Cannabinoid 1 and Transient Receptor Potential Vanilloid 1 Receptors Discretely Modulate Evoked Glutamate Separately from Spontaneous Glutamate Transmission

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Action potentials trigger synaptic terminals to synchronously release vesicles, but some vesicles release spontaneously. G-protein-coupled receptors (GPCRs) can modulate both of these processes. At cranial primary afferent terminals, the GPCR cannabinoid 1 (CB₁) is often coexpressed with transient receptor potential vanilloid 1 (TRPV1), a nonselective cation channel present on most afferents. Here we tested whether CB₁ activation modulates synchronous, action potential-evoked (eEPSCs) and/or spontaneous (sEPSCs) EPSCs at solitary tract nucleus neurons. In rat horizontal brainstem slices, activation of solitary tract (ST) primary afferents generated ST-eEPSCs that were rapidly and reversibly inhibited from most afferents by activation of CB₁ with arachidonyl-2'-chloroethylamide (ACEA) or WIN 55,212-2 [*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone monomethanesulfonate]. The CB₁ antagonist/inverse agonist AM251 [*N*-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide] blocked these responses. Despite profound depression of ST-eEPSCs during CB₁ activation, sEPSCs in these same neurons were unaltered. Changes in temperature changed sEPSC frequency only from TRPV1⁺ afferents (i.e., thermal sEPSC responses only occurred in TRPV1⁺ afferents). CB₁ activation failed to alter these thermal sEPSC responses. However, the endogenous arachidonate metabolite *N*-arachidonyldopamine (NADA) promiscuously activated both CB₁ and TRPV1 receptors. NADA inhibited ST-eEPSCs while simultaneously increasing sEPSC frequency, and thermally triggered sEPSC increases in neurons with TRPV1⁺ afferents. We found no evidence for CB₁/TRPV1 interactions suggesting independent regulation of two separate vesicle pools. Together, these data demonstrate that action potential-evoked synchronous glutamate release is modulated separately from TRPV1-mediated glutamate release despite coexistence in the same central terminations. This two-pool arrangement allows independent and opposite modulation of glutamate release by single lipid metabolites.

Key words: CB₁; NADA; NTS; TRPV1; vesicle pool

Introduction

Synaptic vesicles undergo spontaneous release of their neurotransmitter, and this process was long considered to represent an infrequent, stochastic fusion of primed vesicles from a readily releasable pool (Katz, 1971; Kaeser and Regehr, 2014). For evoked release, activation of voltage-activated calcium channels (VACCs) allows calcium to enter the terminal and bind to synaptotagmin, which activates a core fusion cascade that triggers vesicle exocytosis (Südhof, 2013). Emerging evidence suggests that spontaneous release from some terminals may arise from a separately regulated, unique vesicle pool (Sara et al., 2005, 2011; Atasov et al., 2008; Wasser and Kavalali, 2009; Peters et al., 2010).

The existence of multiple sources of intraterminal calcium offers the potential for separately regulated modes of neurotransmitter release.

Second-order solitary tract nucleus (NTS) neurons receive solitary tract (ST) afferent inputs that either express transient receptor potential vanilloid 1 (TRPV1⁺) or do not (TRPV1[−]; Doyle et al., 2002; Jin et al., 2004; Laaris and Weinreich, 2007). Shocks to the ST activate afferent axons that trigger synchronous release of glutamate [ST-evoked EPSCs (eEPSCs)], a process that is indistinguishable between TRPV1⁺ and TRPV1[−] afferents (Bailey et al., 2006b; Andresen and Peters, 2008). Despite similarities in eEPSCs, TRPV1⁺ afferents display 10-fold higher spontaneous release rates [spontaneous EPSCs (sEPSCs)] than TRPV1[−] afferents, and these events arise from a vesicle pool independent of the evoked pool (Peters et al., 2010). Most ST afferents are TRPV1⁺, and their sEPSC rates closely track temperature in the physiological range (Peters et al., 2010; Shoudai et al., 2010). This thermally driven glutamate release persists when calcium entry through VACCs is blocked (Shoudai et al., 2010; Fawley et al., 2011). This indicates that different sources of calcium independently mobilize separate subsets of glutamate vesicles in ST afferents.

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G-protein-coupled receptors (GPCRs) often modify the vesicle release process through actions at VACCs, adenylyl cyclase, and/or vesicle fusion proteins (Yoon et al., 2007; Brown and Sihra, 2008). CB₁ receptors are one of the most common GPCRs in the CNS and are activated by endocannabinoids derived from lipid metabolites. Natural endocannabinoids closely resemble the chemical structure of vanilloid agonists and can also activate TRPV1 (Pertwee et al., 2010; Di Marzo and De Petrocellis, 2012). CB₁ and endogenous ligands are coexpressed with TRPV1 in the CNS (Cristino et al., 2006, 2008). The synaptic transmission of TRPV1⁺ and TRPV1[−] ST afferents thus serves as a unique model to assess CB₁/TRPV1 interactions in the release of glutamate.

