Title: Physiological temperatures drive glutamate release onto trigeminal superficial dorsal horn neurons

Authors: Tally M. Largent-Milnes, Deborah M. Hegarty, Sue A. Aicher, Michael C. Andresen

Author Affiliations: All authors are affiliated with the Department of Physiology and Pharmacology, Oregon Health & Science University, Mail code: L334, 3181 Sam Jackson Park Road, Portland, OR 97239-3098, United States

Running Head: Temperature drives spontaneous glutamate release in Vc

Address for Correspondence:
T. M. Largent-Milnes
OHSU Department of Physiology and Pharmacology
Mail Code: L334
3181 SW Sam Jackson Park Road
Portland, OR 97239-3098 USA
Email: milnes@ohsu.edu
Phone: (503) 418-2529
Fax: (503) 494-4352

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**Abstract:** Trigeminal sensory afferent fibers terminating in nucleus caudalis (Vc) relay sensory information from craniofacial regions to the brain and are known to express Transient Receptor Potential (TRPs) ion channels. TRP channels are activated by $H^+$, thermal, and chemical stimuli. The present study investigated the relationships between the spontaneous release of glutamate, temperature and TRPV1 localization at synapses in the Vc. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from Vc neurons (n=151) in horizontal brainstem slices obtained from Sprague-Dawley rats. Neurons had basal sEPSC rates that fell into two distinct frequency categories: High ($\geq 10$ Hz) or Low (< 10 Hz) at 35°C. Of all recorded neurons, those with High basal release rates (67%) at near physiological temperatures greatly reduced their sEPSC rate when cooled to 30°C without amplitude changes. Such responses persisted during blockade of action potentials indicating that the High rate of glutamate release arises from presynaptic thermal mechanisms. Neurons with Low basal frequencies (33%) showed minor thermal changes in sEPSC rate that were abolished after addition of TTX, suggesting these responses were indirect and required local circuits. Activation of TRPV1 with capsaicin (100 nM) increased miniature EPSC (mESPC) frequency in 70% of neurons, but half of these neurons had Low basal mEPSC rates and no temperature sensitivity. Our evidence indicates that normal temperatures (35-37°C) drive spontaneous excitatory synaptic activity within superficial Vc by a mechanism independent of presynaptic action potentials. Thus, thermally sensitive inputs on superficial Vc neurons may tonically activate these neurons without afferent stimulation.

**Keywords:** trigeminal nucleus caudalis, temperature, TRPV1, spontaneous release, electrophysiology
**Introduction:** The trigeminal nucleus caudalis (Vc) receives sensory information relayed predominantly by glutamate released from trigeminal primary afferent fibers from the head and neck, including highly specialized tissues like the cornea and dura (Gibbs et al. 2011; Hiura and Nakagawa 2012). Upon peripheral stimulation, action potentials invade Vc central terminals and trigger evoked glutamate release, but substantial release of vesicular glutamate also occurs spontaneously (Jennings et al. 2003; Travagli and Williams 1996). Recently, it has been suggested that evoked and spontaneous glutamate release may arise from different pools of vesicles that are distinctly regulated and have unique physiological importance (Kavalali et al. 2011).

Primary afferent neurons discharge little in the absence of peripheral stimulation or injury (Jankowski et al. 2012; Pitcher and Cervero 2010), and therefore their central terminals might be expected to have similarly low rates of neurotransmitter release in basal conditions. However at central synapses measured in slices, neurotransmitter release occurs spontaneously, and such events are commonly viewed as arising from infrequent and stochastic release from the same pools of vesicles as action potential evoked release (Ermolyuk et al. 2013; Katz 1971). In contrast to peripheral activation characteristics, the rates of spontaneous vesicle release are often much higher from primary afferent terminals (Grudt and Williams 1994; Shoudai et al. 2010; Uta et al. 2010). Indeed, primary afferents that contact second order neurons in the solitary tract nucleus (NTS) often express the Transient Receptor Potential Vanilloid Type 1 Channel (TRPV1) and have substantially higher spontaneous glutamate release rates than neurons with afferents lacking TRPV1 (Peters et al. 2010). The spontaneous EPSC (sEPSC) rates in NTS strongly depend on the ambient temperature for the TRPV1-expressing afferents (Shoudai et al. 2010) and are independent of voltage-activated calcium channels yet strongly modulated by G-protein coupled receptors (GPCRs) (Fawley et al. 2011). The high sensitivity near 37°C contrasts to the relatively weak thermal sensitivity at physiological temperatures of neural excitability and conduction
more generally (Hardingham and Larkman 1998; Klyachko and Stevens 2006; Pyott and Rosenmund 2002).

