release from TRPV1+ afferents. Activation of ST afferents transiently facilitates glutamate release from a TRPV1-operated pool of vesicles unique to TRPV1+ afferents (Peters et al. 2010). Since this asynchronous release is proportional to the amplitude of synchronous EPSCs, we anticipated that GABA<sub>B</sub> activation with baclofen would also reduce asynchronous rates (Figs. 1 and 2). Note that the asynchronous response was measured as the net increment in release above the basal rate. For this series of neurons, the asynchronous component was roughly equal to the basal level (Fig. 3B). This means that during the period following the ST-EPSCs, the total rate of sEPSCs was double the basal rate. The concentration-response relations for the inhibition of the asynchronous release component closely overlapped the relation for inhibition of basal release rates (Fig. 3B). In TRPV1− neurons, basal event rates were not different than the rates during the 1-s poststimulation period, and there was no consistent change in the poststimulus stochastic release period (n = 9, P = 0.2, paired t-test; see Fig. 2).

Given that synchronous, basal, and asynchronous release is, at least in part, dependent on voltage-activated ion channels, our results were consistent with the conclusion that GABA<sub>B</sub> activation might act at conventional voltage-activated ion channels. However, in TRPV1+ afferents, such voltage-dependent processes could not be separated from the potential actions of GABA<sub>B</sub> at TRPV1 itself. To better assess these voltage-independent release processes from TRPV1+ ST afferents, we conducted a series of additional studies, in which we inhibited voltage-activated sodium channels and/or VACCs, both with and without evoking temperature-gated responses.

GABA<sub>B</sub> activation inhibits action potential-independent glutamate release. In ST-evoked synchronous glutamate release, calcium entry and EPSC amplitude depend on VACCs (Mendelowitz et al. 1995), a common site of GABA<sub>B</sub> action. Action potential depolarization of the terminal triggers calcium influx via VACCs, and TTX interrupts this sequence to block ST-evoked EPSCs from both TRPV1+ and TRPV1− afferents (results not shown). In all second-order TRPV1+ neurons, baclofen reversibly reduced spontaneous glutamate release (Fig. 4) to an average of 46% of control (n = 5, P = 0.04, paired t-test). Amplitudes were not significantly changed across neurons (96% control, P = 0.3, paired t-test). Decay time constants were also not different between treatment groups (91% control, P = 0.2, paired t-test). Collectively, these results suggest that GABA<sub>B</sub> strongly inhibited the presynaptic, spontaneous release cascade, independent of terminal action potential activity. However, since the TRPV1 receptors in these terminals are cation channels, their activity might depolarize terminals sufficiently to recruit VACCs, which would then raise intraterminal calcium and support elevated mEPSC rates. To isolate the source of calcium entry, we used the broad-spectrum VACC blocker cadmium.

With action potentials blocked, application of 100 μM cadmium failed to alter mEPSC rates in TRPV1+ NTs neurons (n = 4, P = 0.9; Fig. 5A). Under these conditions, baclofen strongly reduced mEPSC frequency on all neurons (Fig. 5A) in a manner similar to that without cadmium (see Fig. 4). Collectively, the frequency of mEPSCs in the presence of baclofen was reduced to 31% of control (P = 0.045, one-way repeated measures ANOVA). Since event amplitudes were unaltered by cadmium or baclofen (P = 0.4, one-way repeated measures ANOVA), these changes suggest that baclofen acts presynaptically.

Together, these observations demonstrate that presynaptic GABA<sub>B</sub> activation can suppress ongoing basal synaptic gluta-
GABA_B INHIBITS MULTIPLE MODES OF GLUTAMATE RELEASE

Matt J. Peters, Steven A. Beyreuther, and Jeffrey C. Lichtman

In the presence of baclofen, thermal sensitivity was effectively eliminated in these neurons (n = 4, P = 0.9, one-way repeated measures ANOVA; Fig. 6C). Together, our results suggest that GABA_B suppresses glutamate release from the TRPV1-operated vesicle pool, a site downstream from conventional GABA_B targets of calcium entry.

DISCUSSION

Our results suggest a universal expression of GABA_B receptors on ST terminals in caudal NTS that mediate inhibition of multiple modes of glutamate release. The primary process of action potential-synchronized glutamate release from the readily releasable pool of vesicles is triggered by VACC and is indistinguishable between TRPV1+ and TRPV1− (Andersen and Peters 2008; Bailey et al. 2006b; Mendelowitz et al. 1995).
The remarkably similar synchronous, ST-evoked EPSCs for both TRPV1+ and TRPV1− ST afferent terminals shared quantitatively indistinguishable GABA_B-mediated inhibition (EC_{50} \sim 1.2 \mu M). Although the mechanisms underlying highly synchronous release are often lumped together with asynchronous and spontaneous modes of neurotransmitter release, increasing evidence suggests that distinct pools of vesicles are responsible for these different modes of release (Atasoy et al. 2008; Ramirez and Kavalali 2011). In cranial visceral afferents in NTS neurons, a TRPV1-operated pool is distinctly present only in the unmynelinated ST afferent terminals (Andresen and Peters 2008; Peters et al. 2010). Here, we show that in TRPV1+ afferents, GABA_B also inhibited responses associated with the TRPV1-operated pool, including asynchronous EPSCs, action potential-independent mEPSCs, and thermally evoked mEPSCs. Together, our results demonstrate that GABA_B receptors can modify glutamate release at both common and separate stages along these compartmentalized release pathways and thus regulate multiple modes of release within ST terminals.