Here we tested whether CB₁ receptors similarly affected ST-eEPSCs and sEPSCs. CB₁ activation by arachidonyl-2'-chloroethylamide (ACEA) or WIN 55,212-2 [*R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl)] (1-naphthalenyl) methanone monomethanesulfonate (WIN) discretely depressed ST-eEPSCs from TRPV1⁺ and TRPV1[−] afferents without altering the basal sEPSC rates or thermal modulation of sEPSCs from the same afferents. However, *N*-arachidonyldopamine (NADA), an arachidonate derivative (Bisogno et al., 2000; Huang et al., 2002), inhibited ST-eEPSCs via CB₁ activation regardless of TRPV1 expression but facilitated both spontaneous and thermal release only from TRPV1⁺ afferents. Thus, presynaptic CB₁ in ST terminals modified the action potential-evoked release cascade without affecting the release machinery regulating spontaneous release. These results demonstrate a separate and independent regulation of glutamate release from the different vesicle pools without evidence of interactions. The compartmentalization of vesicle pools imparts this synapse with discrete signaling from different pools of a single neurotransmitter.

Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee and conform to the National Institutes of Health guidelines. Male Sprague Dawley rats (150–250 g; Charles River) were used. Brains were removed under deep isoflurane anesthesia (5%), and hindbrain slices were prepared as described previously (Doyle and Andresen, 2001). Briefly, a wedge of ventral brainstem was removed to tilt the hindbrain so that horizontal slices (250 μ m) contained the ST in the same plane as cell bodies in the caudal NTS (VT-1000S vibrating microtome from Leica; and sapphire blade from Delaware Diamond Knives). Slices were submerged in a perfusion chamber in an artificial CSF (ACSF) composed of the following (in mM): 125 NaCl, 3 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 10 glucose, and 2 CaCl₂, pH 7.4 (bubbled with 95% O₂/5% CO₂). The chamber was continuously perfused (1.5–2 ml/min) with ACSF with the temperature held at 32°C within 1°C using an inline heating system (Cell MicroControls). Bath temperature was continuously measured.

Patch-clamp recording. Patch pipettes (2.0–3.6 M Ω) were pulled from borosilicate glass and filled with the following (in mM): 6 NaCl, 4 NaOH, 130 K-gluconate, 11 EGTA, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 2 Na₂ ATP, and 0.2 Na₂ GTP, pH adjusted to 7.3–7.32. NTS neurons were visualized using infrared differential interference contrast optics (Zeiss Axioskop FS2) and selected within $\pm$ 250 μ m rostrocaudal to the caudal end of the fourth ventricle and medial to the ST. Neurons were voltage clamped (−60 mV; Multiclamp 700B; Molecular Devices), and synaptic currents were sampled at 20 kHz and filtered at 6 kHz using pClamp 9.2 software (Molecular Devices). Liquid junction potentials were not corrected. The GABA_A receptor antagonist gabazine (SR-95531 [2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)pyridazinium bromide]; 3 μ M) was present in all experiments. Drugs were purchased from Tocris Bioscience (R&D Systems) or Cayman Chemical. All drugs except gabazine (dissolved in purified water) were dissolved in 100% ethanol so that the final concentration of ethanol in ACSF did not exceed 2 μ l/ml. Ethanol vehicle at this concen-

tration did not alter ST-eEPSC amplitudes ($p = 0.2$, $n = 7$) or sEPSC frequencies ($p = 0.3$, $n = 7$).

ST-eEPSCs define second-order neurons. A concentric bipolar stimulating electrode (200 μ m outer tip diameter; Frederick Haer) was placed on the ST >1 mm from the recorded neuron, and minimal-intensity, constant-current shocks were delivered (5 stimuli at 50 Hz every 6 s, 100 μ s duration) using a Master-8 stimulator (A.M.P.I.). Stimulus shock intensity was increased gradually until a fixed-latency EPSC was evoked consistently at a minimum intensity. The latency was measured from the stimulus shock to the onset of the first EPSC evoked in each burst, and the jitter was then calculated as SD of the latency and averaged across $\geq$ 30 ST shocks. These low-jitter (<200 μ s), consistent-waveform EPSCs were selected for study as a monosynaptic unitary ST afferent input (Doyle and Andresen, 2001; Bailey et al., 2006a). Capsaicin (CAP; 100 nM) tests were conducted at the end of each experiment to verify vanilloid-sensitive (TRPV1⁺) or vanilloid-insensitive (TRPV1[−]) afferents (Doyle and Andresen, 2001; Bailey et al., 2006a; Peters et al., 2010).