Thermally responsive TRPs, including TRPV1, are present in trigeminal sensory afferent fibers and mark the superficial layers in Vc as primary nociceptor terminations (Cavanaugh et al. 2011a; Jennings et al. 2003). Noxiously hot (> 45 °C) peripheral temperatures evoke action potentials in trigeminal afferents (Cuellar et al. 2010) that can be conducted to superficial Vc neurons and are generally attributed to TRPV1 expression (Neubert et al. 2008). Here we measured the thermal sensitivity of spontaneous glutamate release in Vc to test whether temperature sensitivity corresponded to TRPV1 expression centrally. In horizontal slices, we recorded spontaneous EPSCs in neurons in the superficial laminae of Vc. We found two distinct sets of neurons based on their basal sEPSC rate; high rates of spontaneous glutamate release at near physiological temperatures (36°C) was greatly reduced with modest decreases in bath temperature (30-35°C) in one group of neurons. The remaining neurons exhibited low spontaneous glutamate release that was thermally insensitive but TRPV1 expression was not exclusively related to either group. The findings demonstrate substantial heterogeneity in Vc spontaneous glutamatergic transmission and the existence of TRPV1-independent thermal coupling to excitatory transmitter release.

Methods:

Animals: All animal procedures were approved by the Institutional Animal Care and Use Committee and conformed to the guidelines of the National Institutes of Health publication “Guide for the Care and Use of Laboratory Animals”. Hindbrains of male Sprague-Dawley rat pups (P9 - 28; n=120, Charles River Laboratories) were prepared as described previously (Grudt and Williams 1994) under isoflurane
anesthesia (5%, 2 L/min in air). Horizontal slices (200 - 230 μm) were cut with a sapphire knife (Delaware Diamond Knives, Wilmington, DE) mounted in a vibrating microtome (Leica VT-1000S, Leica Microsystems, Bannockburn, IL). Slices were submerged in a perfusion chamber and placed in artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 3 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 10 D-glucose, 2 CaCl₂, bubbled with 95% O₂:5% CO₂; pH 7.4; 293-300 mOsm at 32 °C for 45 - 60 min prior to recording. Bath temperature was controlled within 1°C using an inline heating system (TC2BIP with HPRE2 and TH-10Km bath probe, Cell MicroControls, Norfolk, VA) and was continually measured with a thermistor placed immediately downstream from the slice (Fawley et al. 2011). For light microscopy studies, slices were obtained as described above then immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) using a standard microwave for 5s at 50% power. Sections were rinsed in 0.1M PB and stored in 30% sucrose/30% ethylene glycol storage solution in 0.1M PB at -20°C until immunocytochemistry was performed.

Voltage clamp recordings: Neurons selected for recording had their cell bodies located within the outer lamina (L/IIo) of the Vc (a translucent band ≤ 200 μm medial to spinal trigeminal tract). Patch electrodes were pulled from borosilicate glass (O.D. = 1.5 ± 0.05 mm; I.D. = 1.0 ± 0.05 mm; Garner Glass), fire polished, and had input resistances of 3.5 - 5.0 MΩ when filled with a low Cl⁻ (10mM, E_Cl = -69 mV) intracellular solution consisting of (in mM): 6 NaCl, 4 NaOH, 130 K-gluconate, 11 EGTA, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 2 Na₂ATP, and 0.2 Na₂GTP; pH 7.31-7.33 and 286-294 mOsm. Neurons were visualized using infrared differential interference contrast optics (Zeiss Axioskop FS2, Thornwood, NJ) as previously described (Peters et al. 2010). Neurons were recorded using whole cell configuration and held in voltage clamp at V_H = -60 mV. Signals were sampled at 20 kHz and filtered at 10 kHz using p-Clamp software (version 9.2, Molecular Devices, Sunnyvale, CA) with a Multiclamp 700A, Digidata 1322A analog to digital converter (Axon Instruments, Union City, CA). Under our conditions, both glutamatergic and GABAergic
synaptic currents were inward at a holding voltage of −60 mV. Liquid junction potentials were not corrected. The GABA<sub>A</sub> receptor antagonist, gabazine (GBZ, 3 μM), was present in all experiments to isolate EPSCs. Miniature EPSCs (mEPSCs) were measured in TTX (1 μM) to prevent action potentials. Thermally evoked increases in EPSC frequency were evaluated using small step changes in bath temperature (30-36 °C) by electronically controlling (Master 9, AMPI, Jerusalem, Israel) the inline heating system. Capsaicin (CAP, 100 nM) was added at the end of experiments in the presence of TTX to determine if inputs expressed TRPV1 (TRPV1+).

Current-clamp recordings: In some recordings, neurons were classified as described above based on sEPSCs before switching the recording configuration to current clamp to assess action potential firing. Both EPSP and action potential frequency were recorded during temperature changes in the presence of GBZ to eliminate fast inhibitory transmission and to focus on excitatory mechanisms. Temperatures were controlled as in voltage clamp recordings. Following temperature steps, the configuration was changed back to voltage clamp and TTX added to confirm whether the thermal sensitivity was mediated directly or indirectly.

Drugs: All drugs were added to the aCSF bath solution and perfused for at least 2 min (bath volume 0.5-0.7 mL, flow = 1.9 - 2.1 mL/min). Gabazine (SR-95531), TTX, 6,7-Dinitroquinoxaline-2,3-dione disodium salt (DNQX), and capsaicin (CAP) were purchased from Tocris Bioscience (Ellisville, MO).