Recently, we demonstrated that ST-evoked EPSCs deplete the synchronous, readily releasable pool and conversely, accelerate spontaneous release from the TRPV1-operated pool (Peters et al. 2010). In addition, the higher mEPSC release rates from TRPV1+ terminals arise from an active thermal drive and calcium influx via TRPV1 channels, a mechanism not present in the TRPV1− terminals (Shoudai et al. 2010). The presence of segregated pathways of release within the same terminals suggested the possibility of parallel, minimally overlapping synaptic transduction pathways in ST terminals, a pattern suggested for other central nervous system regions (Ramirez and Kavalali 2011). Our studies focused on whether these different modes of glutamate release, particularly from the unique, TRPV1-operated pool of vesicles of ST afferents, might be modulated differently by a major GPCR, GABA_B.

Synchronous glutamate release from the readily releasable pool is triggered by VACC and is regulated by calcium-sensing proteins, including synaptotagmin 1, which are localized in microdomains near VACCs (Chapman 2008; Kerr et al. 2008; Sun et al. 2007). The G_{\beta\gamma} subunit of GPCRs can directly bind to the VACC itself to inhibit glutamate release, as well as interact with soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins, including synaptotagmin, to disrupt vesicle fusion (Brown and Sihra 2008; Maximov and Sudhof 2005; Yoon et al. 2007). Our evidence suggests that baclofen acts presynaptically at targets in common within both afferent phenotypes to decrease synchronous glutamate release, consistent with GABA_B actions at conventional targets that affect the readily releasable pool.

Since the time of Katz (1971), spontaneous release was thought to reflect a reluctant, stochastic release from the readily releasable pool, the source of vesicles for synchronous release. Baclofen strongly inhibited the frequency of sEPSCs onto TRPV1+ neurons, but such inhibition was less obvious from TRPV1− terminals. The minimal baclofen effects on spontaneous and basal release onto TRPV1− neurons may reflect the 10-fold lower sEPSC rates. The quantitative discrepancy in basal EPSC rates between TRPV1+ and TRPV1− terminals remains surprising, given the indistinguishably similar release processes for ST-EPSC across the two afferent classes, including variance-mean analysis (Andresen and Peters 2008; Bailey et al. 2006b; Peters et al. 2008). The exceptionally low EPSC rates observed at TRPV1− NTS neurons suggest that vesicles in the readily releasable pool are reluctant and might represent a very limited, spontaneous fusion rate from those vesicles destined for the evoked release pathway. Our results show that activation of the GABA_B receptor mechanism in TRPV1+ terminals strongly decreased both basal mEPSC rates, as well as thermal increments in mEPSC rates. These new observations are consistent with the conclusion that the TRPV1-operated pool of vesicles is actively released in basal conditions and thus represents a novel, action potential-independent release pathway that can be modulated by GPCRs. GABA_B receptor activation inhibited this TRPV1-operated glutamate release separately from conventional targets affecting terminal excitability or calcium entry (VACCs). However, we cannot, using these techniques, distinguish between direct inhibition of the TRPV1 from the potential downstream actions on the vesicle release process itself within a common microdomain (Brown and Sihra 2008).

Many important physiological differences exist across the two major classes of cranial visceral afferent neurons, including their TRPV1 expression that dictates the performance modes of glutamatergic synaptic transmission in vivo. In general, the peripheral endings of these primary afferent neurons have a diverse distribution at visceral locations and organs and are activated by mechanical and/or chemical stimuli (Andresen and Kunze 1994; Basbaum et al. 2009; Coleridge and Coleridge 1984; Kunze and Andresen 1991; Thoren 1979). The two broad afferent classes corresponding to TRPV1+ and TRPV1− can be correlated with unmyelinated and myelinated axons, respectively (Jin et al. 2004b), and each expresses intrinsically different ion channel complements (Schild et al. 1994). Unmyelinated cranial visceral afferents commonly have higher thresholds and lower natural discharge rates and are thus generally less active under normal conditions (Jones and Thoren 1977). However, unmyelinated axons outnumber myelinated axons by 8- to 10-fold in cranial visceral afferent nerve trunks (Andresen et al. 1978; Jammes et al. 1982; Kubin and Davies 1995) and often have more powerful reflex actions than A-fibers, despite their lower characteristic discharge frequencies (Fan et al. 1999). With higher physiological thresholds and sparse discharge characteristics, the overwhelming majority of afferent terminals within the caudal NTS, which originate from TRPV1+ sensory neurons, is less likely to discharge action potentials and thus generates fewer synchronous glutamate events under basal conditions compared with the more physiologically active TRPV1− afferent phenotype. In the context of the present studies, modulation of glutamate release by presynaptic GABA_B receptors may then be distinctly different across these afferent classes. In TRPV1− terminals, GABA_B activation should predominantly suppress synchronous transmission, but spontaneous transmission will be reduced from TRPV1+ terminals under physiological conditions. A source of GABA may be local inhibitory neurons that commonly generate an EPSC-IPSC sequence on second-order caudal NTS neurons that reflect the ST-EPSC, followed by disynaptic GABA release on ST afferent activation (Andresen and Yang 1995; Bailey et al. 2008; Mifflin and Felder 1988; Smith et al. 1998). The glutamate release from the TRPV1-operated pool represents a form of synaptic transmission that occurs in the absence of afferent activation and may provide a stochastic,
GABA\textsubscript{B} INHIBITS MULTIPLE MODES OF GLUTAMATE RELEASE


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