ST-eEPSC and sEPSC analyses. Evoked EPSCs (ST-eEPSCs) were examined for >20 successive trials (2 min) to bursts of five ST shocks delivered every 6 s, and the mean peak amplitude was measured (generally the first response, EPSC1). From each stimulus trial, the basal activity was measured as the number of sEPSCs occurring in the 1 s preceding ST activation and collected across trials. Thus, ST-eEPSCs and sEPSCs were assessed at the same time in each cell. Designation of CB₁⁺ ST-eEPSCs required that significant decreases of EPSC1 amplitude occurred within individual experiments (20 trials each) to 7 min application of ACEA (10 μ M), WIN (10 μ M), or NADA (5–10 μ M). For statistical comparisons, values were tested for normal distributions, and appropriate parametric or nonparametric statistics were used, including Kolmogorov–Smirnov (KS) tests of interevent intervals and sEPSC amplitudes, *t* tests (two-group comparisons) or one/two-way repeated-measures (RM) ANOVA with *post hoc* comparisons (generally Tukey's) for more than two groups.

Thermally evoked sEPSCs. Bath temperature was controlled within 1°C using the inline heating system. Previous experiments indicate that ST afferents associated with substantial asynchronous EPSCs are indicative of TRPV1 expression (Peters et al., 2010), and we incorporated thermal tests in selected experiments when TRPV1 was present. In these protocols, ST-eEPSCs were measured initially at 32°C. For thermal tests, sEPSC activity was recorded during slow ramp increases in bath temperature to 36°C, followed by a slow ramp return to 32°C. The rate of temperature change was kept to 4°C for 3 min to evoke reproducible steady-state sEPSC rates. The sEPSC responses to the ramp increases and decreases in temperature were analyzed separately. Bath temperature values and sEPSC rates were averaged across the same 10 s intervals (Clampfit; Molecular Devices). Arrhenius relations were calculated as plots of the log of the event frequency versus the temperature [1000/*T* (°K)], and this relation was fitted by linear regression with the slope as a measure of the thermal sensitivity. All thermally responsive neurons responded to CAP and were thus TRPV1⁺. The sEPSCs were collected and analyzed in 10 s bins using MiniAnalysis (Synaptosoft) with synaptic events >10 pA detected. To test for CB₁ actions, ST-evoked and thermal responses were recorded before and during the application of 10 μ M ACEA, 10 μ M WIN, or 5–10 μ M NADA as an RM design. The CB₁ antagonist/inverse agonist AM251 [*N*-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; 10 μ M (Pertwee et al., 2010)] was tested against the agonist in selected experiments. Thermal responses were not assayed in neurons receiving TRPV1[−] ST afferents, because previous tests established their very low thermal sensitivity (Peters et al., 2010; Shoudai et al., 2010). In some experiments, miniature EPSCs (mEPSCs) were measured in the presence of 1 μ M TTX.

Results

CB₁ activation depresses evoked release regardless of TRPV1
ST shocks evoked fixed-latency, monosynaptic eEPSCs in horizontal brainstem slices that were similar for neurons receiving TRPV1⁺ or TRPV1[−] afferents (ST-eEPSCs; Fig. 1; Andresen et al., 2012). The TRPV1 agonist CAP (100 nM) identified TRPV1⁺ afferents (Fig. 1C) by blocking evoked transmission but did not

Figure 3. CB₁ activation failed to alter sEPSCs despite depression of eEPSCs from the same afferent. In TRPV1[−] (**A–C**) or TRPV1⁺ (**D–I**) ST afferents, ACEA (10 μ M, blue) did not alter basal sEPSC rates (**A, D**) but reduced ST-eEPSCs (**B, E**) from control (Ctrl, black). Across afferents, ACEA did not affect basal sEPSC frequency (**C**, $p = 0.2$, paired t test) or amplitude ($p = 0.3$, paired t test) from TRPV1[−] or TRPV1⁺ (**F**; frequency, $p = 0.1$; amplitude, $p = 0.6$, paired t tests) afferents. Note the substantially higher sEPSC rates characteristic of TRPV1⁺ compared with TRPV1[−] ($p = 0.01$, t test). **G**, sEPSC frequency (10 s bins black/filled gray) from TRPV1⁺ afferents tracked changes in bath temperature (red), but ACEA (blue box) had no effect. **H**, Temperature sensitivity was determined by linear regression fits of the log sEPSC frequency versus temperature [1000/(°K)] from increasing temperature ramps in control (black inverted triangles) and ACEA (blue circles). **I**, Across neurons, temperature sensitivities were unaltered by CB₁ activation ($p = 0.8$, paired t test).