Light Microscopy: Immunocytochemistry for light microscopic peroxidase detection of TRPV1 in the Vc was performed using a method modified from that previously described (Hegarty et al. 2007). Following incubation in 0.5% bovine serum albumin (BSA) in 0.1 M Tris solution (TS) for 30 min, 200 μm horizontal sections were incubated in the goat anti-TRPV1 primary antibody (1:500, Santa Cruz Biotechnology, Inc. Cat # sc12498, Dallas, TX; 0.1% BSA, 0.25% Triton X-100 (Sigma, St. Louis, MO) in 0.1 M TS) for 2 nights at
4 °C with continuous agitation. Bound TRPV1 primary antibody was visualized by incubating the Vc tissue sections in biotinylated horse anti-goat IgG secondary antibody (1:400, Vector Laboratories Cat # BA-9502, Burlingame, CA; 0.1% BSA in 0.1M TS) for 30 min followed by 30 min incubation in Avidin–Biotin (Elite Vectastain ABC kit; Vector Laboratories), then diaminobenzidine–hydrogen peroxide (DAB–H₂O₂) solution for 2 min. Tissue sections were mounted on slides, serially dehydrated in increasing ethanol concentrations and xylenes, then coverslipped using DPX mounting medium (Sigma). Images from sections containing Vc and spV were collected using an Olympus BX51 microscope equipped with a DP71 camera (Olympus America, Center Valley, PA) and associated software.

Data Analysis: Data (frequency, amplitude, bath temperature) were analyzed offline using MiniAnalysis (Synaptosoft Systems) and O-Phys (courtesy of Jason Frazier, University of Florida) in OriginLab 8.6. Individual cells were evaluated for normal distribution using the appropriate parametric (Student’s T-test) or nonparametric tests (Kolmogorov-Smirnov, K-S). Temperature sensitivity was determined by one-way analysis of variance (ANOVA; post hoc, Student-Newman-Keuls), 2-way ANOVA (Bonferroni post-hoc), and by calculating Arrhenius relationships with the thermal coefficient - Q₁₀ - (Ni et al. 2006). Data were plotted then fitted by linear regression, and the slope values (slope) and the correlation coefficient (R²) calculated to quantify thermal sensitivity. Group data are presented as mean ± standard error of the mean (SEM) and were statistically compared using either the Student’s T-test (2 groups) or repeated measures ANOVA (> 2 groups). Statistical significance was set at *p ≤ 0.05.

Results: Spontaneous EPSCs (sEPSCs) were evident in all Vc neurons (Figure 1). At a bath temperature of 36°C, the frequencies of sEPSCs were quite high in most neurons (Figure 1A) but cooling to 30°C markedly reduced this rate. Changes in bath temperature were closely tracked in the sEPSC rate in such thermally responsive neurons (e.g. increases in temperature evoked increases in sEPSC rate, Figure 1C). In the remaining neurons, the basal sEPSCs rates were substantially lower even at warm, physiological...
temperatures and poorly tracked changes in bath temperatures (Figure 1B, D). Thus, the basal sEPSC rates identified two distinct groups of Vc neurons with inputs that had high and low rates of release (Figure 2A): High (≥ 10 Hz) or Low (< 10 Hz) neurons, respectively, when compared at 35°C. Note that High neurons responded to changes in temperature with substantial changes in sEPSC rate while Low neurons showed little change in sEPSC rate. This separation by frequency extended to the full temperature-sEPSC rate relations for individual neurons such that the sEPSC rate of High neurons did not overlap with relations of individual Low neurons and vice versa (Figure 2A); therefore, the standard recording temperature was set at 32°C. The non-N-methyl-D-aspartate (non-NMDA) receptor selective antagonist DNQX (5-20 μM in GBZ; n = 4) blocked 99.9 ± 0.01 % of sEPSCs in either type of neuron at all temperatures. Temperature changes did not alter the amplitudes of sEPSCs in the two groups of neurons (Figure 2B), suggesting that temperature acts at a presynaptic site to selectively promote the frequency of glutamate release. The magnitude of the changes in sEPSC frequency also suggests that temperature is the predominant factor driving tonic glutamate release onto superficial Vc neurons at normal temperatures.

To test the physiological relevance of these sEPSCs, we turned to current clamp conditions to record action potentials. At 36°C, most High neurons discharged spontaneously and lowering the temperature of the bath decreased and then eliminated the action potential frequency (Figure 3A). As might be expected from the synaptic recordings, Low neurons displayed modest instantaneous action potential discharge rates that decreased to silence by lowering the temperature (Figure 3B). This subset of cells (n=21) was initially classified as High (12.9 ± 1.1 Hz, n=11) or Low (2.1 ± 0.4 Hz, n=10) under the voltage clamp protocols. Under current clamp, 67% of recorded superficial Vc neurons had temperature sensitive action potential responses that resembled their sEPSC responses (i.e. n_{High}=7, n_{Low}=7). Action potentials in the remaining 7 neurons were not temperature sensitive (n_{High}=4; n_{Low}=3), evidence of the
specific and restricted site of action of high thermal sensitivity. Note that the action potential response to current injection was not altered by temperature changes - a finding that is consistent with a presynaptic action and postsynaptic electrical excitability. These data suggest that thermally evoked, spontaneous glutamate release is sufficient to initiate postsynaptic action potentials and therefore this information will be conducted within these circuits.

