

NMDA Receptors Potentiate Activity-dependent Dendritic Release of Neuropeptides from Hypothalamic Neurons

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Abbreviated title: Activity-dependent neuropeptide dendritic release

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KEY POINT SUMMARY

- Using “sniffer” cell biosensors, we evaluated the effects of specific firing patterns and frequencies on activity-dependent somatodendritic release of VP from PVN neurones. This is an Accepted Article that has been peer-reviewed and approved for publication in the The Journal of Physiology, but has yet to undergo copy-editing and proof correction. Please cite this article as an 'Accepted Article'; [doi: 10.1113/JP277167](https://doi.org/10.1113/JP277167).

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- Somatodendritic release of VP was rarely observed during continuous firing but was strengthened by clustered activity. Moreover, release evoked at any given frequency was robustly potentiated by NMDAR-mediated firing.
- Differently from axonal release, NMDAR activation was necessary for somatodendritic release to occur at physiological firing frequencies, acting thus as a gating mechanism by which activity-dependent release from these two neuroneal compartments could be independently regulated.
- The NMDAR-mediated potentiation was independent of a specific firing pattern and was not accompanied by increased spike broadening, but correlated with higher dendritic Ca^{2+} levels.
- Our studies provide fundamental novel information regarding stimulus-secretion coupling at somatodendritic compartments, and shed light into mechanisms by which activity-dependent release of neuronal signals from axonal terminals and dendrites could be regulated in a spatially compartmentalized manner.

ABSTRACT

Dendrites are now recognized to be active transmitting neuronal compartments subserving complex brain functions, including motor behaviors and homeostatic neurohumoral responses. Still, the precise mechanisms underlying activity-dependent release of dendritic signals, and how dendritic release is regulated independently from axonal release, remains largely unknown. We used “sniffer” biosensor cells to enable the measurement and study of activity-dependent dendritic release of vasopressin (VP) from hypothalamic neurones in brain slices. Sniffer_{VP} responses were dose-dependent, with a threshold detection level of 0.5 nM for VP, being thus a highly sensitive tool to detect endogenous physiological levels of the neuropeptide. Somatodendritic release of VP was rarely observed in response to a burst of action potentials fired in continuous mode, but was strengthened by clustered firing activity. Moreover, release evoked at any given frequency was robustly potentiated when firing was triggered by NMDA receptor (NMDAR) activation. Differently from axonal release, NMDAR activation was necessary for dendritic release to occur at physiological firing frequencies. Thus, we propose that NMDARs may act as a gating mechanism by which activity-dependent release from these two neuronal compartments can be independently regulated. The NMDAR-mediated potentiation of dendritic release was independent of a particular action potential waveform, firing pattern evoked, or a more pronounced spiked broadening, but correlated with higher dendritic Ca²⁺ levels. Overall, our studies provide fundamental novel information regarding stimulus-secretion coupling at neuronal dendrites, and shed light into mechanisms by which activity-dependent release of neuronal signals from axonal terminals and dendrites can be regulated in a spatially compartmentalized manner.

INTRODUCTION

Transfer of information in the brain was classically considered a unidirectional process, in which dendrites acted as passive receptive fields, integrating and transferring signals to the soma and then to the axon, leading ultimately to release of a chemical transmitter. It is now well accepted however that dendrites possess active properties, that they can transmit action potentials in the reverse direction (Stuart *et al.*, 1997), and can also actively release signals (Cheramy *et al.*, 1981; Toida *et al.*, 1994; Ludwig & Leng, 2006; Kennedy & Ehlers, 2011; Rice & Patel, 2015), particularly neuropeptides from dendritic large dense-core vesicles (LDCVs). Thus, dendrites act not only as receivers, but also as transmitting cell compartments in information processing in the brain.

Somatodendritic release of neuropeptides is now recognized to play critical physiological roles, including autoregulation of neuronal activity (Ludwig & Leng, 1997, 2006), generation of multimodal homeostatic responses (Son *et al.*, 2013), and complex behavioral responses as well (Engelmann *et al.*, 1994; Ludwig & Leng, 2006; Albers, 2015). Nonetheless, most available knowledge on stimulus-secretion coupling originated almost exclusively from studies on synaptic vesicles and classical neurotransmitters at axonal terminals. Thus, fundamental information regarding the precise mechanisms governing stimulus-secretion coupling of dendritic LDCVs in the CNS remains largely unexplored.