activity, and activation of CB₁ with ACEA remarkably failed to alter these rates (Fig. 3*A, D*). So despite substantial inhibition of evoked release from CB₁⁺ ST afferents (Fig. 3*B, E*), sEPSC rates from either afferent class were unaffected (Fig. 3*C, F*). Similarly, WIN reduced ST-eEPSC amplitudes without altering sEPSCs rates or amplitudes from either TRPV1 type (all p values > 0.2, paired t tests). AM251 alone did not alter basal TRPV1⁺ sEPSCs rates ($p = 0.9$, paired t test). Furthermore, in the absence of action potentials (in TTX), neither mEPSC frequencies ($p = 0.5$, $n = 4$, paired t test) nor amplitudes ($p = 0.2$, paired t test) from TRPV1⁺ afferents were inhibited by CB₁ activation (additional data not shown). Despite the inhibition of evoked glutamate release (i.e., ST-eEPSCs), the ongoing basal glutamate release (i.e., sEPSCs) was not altered from the same afferents. These observations suggest that CB₁ discretely regulates evoked glutamate release without disturbing the spontaneous release process.

CB₁ fails to alter thermal regulation of sEPSCs

Under baseline conditions, spontaneous glutamate release is substantially higher from TRPV1⁺ ST afferents (Shoudai et al., 2010). Although this might suggest that the high release rate is a passive process, cooling below physiological temperatures substantially reduces the sEPSC rate only in TRPV1⁺ neurons and indicates an active role for thermal transduction in TRPV1⁺ terminals (Shoudai et al., 2010). To test whether CB₁ activation modified this active thermal release process, we compared the sEPSC rate changes to thermal challenges. In CB₁⁺ TRPV1⁺ afferents (Fig. 3*B, E*), small changes in bath

Figure 4. NADA activated both CB₁ and TRPV1 with opposite effects on glutamate release. NADA (5 μ M, green) inhibited ST-eEPSCs whether TRPV1 was present (**D**) or not (**A**). Across neurons receiving TRPV1⁺ afferents ($n = 10$), NADA (5–10 μ M) reduced ST-eEPSCs by $34 \pm 4\%$ ($*p < 0.01$, two-way RM-ANOVA) without affecting ST-eEPSC2–eEPSCs ($p > 0.2$, two-way RM-ANOVA). NADA (5–10 μ M) similarly reduced synchronous release from TRPV1[−] afferents ($n = 4$), both ST-eEPSC1 ($33 \pm 6\%$, $p < 0.0001$, two-way RM-ANOVA) and ST-eEPSC2 ($27 \pm 12\%$, $p < 0.01$, two-way RM-ANOVA). However, NADA increased basal sEPSC rates only from TRPV1⁺ afferents (**B, C**; TRPV1⁺, $*p = 0.02$; **E, F**; TRPV1[−], $p = 0.3$, paired t tests), indicating a functionally independent effect of CB₁-induced depression of eEPSCs versus the enhanced sEPSC release mediated by TRPV1. NADA (5–10 μ M) also facilitated thermal sensitivity from TRPV1⁺ afferents (**G–I**). **G**, Bath temperature (red) and sEPSCs (black) were binned (10 s), and the sensitivity (**H**) was determined as described in Figure 3*H*. The sensitivities were averaged across neurons (**I**; $*p = 0.03$, paired t test). Ctrl, Control.

temperature modified the sEPSC rate (Fig. 3*G*), and the average ($n = 5$) thermal sensitivity relationship for sEPSC rates was unaffected by ACEA (Fig. 3*H, I*). The lack of effect of CB₁ activation on thermally regulated spontaneous glutamate release—despite effectively depressing action potential-evoked glutamate release—suggests that the second-messenger cascade activated by CB₁ failed to alter spontaneous release or its modulation by temperature.