Spontaneous release can either arise from mechanisms within presynaptic terminals or be generated by local circuit pathways (Kaeser and Regehr 2014). To test whether conducted presynaptic action potentials might contribute to temperature-induced sEPSC responses, we generated thermally responsive sEPSCs and then added TTX (1 µM), a voltage-gated Na⁺ channel blocker, to the aCSF and repeated the temperature challenges (Figure 4A). On average in High neurons (n=13), the sEPSC rates without TTX were not different from the rates of miniature EPSCs (mEPSCs) measured in TTX in these same neurons (p > 0.05, Student’s T-test; Figure 4B). Note that thermally driven changes in mEPSC rate, like sEPSCs, closely tracked bath temperature and were readily reversible (Figure 4A). These observations suggest that, in High neurons, thermal sensitivity is intrinsic to the glutamate terminals directly contacting the recorded neurons (Figure 4B). In Low neurons (n=5), TTX did not alter basal sEPSC rates (sEPSCs: 1.6 ± 0.8 Hz; mEPSCs: 2.2 ± 1.5 Hz, p = 0.91, Student’s T-test).

In some Low neurons, the warmest temperatures increased the sEPSC rates significantly above sEPSC rates recorded at lower temperatures (Figure 1B). Note, however, that the average absolute frequencies of sEPSCs were quite low and any increases at warmer temperatures were small and often inconsistent (Figure 1D, 2A, 4C). Application of TTX reduced the sEPSC rates and eliminated temperature-dependent changes in Low neurons (Figure 4D). This result suggests that sEPSC rates at 36°C arose from conducted action potentials (Figure 4D). Thus, glutamate terminals directly contacting Low neurons are not
themselves intrinsically thermosensitive but the neurons received conducted action potentials from other thermosensitive neurons (e.g. High neurons). Our evidence suggests that physiological temperatures directly generate a high tonic level of glutamate release at the majority of central terminals within the superficial lamina of Vc that can activate local circuits.

To better understand intrinsic thermal synaptic responses at Vc neurons, all subsequent tests measured mEPSCs in TTX (n=54). On average, three-quarters of Vc neurons were classified as High with mEPSC rates 2.8 fold greater at 32°C than the remainder, which were Low neurons (Figure 5A). The thermal relations for mEPSC frequencies of High neurons (n=36) were well fit by a simple, least-squares linear regression (Slope = 2.0 ± 0.2 Hz/°C, R²= 0.94, Figure 5B) and showed substantial temperature sensitivity (Q₁₀=17.8). In contrast, the average mEPSC rate in Low neurons (n=18) was not altered by temperatures between 30 - 36 °C as evidenced by a horizontal slope (0.0 ± 0.1 Hz/°C; R² = -0.20) and thermal coefficient (Q₁₀ = 3.2 ± 1.2; Figure 5A, B). However, it should be noted that at the lowest temperatures tested (30°C), the mEPSC rate of High, temperature-sensitive neurons equaled that of Low, temperature-insensitive neurons, i.e. cooling made these very different neurons appear similar.

Histological plots of the location of High and Low neurons showed an indistinct, intermixed distribution within the superficial laminae of Vc (Figure 5C). Staining for TRPV1 showed a band of immunoreactivity largely limited to within the superficial lamina of Vc and this TRPV1 distribution largely overlapped with that of the recorded neuron locations (Figure 5D). This proximity suggested that capsaicin sensitivity may correlate with thermal sensitivity as previously observed in NTS (Shoudai et al. 2010). While the majority of Vc neurons (67%, n = 36 of 54; Figure 5E) were thermally responsive to our tests, these temperatures were well below the conventional minimum threshold of 42°C for TRPV1 activation. Exposing neurons to capsaicin (CAP, 100 nM; TTX) significantly increased the frequency of mEPSCs in
nearly three-quarters of Vc neurons by an average of ≥5-fold (32°C, TTX: 6.3 ± 2.9 Hz, TTX+CAP: 45.8 ± 9.1 Hz, \(*p < 0.01\), Student’s T-test). Event amplitudes were unchanged during CAP in most Vc neurons (73%, n = 19 of 26; Figure 5E) and the holding current was not significantly altered (TTX: -55.0 ± 24.3 pA; CAP: -95.4 ± 34.6 pA, \(p = 0.31\)). Both of these observations are consistent with a presynaptic localization of and action at TRPV1 receptors.