Magnocellular neurosecretory cells (MNCs) of the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei are one of the best-characterized prototypes of dendritic neurotransmitter release (Morris & Ludwig, 2004; Ludwig & Leng, 2006). In addition to releasing their vasopressin (VP) and oxytocin (OT) cargo from neurohypophysial axon terminals, MNCs also store and release neuropeptides from their somata and dendrites. Somatodendritic neuropeptide release from MNCs is activity-dependent, involves Ca^{2+} -dependent exocytosis of LDCVs, and importantly, it can be regulated autonomously from axonal release (Ludwig *et al.*, 2005; Ludwig & Leng, 2006). Somatodendritic release of VP

and OT acts as a powerful feedback signal by which MNCs autoregulate their own activity (Ludwig & Leng, 1997; Gouzenes *et al.*, 1998). Moreover, we recently showed that somatodendritically released VP acts as a diffusible interpopulation signal to coordinate the activity of neurosecretory and sympathetic neurones, and to mediate the generation of multimodal homeostatic responses by the PVN (Son *et al.*, 2013). Despite the physiological relevance of dendritically released OT and VP, information about the precise mechanisms that regulate their release is missing, due in part to the lack of efficient tools available to measure neuropeptide release with high spatiotemporal resolution and sensitivity.

In the present study, we used MNCs as a model system to study stimulus-secretion coupling mediating dendritic release of LDCVs. To monitor somatodendritic release of VP *in situ* and in real time, we used “sniffer” biosensor cells consisting of genetically engineering Chinese ovarian hamster (CHO) cells that expressed VP or OT receptors along with a fluorescent Ca^{2+} indicator. Using sniffer biosensors in conjunction with patch-clamp electrophysiology and confocal Ca^{2+} imaging in PVN slices from eGFP-VP transgenic rats, we assessed the impact of varying firing frequencies and firing patterns on dendritic release of VP. Moreover, given that glutamate, acting mostly on NMDA receptors (NMDARs), is a critical neurotransmitter driving firing activity in MNCs (Hu & Bourque, 1992; Fleming *et al.*, 2011), and based also on the fact that NMDAR activation was previously shown to efficiently evoke dendritic release of VP and OT (de Kock *et al.*, 2004; Son *et al.*, 2013), we assessed the role of NMDAR-evoked firing in regulating the efficacy of dendritic release of VP from MNCs. We found that neuropeptide dendritic release efficacy is dependent on the pattern (but not the total number or frequency) of somatic firing activity. We also uncover that for any given frequency, firing activity evoked by NMDA receptor (NMDAR) activation robustly boosted dendritic release of VP and shortened its release latency, an effect that correlated with larger dendritic Ca^{2+} signaling compared to other firing modalities. Differently from what was previously shown for axonal release, our studies demonstrate that activation of NMDARs was necessary for dendritic release to occur at physiological firing frequencies

typically displayed by VP neurones. Thus, we propose that NMDARs in MNCs may act as a “gating” mechanism, contributing to the autonomous activity-dependent regulation of neuropeptide release from dendritic and axonal compartments.

METHODS

Animals. All procedures were performed in agreement with guidelines of the Augusta University and Georgia State University Institutional Animal Care and Use Committee and were approved by the respective committees (approval reference no. 2008-0133). Male heterozygous transgenic eGFP-VP Wistar rats (3-5 weeks old, n= 32) were used (Ueta *et al.*, 2005). Rats were housed in rooms with constant temperature of 22-24°C and under a controlled light/dark cycle (12 h: 12 h), with normal rat chow and drinking water *ad libitum*.