NADA oppositely modulates evoked and TRPV1-operated glutamate release

Endocannabinoids and endovanilloids share similar structural motifs (Di Marzo et al., 1998), and some arachidonate derivatives, including NADA, activate both CB₁ and TRPV1 (Marinelli et al., 2003, 2007; Matta and Ahern, 2011). As expected, NADA depressed ST-eEPSC amplitudes for CB₁⁺ ST afferents similarly whether they were TRPV1⁺ or TRPV1[−] (Fig. 4*A, D*). Although NADA did not alter the rate of ST-evoked failures from TRPV1⁺ ($p = 0.08$, two-way RM-ANOVA) or TRPV1[−] ($p = 0.4$, two-way RM-ANOVA) afferents, it effectively mimicked CB₁-selective agents to depress action potential-evoked release of glutamate. NADA simultaneously increased ongoing basal release rates only from afferents with TRPV1⁺ (Fig. 4*E, F*) but not from TRPV1[−] ST afferents (Fig. 4*B, C*). In addition, NADA facilitated thermally

Figure 5. Afferents lacking CB₁ receptors served as a natural control for NADA actions. Representative current traces are from second-order NTS neurons that received only TRPV1⁺ afferent(s). **A**, ST shocks evoked ST-eEPSCs from this TRPV1⁺ afferent that were unaltered by ACEA (10 μM, blue; $p = 0.9$, paired t test) identifying the afferent as CB₁⁻. **B**, The sEPSC rates from the same afferent (ctrl, black) were unaffected by ACEA (blue; $p = 0.8$, KS test). **C**, Across CB₁⁻ afferents ($n = 5$), neither the ST-eEPSC amplitude ($p = 0.6$, paired t test) nor the frequency of sEPSCs ($p = 0.9$, paired t test) were affected by CB₁-specific activation by ACEA. **D**, Similarly, a different second-order neuron with TRPV1⁺ afferents had no ST-eEPSC response to NADA (green, 5 μM; $p = 0.3$, paired t test) and was thus void of CB₁. **E**, Nonetheless, NADA nearly doubled the rate of sEPSCs ($p = 0.001$, KS test). **F**, Across CB₁⁻ afferents tested with NADA ($n = 4$), the ST-eEPSC amplitude was unaffected by NADA ($p = 0.9$, paired t test) but showed increased sEPSC rates ($*p = 0.04$, paired t test). **G**, NADA enhanced the sEPSC frequency (10 s bins black/filled gray) response to increases in bath temperature (red). X-axis breaks mark ST-eEPSC measurements. **H**, Across afferents, NADA increased temperature sensitivity by 30%. These results suggest that NADA acts on sEPSC regulation through TRPV1 regardless of CB₁ expression.

triggered sEPSCs rates in neurons receiving TRPV1⁺ ST afferents (Fig. 4G–I). TRPV1⁺ afferents that lacked suppression of ST-eEPSCs in response to CB₁ agonist (CB₁⁻) served as naturally occurring “controls” for CB₁ actions (Fig. 5). NADA only enhanced basal and thermally triggered sEPSCs without altering ST-eEPSC amplitudes from these CB₁⁻/TRPV1⁺ afferents, which is consistent with endocannabinoid actions solely at TRPV1.

In afferents with both receptors (CB₁⁺/TRPV1⁺; Fig. 6), the TRPV1 antagonist capsazepine blocked NADA enhancement by NADA but did not prevent the ST-eEPSC depression (Fig. 6A–D). Likewise, the TRPV1 antagonist 5'-iodoresiniferatoxin (iRTX) blocked NADA-mediated increases in sEPSCs (control, 16.0 ± 4.6 Hz vs NADA + iRTX, 14.9 ± 5.0 Hz; $n = 5$, $p = 0.6$, one-way RM-ANOVA). These actions of TRPV1 antagonists indicate that NADA acted on spontaneous release by binding to the vanilloid binding site on TRPV1 receptors. Conversely, AM251 blunted NADA-induced inhibition of the ST-eEPSC but failed to prevent NADA from increasing the sEPSC rate (Fig. 6E–H). This

Figure 6. Antagonists for TRPV1 [capsazepine (CPZ), blue] and CB₁ (AM251, gray) selectively blocked the NADA-induced effects associated with each respective receptor. **A**, Representative traces from a TRPV1⁺ afferent demonstrates that 10 μM CPZ (blue) did not block the NADA-induced reduction (green) in ST-eEPSC amplitude compared with control (Ctrl, black). This demonstrates the lack of direct action of TRPV1 on action potential-evoked glutamate release and reinforces the role of CB₁ receptors in reducing ST-eEPSC amplitude. **B**, Across neurons, CPZ had no effect alone and did not block NADA-induced reduction of ST-eEPSC1 ($**p = 0.02$, one-way RM-ANOVA). **C**, In contrast to eEPSCs, sEPSC traces from the same NTS neuron as A demonstrated that CPZ blocked the increase induced by NADA, suggesting action via TRPV1. **D**, Across neurons, CPZ had no effect on sEPSCs and prevented NADA enhancement ($p = 0.5$, one-way RM-ANOVA). **E**, Traces from a different TRPV1⁺ ST afferent demonstrate that AM251 (20 μM) blunts the effect of NADA (10 μM, green) on ST-eEPSC (ST1). **F**, Across afferents, NADA (5–10 μM) reduced the amplitude of ST-eEPSC1 by 22% ($**p < 0.05$, two-way RM-ANOVA), but when it was coapplied with AM251 (10–20 μM), there was only an 11% reduction ($*p < 0.05$, two-way RM-ANOVA). This demonstrates that NADA reduced evoked glutamate via CB₁. **G**, Traces from the same NTS neuron as E demonstrate that this CB₁ antagonist did not block NADA-induced increases in sEPSC rates. **H**, Across afferents, NADA increased sEPSC rates ($**p < 0.001$, two-way RM-ANOVA) regardless of AM251 ($*p = 0.01$, two-way RM-ANOVA), supporting previous observations that NADA increases sEPSCs via TRPV1.