To examine the association of TRPV1 with thermal release of glutamate, we next tested CAP and temperature challenges in the same Vc neurons. In detailed studies, twenty Vc neurons completed the full test protocol on mEPSCs (Figure 6 and 7). CAP activated mEPSCs indicating that approximately 60% of the neurons received TRPV1- expressing inputs (TRPV1+ neurons) (Figure 6). Surprisingly, only about half of these TRPV1+ neurons had High basal mEPSC rates and significant temperature sensitivity (Figure 6A, C, D, n=6). The remaining TRPV1+ neurons were thermally-insensitive, Low neurons with minimal basal mEPSC rates (Figure 6B, E, F; n=6). CAP increased mEPSC rates similarly in these subsets of High and Low neurons (Figure 6C and 6E, respectively). The remaining 40% of neurons in this group were TRPV1- (insensitive to CAP) but, like the dichotomy observed in TRPV1+ recordings, these TRPV1- inputs were either temperature-sensitive (Figure 7A, C, D; n=6) or not (Figure 7B, E, F; n=2). TRPV1- neurons included both High and Low based on basal mEPSC rates (Figure 7, 8). Interestingly, the temperature required to increase mEPSC rate during warming in neurons with thermally sensitive inputs occurred at a lower value in TRPV1+ neurons (34°C) than in TRPV1- neurons (36°C; Figure 6D, 7D, \(p < 0.05\)). Such paired observations of Vc neurons indicate that neither high intrinsic mEPSC rate nor high temperature sensitivity was exclusively associated with TRPV1 expression and vice versa, resulting in the highly heterogeneous groups of glutamatergic synapses (Figure 8).

Spontaneous glutamate release increase with age in neurons in the spinal dorsal horn (Baccei et al., 2003). In Vc neurons, we compared mEPSC rates between postnatal development ages: P7-13 (week 2),
P14-20 (week 3), and P21-28 (week 4). At physiological temperatures (36ºC), the absolute mEPSC rates were significantly higher in recordings from P14-20 (n=15) and P21-28 (n=8) compared to those at P7-13 (n= 15; p < 0.05, 2-way ANOVA; Figure 9A). No differences in mEPSC rates were observed from thermally insensitive inputs (Temp-; p > 0.05, 2-way ANOVA; Figure 9B). Event amplitudes were similar with temperature or age (p > 0.05, 2-way ANOVA; Figure 9C). The proportion of neurons that were Temp+ and Temp- was uniform across this developmental range (60-65%; Figure 9D). Lastly, CAP evoked similar significant increases in mEPSCs rates across all age groups (*p < 0.01 versus TTX, p = 0.31 between ages, 2-way ANOVA, Bonferroni post-hoc; Figure 9E). Together, these data suggest that similar cohorts of temperature-sensitive afferents contact Vc neurons but that the thermal mechanism matures during the first 2 weeks of life.

Discussion: Transmission of sensory information from craniofacial regions to Vc neurons relies on the release of glutamate. Measuring spontaneous and miniature EPSCs of Vc neurons, we found that: (1) basal rates of glutamate release identified two distinct neuron populations, either High or Low activity Vc inputs; (2) the starkly higher mEPSC rate of High neurons near physiological temperature (36°C) was eliminated by cooling neurons to 30-32°C; (3) thermally triggered glutamate release upon warming was responsible for most spontaneous release at High neurons via a presynaptic action in which amplitudes were unchanged; (4) thermal sensitivity of Low neurons was minor and indirect (eliminated by TTX); and (5) a non-TRPV1-mediated mechanism drives thermally regulated glutamate release in some TRPV1- Vc neurons. Our tests suggest at least four distinct groups of Vc neurons based on the intrinsic characteristics regulating glutamate release within the superficial laminae of Vc. These glutamate terminals may be a new, central integrative locus in Vc in which presynaptic mechanisms excite
postsynaptic neurons even in the absence of conducted afferent discharge. This ongoing activity in normal conditions might be modified by other signaling mechanisms including GPCRs.

Rate of spontaneous glutamate release classifies Vc synapses: Spontaneous neurotransmitter release has conventionally been thought to reflect a low probability, spontaneous expulsion of synaptic vesicles from a pool of readily releasable vesicles primed for release by action potentials (Kaeser and Regehr 2014). Growing evidence suggests that spontaneous release in some central synapses represents neuronal communication independent of evoked release (Glitsch 2008; Kavalali et al. 2011). Basal release is exceedingly low (<0.02 Hz) at many central neurons (Kavalali et al. 2011). In the superficial Vc, in contrast, basal EPSC frequencies ranged from 1 to nearly 20 Hz (Grudt and Williams 1994; Inoue et al. 2012; Jennings et al. 2003). We report that, within this broad range of basal sEPSC rates, Vc neurons distinctly divided into either High (≥ 10 Hz) or Low (< 10 Hz) but this distinction disappeared with subphysiological cooling. Modest temperature changes altered the sEPSC rates only in High Vc neurons and likewise altered postsynaptic action potential rates. In TTX, only mEPSC frequency changed indicating an intrinsic presynaptic mechanism with high thermal sensitivity (High Q10=17.8) that drives to glutamate release. Since cooling to 30°C rendered the two populations indistinguishable, it is unlikely that differing numbers of synaptic contacts was responsible for the High/Low distinctions (Sedlacek et al. 2007). This thermally sensitive release mechanism in Vc resembles that of TRPV1+ solitary tract afferents in medial NTS neurons (Shoudai et al. 2010).