Slice preparation. Hypothalamic brain slices were prepared according to methods previously described (Son *et al.*, 2013). Briefly, rats were anesthetized with pentobarbital (50 mg/kg ip); brains dissected out and hypothalamic coronal slices (240 μ m) containing the PVN were cut in an oxygenated ice-cold artificial cerebrospinal fluid (aCSF), containing in mM: 119 NaCl, 2.5 KCl, 1 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, 20 D-glucose, 0.4 ascorbic acid, 2 CaCl₂, and 2 pyruvic acid; pH 7.3; 295 mOsm). Slices were placed in a holding chamber containing aCSF and kept at room temperature until used.

Electrophysiology. Hypothalamic slices were transferred to a recording chamber and superfused with continuously bubbled (95% O₂-5% CO₂) aCSF (30°C-32°C) at a flow rate of ~3.0 ml/min. Thin-walled (1.5-mm OD, 1.17-mm ID) borosilicate glass (G150TF-3; Warner Instruments, Sarasota, FL) was used to pull patch pipettes (3-5 M Ω) on a horizontal micropipette puller (P-97; Sutter Instruments, Novato, CA). The internal solution contained

the following (in mM): 135 potassium gluconate, 0.2 EGTA, 10 HEPES, 10 KCl, 0.9 MgCl₂, 4 Mg²⁺ATP, 0.3 Na⁺GTP and 20 phosphocreatine (Na⁺); pH was adjusted to 7.2-7.3 with KOH. Unless otherwise indicated, all recordings were obtained from identified VP neurones in the lateral magnocellular subdivision of the PVN, using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), and with a combination of fluorescence and infrared differential interference contrast (DIC) illumination. Recorded neurones were verified as MNCs based on the presence of a robust transient outward rectification upon depolarization from hyperpolarized membrane potentials (Luther and Tasker, 2000). The voltage output was digitized at 16-bit resolution and at 10 kHz and was filtered at 2 kHz (Digidata 1440A; Axon Instruments). Data were discarded if the series resistance was not stable throughout the entire recording (> 20% change). Firing activity was evoked either via current injection through the patch pipette (10-100 pA) or by focally delivering NMDA via a patch pipette positioned within 10-20 µm from the recorded cell (picospritzer, 3-5 PSI, 10 µM NMDA).

For direct current injection, firing activity was evoked either in a continuous mode (CF) (depolarizing pulses of increasing duration, between 0.05 s and 3 s) or in a repetitive bursting mode (RBF), consisting of 5 consecutive stimuli, 0.5 s duration each, 0.2 s interval. We selected this particular protocol based on our previous work showing that 5 consecutive bursts were necessary to evoke an interpopulation crosstalk between VP MNCs and presympathetic PVN neurons, mediated by somatodendritically-released VP (Son *et al.*, 2013). For NMDA applications, the duration of the puff was varied (100-1000 ms) in order to achieve similar number of action potentials evoked via direct current injection). Following this procedure, we found that moving the puff pipette 20-25 µm (i.e., about 1-2 MNC body diameter) away from its original position failed to evoke a Ca²⁺ response in the patched neurone. Moreover, in a few control cases (n=4) in which slices were preloaded with the membrane permeant Ca²⁺ sensitive dye Fluo-5 AM (10 µM), using similar approaches as previously reported (Filosa et al., 2011), and in which a cluster of viable and loaded MNCs were close to each other, we found that Ca²⁺ responses were elicited only in the MNCs to

which the puff pipette was targeted. These control experiments, along with our results showing that somatodendritic release of VP in response to NMDA puff was almost completely blocked when the patched VP neurones were dialysed with BAPTA (see more in *Results*), support that a single MNCs was sufficiently stimulated with NMDA to evoke a detectable Ca^{2+} response.

In some cases, the experimentally recorded burst of action potentials evoked was then applied as a voltage-clamp command to the same recorded neurone. The number and frequency of spikes evoked (spikes/sec), for the entire duration of each stimulating protocol was calculated. Spike broadening during a train of action potentials was calculated by measuring individual spike width (at 50% of peak), which were then normalized to the spike width of the first action potential in the train. Plots of relative spike width as a function of the spike number in the train were generated and then fit with a linear regression to calculate the rate of spike broadening. All electrophysiology analysis was performed using Clampfit (Axon Instruments) or MiniAnalysis (Synaptosoft) software. The number of evoked action potentials and their frequency (number of spikes/time) are reported. For RBF, reported values represent the total number of spikes in the 5 elicited bursts, and the frequency represents the mean frequency calculated for each burst. As indicated throughout the text, drugs were either bath-applied, focally-applied with a picospritzer, or delivered as a bolus (0.5 ml) into the perfusion line.