result suggests that NADA acts on evoked release by activating the CB₁ receptor. Thus, NADA has dual opposing actions on glutamate release within single afferents attributed separately to CB₁ and TRPV1 activations. The independence and selectivity of the actions suggests that CB₁ and TRPV1 signaling function without crosstalk between the two mechanisms (De Petrocellis et al., 2001; Evans et al., 2007). Such findings are consistent with complete functional isolation of CB₁ and its second-messenger system from TRPV1-mediated responses.

Discussion

In this study, we demonstrate that CB₁ and TRPV1 separately targeted different forms of glutamate release from ST primary afferent terminals. CB₁ activation inhibited evoked neurotransmission, and its actions were limited to aspects of action potential-evoked release (decreases in ST-eEPSC amplitude and increases in failure rates) without disturbing spontaneous vesicular release (including the TRPV1-operated form) from the same afferents. Although central terminals within the NTS express VACCs and may additionally express TRPV1 (Mendelowitz et al., 1995; Andresen et al., 2012), the actions of CB₁-selective agents were consistent across multiple subsets of CB₁⁺ afferents regardless of TRPV1 expression. In contrast, the endocannabinoid NADA triggered both inhibitory CB₁ actions on evoked release but also augmented spontaneous and thermal release of glutamate (sEPSCs) by activating TRPV1. We found no evidence that the pronounced

CB₁ action on the evoked release process affected spontaneous and TRPV1-mediated glutamate release and vice versa. Despite being a GPCR with intracellular second messengers, CB₁ discretely targeted evoked glutamate release without actions on spontaneous release. These data are consistent with two noncompeting pools of vesicles within ST cranial afferent terminals that can be independently modulated.

Our study focused on ST transmission of cranial visceral afferents arising from two afferent phenotypes based on differences in TRPV1 expression. Both myelinated (TRPV1[−]) and unmyelinated (TRPV1⁺) primary visceral afferents use similar mechanisms for evoked release that generate a characteristically strong frequency-dependent depression of ST transmission (Bailey et al., 2006b; Andresen and Peters, 2008; Peters et al., 2008). Several GPCRs modulate evoked ST-eEPSCs regardless of TRPV1 status (Appleyard et al., 2005; Bailey et al., 2006b; Peters et al., 2008; Fawley et al., 2011). In the present studies, three different CB₁ agonists—ACEA, WIN, and NADA—similarly depressed ST-eEPSCs regardless of TRPV1 status, and the CB₁-selective antagonist/inverse agonist AM251 blocked these actions. AM251 showed no effects when administered alone in NTS slices, a finding that rules out tonic excitatory actions reported in some sensory neurons (Patil et al., 2011). CB₁ activation attenuated eEPSCs from most ST afferents, suggesting a similar widespread presynaptic CB₁ expression among ST afferents. These CB₁ actions on evoked release likely arise from inhibition of VACCs in ST axons directly linked to highly synchronous release (Mendelowitz et al., 1995; Brown et al., 2004; Castillo et al., 2012).

ST-evoked transmission relies on EPSCs recruited at minimal stimulus strength with latency and amplitude characteristics consistent with responses evoked by a single axon (Doyle and Andresen, 2001; McDougall et al., 2009). Detailed studies have indicated that, in basal conditions, ST-eEPSCs average a 90% probability of glutamate release from the readily releasable pool of vesicles regardless of TRPV1 expression (Bailey et al., 2006b). The uncommonly high release probabilities of ST afferents likely contribute to the near zero failure rates for the first shock (McDougall et al., 2009; McDougall and Andresen, 2013). The CB₁-mediated depression of the release probability likely reflects actions within the synaptic terminal and was most evident in the CB₁-induced increase in ST-eEPSC1 amplitude variance. This CB₁ effect follows from the steep parabolic relation between variance and amplitude for this high release synapse (Bailey et al., 2006b). The lack of CB₁ effects on consequent ST-eEPSCs (ST-eEPSC2–eEPSC5) likely reflects a mixing of these two mechanisms in which a CB₁-mediated decrease in release probability attenuates vesicle depletion and consequently means that more vesicles are available for release on the second shock. A lower probability of release combined with less frequency-dependent depression during CB₁ activation might result in net responses that were unchanged in both afferent types (Fig. 1*D,I*).