Origins of central thermal sensitivity: Most biological processes including neurotransmission are affected by temperature but often only weakly (Q10 < 3) or not at all between 33 and 38°C (Klyachko and Stevens 2006). In contrast, the high thermal coefficient of glutamate release on Vc neurons suggests a relatively narrow set of potential molecular drivers – chiefly the most sensitive thermoTRPs (Caterina 2007). The best-characterized member of these is TRPV1. TRPV1 is expressed throughout the trigeminal afferents...
extending to their presynaptic terminals in the superficial Vc thereby identifying primary afferent fibers (Bae et al. 2004; Braz and Basbaum 2010; Cavanaugh et al. 2011a; Cavanaugh et al. 2011b; Davies and North 2009; Jennings et al. 2003). In our system, 70% of Vc neurons were CAP sensitive (i.e. TRPV1+). Such TRPV1+ neurons in Vc were evenly divided in their afferent sensitivity to physiological temperatures and basal rates of release. Thermal sensitivity of afferents in some Vc neurons might reasonably be linked to a TRPV1 similar to those in the NTS or dorsal motor nucleus of the vagus (Anwar and Derbenev 2013; Shoudai et al. 2010). However some TRPV1+ Low Vc neurons failed our modest thermal challenges; yet we cannot rule out the possibility that TRPV1 and thermal sensitivity was active at > 42°C which is more typical of TRPV1 in heterologous expression systems and dorsal root ganglion neurons (Caterina et al. 1997). An additional set of neurons had quantitatively similar thermal sensitivity as TRPV1+ neurons but tested negatively with capsaicin. The presence of thermally sensitive sEPSCs without TRPV1 points to expression of another thermosensor as responsible in this subpopulation in trigeminal inputs. A comparative analysis of TRP mRNA in trigeminal ganglion (TG) and dorsal root ganglion revealed regionally distinct patterns of expression individual TRP channels (Vandewauw et al. 2013). Transcripts in TG identified TRPV4, TRPM5 and TRPM3 – all of which are activated at physiological temperatures (Caterina 2007; Talavera et al. 2005; Vriens et al. 2011). Unfortunately, these additional thermoTRPs are difficult to study functionally outside of expression systems due to lack of selective agonists and antagonists and thus their evaluation will necessitate genetic models and other strategies.

Sources of afferent heterogeneity: The marked heterogeneity in Vc synaptic responses likely reflects differences in terminal phenotype. We classified one fundamental aspect of primary afferent neurons (TRPV1+), but our data support at least four sub-classifications of synaptic responses. Primary trigeminal
afferents are quite diverse (Hegarty et al. 2010; Kobayashi et al. 2005) and, therefore, the variety of responses is not surprising. Many peripheral sensory responses, however, can show primary afferent nerve activation with decreases in temperature described in the spinal cord (Wrigley et al. 2009) and during extracellular in vivo recordings from the Vc (Kurose and Meng 2013) – something that we rarely observed in the central responses (1 of 151 recordings). Remarkably, increases in temperature directly increased glutamate release in most of the Vc neurons tested. In addition to primary trigeminal afferents, Vc neurons receive input from cervical dorsal root ganglia neurons or IX and X cranial nerves with soma in the jugular and nodose ganglia (Beckstead and Norgren 1979; Pfaller and Arvidsson 1988). This raises the interesting possibility that the peripheral and central portions of primary afferents may have different thermal thresholds and sensitivities that correspond with their systemic source. Alternatively, these temperature-sensitive and –insensitive, TRPV1- neurons may originate from central neurons creating a complex synaptic arrangement. Glutamate released in Vc may arise from interneurons (Kato et al. 2009) comprising inter-nuclear and -laminar connections (Hamba and Onimaru 1998; Han et al. 2008; Onodera et al. 2000), as well as descending pathways (Aicher et al. 2012; Sato et al. 2013). Knowledge of the degree of synaptic convergence of such afferent nerves is limited in Vc but the varied thermal and CAP responses could reflect these diverse sources (Zanotto et al. 2007). Combining anatomic approaches with patch clamp recordings may help identify distinct Vc synapses and pathways (Gracheva et al. 2011; Malin et al. 2011).