Sniffer_{VP} and sniffer_{OT} cells. Chinese hamster ovarian (CHO) cells were transfected with pcDNA3.1+ containing human V1a or OT receptors cloned in at EcoRI (5') and XhoI (3') (plasmid obtained from Missouri S&T cDNA Resource Center) using lipofectamine and stable over-expression was achieved by geneticin (500 mg/ml) selection (Pinol *et al.*, 2014). V1aR/OTR-expressing CHO cells (sniffer_{VP} and sniffer_{OT} cells, respectively) were then plated and transiently transfected to express the red fluorescent genetically encoded Ca^{2+} indicator

(R-GECO; GenScript) with Eugene HD reagent. To examine the selectivity, affinity and responsiveness of these V1aR/OTR-expressing CHO cells, we measured their Ca^{2+} dose-response relationship following exogenously delivered VP and OT (10-100 nM). To study sniffer cell responsiveness to activity-dependent, endogenously released VP and OT, sniffer cells were treated with trypsin (0.05 %), re-suspended in aCSF and then plated directly onto a brain slice containing PVN eGFP-VP neurones. PVN neurones were stimulated as described above, and fluorescent Ca^{2+} responses were monitored in surrounding sniffer cells contained within the lateral magnocellular subdivision of the PVN. In a series of pilot studies, we determined that ~50-70% of the plated sniffer cells that were detectable based on their basal fluorescence level were responsive to their respective agonists (e.g., OT or VP, see **Fig.1A**). It is also important to know that Ca^{2+} responses were also evoked following different stimulation protocols in sniffer cells that had no detectable basal fluorescence (e.g., **Fig.2C**, cell #1). Finally, the density of plated sniffer cells varied across the slice. To circumvent these limitations and be consistent across studies, we restricted our experiments to patched eGFP-VP neurones that had at least 5 fluorescently visible sniffer cells in their vicinity (~10 on average). The overall mean distance from the center of the patched eGFP-VP somata to the responsive sniffer_{VP} cells was $46.6 \pm 6.7 \mu\text{m}$ (range 5-122 μm).

Confocal Ca^{2+} imaging and analysis of sniffer cells and patched MNCs. Sniffer_{VP} and sniffer_{OT} cells, as well as Fluo-5F pentapotassium salt (50 μM ; Invitrogen)-loaded MNCs (Stern & Potapenko, 2013), were imaged using the Andor Technology Revolution system (iXON EMCCD camera with the Yokogawa CSU 10, confocal scanning unit), at a rate of 4 Hz, using an excitation light of 488 nm and emitted light at > 495 nm (Fluo-5F). Basal fluorescence in sniffer cells was variable, likely due to multiple factors including variability in plasmid transfection efficacy and actual basal cytoplasmic Ca^{2+} levels, among others. Thus, for quantitative measurements, we used fractional fluorescence (F/F_0), a standardized and widely used approach to quantitatively assess relative changes in fluorescence. F/F_0 within

each sniffer cell was determined by dividing the fluorescence intensity (F) within a region of interest by a baseline fluorescence value (F_0) determined from 50 images before stimulation (Stern & Potapenko, 2013). Importantly, despite this intrinsic variability, we found in a subset of experiments no correlation between basal sniffer cell fluorescence and the magnitude of their Ca^{2+} response to the various firing modalities used in this study ($r^2 = 0.02$, $n = 31$). We also noticed that some sniffer cells showed intrinsic oscillatory Ca^{2+} activity. Thus, before each experiment, baseline images traces were obtained (5-10 mins) to identify oscillatory cells, which were discarded from analysis. Imaging data were analyzed using ImageJ software (NIH).