CB₁ activation interrupted the usually faithful conversion of ST action potentials to eEPSCs by increasing synaptic failures only in TRPV1⁺ afferents. TRPV1⁺ ST afferents characteristically have much higher use-dependent failure rates compared with TRPV1[−] afferents (Andresen and Peters, 2008), and this difference between myelinated (TRPV1[−]) and unmyelinated (TRPV1⁺) primary cranial afferents may reflect critical differences in ion channel expression (Schild et al., 1994; Li et al., 2007). Our observation that transmission along TRPV1[−] afferents was inherently more reliable with lower failures, and an intrinsically higher safety margin may account for the inability of ACEA or WIN to augment failures in TRPV1[−] ST afferents. GP-

Figure 7. Schematic illustration of CB₁ (blue) and TRPV1 (red) activation to mobilize separate pools of glutamate vesicles. **A**, The GPCR CB₁ depresses glutamate release from the readily releasable pool of vesicles (gray) measured as ST-eEPSCs. Calcium entry through VACCs primarily regulates this vesicle pool. CB₁ action on ST-eEPSCs is equivocal whether ACEA, WIN (dark blue pie), or NADA (bifunctional agent acting at both CB₁ and TRPV1 sites, blue pie/orange key) activates the receptor. **B**, CB₁ also interrupts action potential-driven release when activated by ACEA or WIN, likely by blocking conduction to the terminal. **C**, Calcium sourced from TRPV1 drives spontaneous EPSCs from a separate pool of vesicles (red) on TRPV1⁺ afferents. NADA activates TRPV1, likely through its ligand binding site (pink), to potentiate basal and thermal-activated [heat (flame)] sEPSCs via the temperature sensor (maroon bent hash marks). **D**, Although the endogenous lipid ligand NADA can activate both CB₁ and TRPV1, selective activation of CB₁ with ACEA or WIN only suppresses voltage-activated glutamate release with no interactions either directly or indirectly with TRPV1. Likewise, TRPV1 activation with NADA does not interact with CB₁ or affect ST-eEPSCs, demonstrating that the two pools of glutamate release can be independently regulated.

CRs, including the vasopressin V_{1a} receptor on ST afferents in the NTS, are found relatively distant from the terminal release sites and affect the failure rate independent of changes in the release probability (Voorn and Buijs, 1983; Bailey et al., 2006b). Thus, CB₁-induced increases in conduction failures may well reflect similar conduction failures at relatively remote CB₁ receptors (Bailey et al., 2006b; McDougall et al., 2009). The difference we observed in ST-eEPSC failures with activation of CB₁ by NADA may relate to the lower affinity of NADA for CB₁ compared with the selective agonists tested (Pertwee et al., 2010). Thus, the two actions of CB₁ receptor activation are attributed to distinctly separate sites of action: one that decreases release probability (i.e., within the synaptic terminal) and the other affecting conduction (i.e., along the afferent axon) that induces failures of excitation.

A major difference in ST transmission is the presence of TRPV1 in unmyelinated ST afferents (Andresen et al., 2012). In contrast to ST-eEPSCs, elevated basal sEPSCs and thermal-mediated release from TRPV1⁺ afferents are independent of VACCs and instead depend on calcium entry that persists in the presence of broad VACC blockers, such as cadmium (Jin et al., 2004; Shoudai et al., 2010; Fawley et al., 2011). Because sEPSCs depend on external calcium levels (Peters et al., 2010), TRPV1

appears to provide a second calcium source for synaptic release independent of VACCs (Fig. 7). However, the calcium sourced through TRPV1 does not affect evoked glutamate release. Raising the bath temperature (33–38°C) strongly activated TRPV1-dependent sEPSCs (Shoudai et al., 2010) but not the amplitude of evoked release (Peters et al., 2010). Likewise, when CB₁ was absent (CB₁[−]) or blocked, NADA increased spontaneous and thermal-evoked sEPSCs with no effect on ST-eEPSCs, providing additional evidence that TRPV1-mediated glutamate release is separate from evoked release. The actions of NADA together with temperature are consistent with the polymodal gating of TRPV1 via binding to a separate CAP binding site, as well as temperature actions at a thermal activation site within TRPV1 (Caterina and Julius, 2001). Although other channels may contribute to temperature sensitivity including non-vanilloid TRPs (Caterina, 2007), TRPV1 block with capsazepine or iRTX prevented NADA augmentation of sEPSC responses, indicating a TRPV1-dependent mechanism. Together, our data suggest that presynaptic calcium entry via TRPV1 has access to the vesicles released spontaneously but does not alter release by action potentials and VACC activation (Fig. 7).