**Physiological implications and pharmacological considerations**: The physiological ramifications of spontaneously released glutamate are unclear. The very small temperature shifts and the physiological range suggest that central brain temperature will significantly impact spontaneous excitatory transmission, and those signals will generate postsynaptic action potentials at normal temperatures in
Vc. Our observations that thermal sensitivity increases with age further indicating an important role for such tightly regulated spontaneous glutamate release in Vc synaptic relay that is not an artifact or paradigm-dependent. Recent evidence has suggested that such synaptically active terminals, even in the absence of evoked afferent activity, serve to shape and maintain synaptic connections (McKinney et al. 1999) and can be regulated in isolation from evoked release (Kavalali et al. 2011). Since trigeminal afferents share some synaptic characteristics with both solitary tract and spinal afferents, it is plausible that spontaneous release is subject to modulation independent of evoked release; studies combining examination of electrically evoked and thermally regulated spontaneous release within recordings will answer this question directly. This thermally driven, spontaneous release is modulated by GPCRs (Fawley et al. 2011) suggesting that despite being autonomous, it can be influenced by other neural inputs and may participate in network signaling activity to affect other brain regions.

Conclusions: Prevailing normal temperatures tonically activate thermally sensitive afferents in most superficial Vc neurons without action potential-induced afferent activation. Thus, synaptic transmission in the superficial lamina of Vc is strikingly diverse compared to other regions that receive primary afferent input such as the spinal dorsal horn (Bereiter et al. 2000) and the NTS (Shoudai et al. 2010). Distinct properties of spontaneous glutamate release including basal rate, thermal sensitivity, and capsaicin responses identified subpopulations of Vc synapses. The autonomous nature of spontaneous glutamate signaling may provide unique synaptic signals differentiating particular circuits that may be altered in pathological states and subject to pharmacological regulation.

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Figure Legends

Figure 1: Physiological temperatures drive spontaneous glutamate release in Vc. Traces representing basal and temperature responses (30–36°C) in Vc neurons with (A) High spontaneous activity and (B) Low spontaneous activity. Individual diary plots of sEPSC frequency (black bars) and bath temperature (red line) over the first 15 min of recording are shown in (C) High and (D) Low, for cells shown in (A) and (B), respectively. sEPSC rate tracked with increases or decreases in temperature accordingly.

Figure 2: Temperature acts presynaptically to drive glutamate release. (A) Neurons segregated into 2 distinct groups: High (red squares, n = 7) with sEPSC rates ≥ 5Hz or Low (blue squares, n = 5) sEPSC rates < 5Hz at 32 °C. Significant increases in sEPSC rate were observed at temperatures ≥ 34 °C regardless of basal rate (p < 0.001, ANOVA). (B) Amplitudes were not different between High and Low neuron groups (p > 0.05, Student’s T-test), and temperature did not significantly change event size (p > 0.05, ANOVA). Large symbols represent the mean value (± SEM); individual cells are represented by colored lines.

Figure 3: Temperature-evoked sEPSCS generate postsynaptic action potentials. Representative current clamp recordings of superficial Vc neurons with sEPSC rates classified as (A) High, ≥ 5Hz (n=7) or (B) Low < 5Hz (n=7) sEPSC rates at 32 °C . As temperature increased from 30 to 36°C, the number of action potentials generated increased regardless of basal sEPSC rate; the magnitude of elicited AP responses was dependent on sEPSC category as observed at 36°C (right panels).

Figure 4: Blockade of voltage-gated Na+ channels in High and Low neurons reveals selective drive of thermally evoked glutamate release. (A) The histogram of a representative High neuron where sEPSC frequency tracks closely and is reversible with bath temperature in control solution (left, 0 - 5 min). Addition of tetrodotoxin (TTX, 1 µM) to block voltage-gated Na+ channels isolated activity-independent
glutamate release and did not alter the rate of thermally evoked EPSCs (6 - 10 min). (B) mEPSC frequency from High neurons (n = 13) is not significantly different in the control solution (red squares) versus TTX (red circles) (p > 0.05, Student’s T-test). Increases in EPSC rate were intact after exposure to TTX suggesting High neurons were directly activated by temperature. (C): A histogram of EPSC frequency and bath temperature for a Low neuron in control solution (sEPSCs, 0 - 5 min) and in TTX (mEPSCs, 6 - 10 min). (D) For Low neurons, inclusion of TTX (blue circles) in the bath solution significantly attenuated thermally evoked responses, but not basal release in control solution (blue squares) suggesting that Low neurons (n = 5) were insensitive to temperature changes from 30-36°C (**p ≤ 0.01, ANOVA). Data in (B, D) are shown as mean (± SEM).

**Figure 5: Glutamate release in Vc is either temperature-sensitive or -insensitive. (A)** TTX-isolated, direct inputs to Vc for High (red, n = 36) or Low (blue, n = 18) neurons; mEPSC frequency is temperature-sensitive or –insensitive, respectively. Significant increases in mEPSC rate over the basal frequency at 32°C were observed when the bath was ≥ 34 °C (*p ≤ 0.05, ANOVA) for High inputs. Increasing or decreasing bath temperature to 36 or 30, respectively, from 32°C did not significantly change mEPSC rates in Low neurons (blue bar, p > 0.05, ANOVA). Data are represented as mean (± SEM) (B) Linear regression analysis of Vc neurons confirmed thermal sensitivity of spontaneous glutamate release from High neurons (Red) from Low neurons (Blue). Dotted lines represent the 95% confidence interval. (C) Localization map of recording sites for temperature-sensitive (red circles) and temperature-insensitive (blue triangles) neurons. Thermally defined populations overlapped throughout the Vc. Scale bar = 100 µm. (D) TRPV1 immunoreactivity is restricted to the superficial lamina of Vc in the horizontal slice, paralleling the recording zone in (C); Scale bar = 250 µm. (C, D) are orientated such that rostral is to the left and midline is at the bottom. spV: spinal trigeminal tract, Vi: trigeminal nucleus interpolaris, Vc: trigeminal nucleus caudalis. (E) Summary pie charts Temperature (left, n=54) and CAP (right, n=26)
responsive neurons in Vc. Color coding is such that Red: Temperature-sensitive (Temp+); Blue: Temperature-insensitive (Temp-); White: CAP-sensitive (CAP+); Yellow: CAP-insensitive (CAP-)

Figure 6: TRPV1 expression does not correlate to intrinsic rates of release or thermal sensitivity

Representative traces of superficial Vc mEPSC recordings with High (A, red bars) or Low (B, blue bars) basal release rates in TTX at 32 °C (top), 36 °C (middle), and in CAP 100nM (bottom). Inputs were considered to be capsaicin sensitive and therefore TRPV1 expressing if application of CAP resulted in a mEPSC rate more than twice that observed in TTX, 32 °C. CAP evoked a ≥ 5 fold increase in mEPSC rate High (C, n=6). In these same recordings, temperature manipulation directly induced increases or decreases in mEPSC rate when warming of cooling steps, respectively were applied to High neurons (D). Despite similar responses to CAP application (E), Low neurons with TRPV1 did not have altered rates of glutamate release across 30-36 °C (F). Capsaicin (100nM, CAP) was applied at the end of Vc recordings in the presence of TTX (Student’s T-test). CAP effects on mEPSCs were analyzed independently from thermally evoked responses (ANOVA). Data in C-F represent the Mean ± SEM. Statistical significant is denoted by *p ≤ 0.05, **p ≤ 0.01.

Figure 7: Thermal sensitivity persists in neurons lacking TRPV1. Representative traces of superficial Vc mEPSC recordings in sensitive to capsaicin with High (A, red bars) or Low (B, blue bars) basal release rates in TTX at 32 °C (top), 36 °C (middle), and in CAP 100nM (bottom). Inputs were considered to be capsaicin insensitive if application of CAP did not increase the mEPSC rate more than twice that observed in TTX, 32 °C. CAP failed to evoke enhanced mEPSC rates in 8 recordings. In TRPV1- recordings with a High mEPSC rate (C, n=8), temperature changes directly influence mEPSC rate when warming of cooling steps were applied, respectively (D). (E, F) Rarely, Low neurons were encountered without TRPV1 that did not have altered rates of glutamate release across 30-36 °C (F). Capsaicin
(100nM, CAP) was applied at the end of Vc recordings in the presence of TTX (Student’s T-test). CAP effects on mEPSCs were analyzed independently from thermally evoked responses (ANOVA). Data in C-F represent the Mean ± SEM. Statistical significant is set *p ≤ 0.05.

**Figure 8: Vc synaptic populations with respect to temperature sensitive glutamate release and TRPV1 expression.** Summary of the proportion of neurons with mEPSC rates sensitive to modest temperature (Temp+) changes and/or CAP application (TRPV1+). CAP sensitivity was not predictive of thermal sensitivity such that 4 populations were identified: Temp+/TRPV1+ (red), Temp-/ TRPV1 + (blue), Temp+/ TRPV1- (orange), and Temp-/TRPV1- (green). Capsaicin (100nM, CAP) was applied at the end of Vc recordings in the presence of TTX and subsequent effects on mEPSCs were analyzed independently from thermally evoked responses.

**Figure 9: Thermally sensitive miniature glutamate release is enhanced during postnatal development.**

(A) The rate of thermally-evoked miniature glutamate release is significantly greater in rats older than 14 days (grey P14-20, n=15; black P21-28 days, n=8) compared to P7-13 (white, n=15) at 36°C (*p < 0.05, 2-way ANOVA). (B) In contrast, postnatal development does not change the rate of miniature release onto neurons from thermally insensitive inputs (P7-13, n=8; P14-20, n=9; P21-28, n=4). (C) Amplitudes of mEPSCs were similar across developmental ages and input types. (D) The percentage of Vc cells with either temperature-sensitive (Black, Temp+) or –insensitive (white, Temp-) inputs was unchanged across the development age range from P7 to P28. . (E) Responses of mEPSC rates to capsaicin (100nM) were similar regardless of age (P7-13, n=8; P14-20, n=16; P21+, n=3; p > 0.05, ANOVA). CAP increased rates in all TRPV1+ neurons *p < 0.05). All experiments were performed in TTX (1µM) and GBZ (3µM).
High, Temp+, TRPV1+, n=6
High, Temp+, TRPV1-, n=6
Low, Temp-, TRPV1+, n=6
Low, Temp-, TRPV1-, n=2