Our studies highlight a unique mechanism governing spontaneous release of glutamate from TRPV1⁺ afferents (Fig. 7). In the NTS, TTX did not alter the rate of sEPSCs activity and demonstrates that very little spontaneous glutamate release originates from distant sources relayed by action potentials (Andresen et al., 2012). Focal activation of afferent axons within 250 μm of the cell body generated EPSCs with characteristics indistinguishable from ST-evoked responses in the same neuron (McDougall and Andresen, 2013) and suggests that afferent terminals dominate glutamatergic inputs to second-order neurons, such as the ones in the present study. So although additional, non-afferent glutamate synapses certainly exist on NTS neurons—as evident in polysynaptic-evoked EPSCs that likely represent disynaptic connections (Bailey et al., 2006a)—their contribution to our sEPSC results is likely minor.

Our study adds to emerging data that challenge the conventional view that vesicles destined for action potential-evoked release of neurotransmitter belong to the same pool as those released spontaneously (Sara et al., 2005, 2011; Atasoy et al., 2008; Wasser and Kavalali, 2009; Peters et al., 2010). At synapses with single, common pools of vesicles, depletion by high frequencies of stimulation depressed spontaneous rates (Kaesler and Regehr, 2014). In contrast, the high-frequency bursts of ST activation transiently increased the rate of spontaneous release only from TRPV1⁺ afferents (Peters et al., 2010). The single pool concept of glutamate release would predict that a singular presynaptic GPCR would modulate all vesicles in the terminal similarly. However, our results clearly indicate that the GPCR CB₁ only modulates a subset of glutamate vesicles (eEPSCs). The separation of the mechanisms mediating spontaneous release from action potential-evoked release at ST afferents is consistent with separately sourced pools of vesicles that supply evoked or spontaneous release for cranial visceral afferents.

The discreteness of CB₁ from TRPV1 actions in ST transmission was surprising with respect to other primary sensory afferent neurons. The functional isolation and lack of cross-talk between CB₁ and TRPV1 when coexpressed in ST afferents suggests quite different compartmentalization than in neurons from the spinal cord dorsal root ganglion and dorsal horn (De Petrocellis et al., 2001; Matta and Ahern, 2011). Because ST-evoked and spontaneous transmissions appear to

arise from separate pools, this raises the possibility that the vesicles may be physically separated with different compartmentalization within microdomains or nanodomains, as suggested for VACCs (Bucurenciu et al., 2008; Neher and Sakaba, 2008). Larger-scale separations may occur, such as different boutons for spontaneous and evoked release similar to the neuromuscular junction (Melom et al., 2013; Peled et al., 2014). Little is known about vesicle organization of ST afferent synaptic terminals. The fundamental segregation of the evoked release mechanism from the TRPV1-operated pool indicates that different lipid mediators may adjust ongoing glutamate release for fast synaptic transmission distinct from spontaneous release. Because spontaneously released glutamate is suggested to play a key role in synapse maintenance/stabilization and tasks such as postsynaptic gene transcription (McKinney et al., 1999; Nelson et al., 2008; Kaesler and Regehr, 2014), this distinct and separate regulation of spontaneous release provides a mechanism to modulate a wide range of cellular functions independent of afferent action potentials. TRPV1 consequently serves as an essential modulation target because it provides a calcium source to drive spontaneous release independent from afferent activity or voltage.

It is not clear how spontaneous release of glutamate in the NTS and the modulatory differences that we observe in evoked glutamate translates to physiological functions. Both TRPV1 and CB₁ in the NTS modify basic homeostatic functions. TRPV1 plays a key role in neonatal respiratory regulation with small temperature shifts within the NTS (Xia et al., 2011). CB₁ receptors broadly inhibit cardiovascular and gastrointestinal functions (Van Sickle et al., 2003; Brozoski et al., 2005; Evans et al., 2007). The importance of endocannabinoid/endovanilloid signaling might be amplified or have more pronounced consequences in disease states in which there are chronic shifts in lipid profiles (e.g., hyperglycemia and obesity; Matias et al., 2008). The CB₁/TRPV1 mechanisms and their interactions with lipid signaling may have potential implications in multisystem, homeostatic dysfunction that accompanies inflammatory states (Pingle et al., 2007), obesity (Marshall et al., 2013), and/or early development (Xia et al., 2011).

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