Neuronal dendritic Ca^{2+} responses (F/F_0) to the various stimulation protocols evoked in each neurone were quantified and normalized by the number of action potentials evoked. To better display changes in fluorescence levels, images were converted to pseudocolor using ImageJ.

Drugs. All drugs, with the exception of tetrodotoxin (TTX, Alomone Labs, Jerusalem, Israel), were purchased from Sigma Aldrich.

Statistical analysis. All values are expressed as means $\pm$ SE. Student's paired t tests were used to compare the effects of drug treatment. One- or two-way ANOVA tests with Bonferroni or Dunnet's post hoc tests were used as stated. Differences were considered significant at $p < 0.05$ and n refers to the number of sniffer cells. EC50 values were obtained by fitting dose-response data with a nonlinear function. All statistical analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

Quantitative detection of VP and OT with sniffer biosensor cells

Chinese ovarian hamster (CHO) cells that express V1a VP receptors (V1aRs) or OT receptors, along with the red fluorescent Ca^{2+} indicator R-GECO were used as VP or OT sensitive sniffer cells (sniffer_{VP} and sniffer_{OT}), respectively. Binding of VP to V1aRs is known to evoke an IP3-dependent release of Ca^{2+} from intracellular stores (Sabatier *et al.*, 2004), which is then visualized as an increase in R-GECO fluorescence within the sniffer_{VP} cell. To determine the efficiency and sensitivity of this system, sniffer_{VP} cells were first exposed to varying concentrations of VP. As shown in **Fig.1A**, VP evoked robust increases in sniffer_{VP} Ca^{2+} levels that were reproducible, with no significant changes in the magnitude of the response over time ($p = 0.8$, one-way ANOVA, $n = 4$, **Fig.1B1**). VP effects were dose-dependent, with an EC₅₀ of 7.2 nM and an apparent threshold response of 0.5 nM (**Fig.1B2**). The magnitude of the sniffer_{VP} Ca^{2+} response was also dependent on the proximity of the VP source to the CHO_{VP} cell ($p < 0.03$, one-way ANOVA, **Fig.1B3**).

Both VP and OT neuropeptides can bind V1aRs, though the sensitivity of the latter to OT is much lower compared to VP (Manning *et al.*, 2012). We found that at a concentration of 5 nM and 50 nM, OT failed to evoke Ca^{2+} responses in sniffer_{VP} cells. Even at 500 nM, OT responses were only $4.9 \pm 1.8\%$ of the response evoked by 50 nM VP on the same sniffer_{VP} cells (500 nM OT: $9.5 \pm 2.5 \text{ F/F}_0^* \text{s}$, $n = 10$ vs. 50 nM VP: $163.0 \pm 7.5 \text{ F/F}_0^* \text{s}$, $n = 29$, $p < 0.0001$). Conversely, a much more robust response to OT (5 nM) was observed in sniffer_{OT} cells ($180.3 \pm 27.2 \text{ F/F}_0^* \text{s}$, $n = 25$). Thus, sniffer_{VP} cells display a high sensitivity and selectivity for VP. These results are in large agreement with a recent work published at the time of submission of this manuscript, in which similar sniffer cells were generated using human embryonic kidney (HEK) cells transfected with GCaMP6m as the Ca^{2+} sensor (Zaelzer *et al.*, 2018), and further confirm the use of the sniffer cell approach as an efficient and sensitive tool to quantitatively detect oxytocin and vasopressin release.

Detection of activity-dependent somatodendritic release of neuropeptides from the population of VP PVN neurones using sniffer biosensor cells

To determine if in our conditions sniffer_{VP} cells could detect a mean endogenous “population” somatodendritic release of VP, sniffer_{VP} cells were enzymatically dispersed and plated onto PVN slices containing genetically identified VP neurones (i.e., eGFP-VP, **Fig.2A**, see more details in *Methods*) that were challenged with an osmotic stimulation (bolus, mannitol, +10 mOsm), a condition known to evoke activity-dependent somatodendritic release of VP (Ludwig *et al.*, 1994; Ludwig *et al.*, 1995). We simultaneously monitored the electrical activity of a patched eGFP-VP neurone in the PVN, which acted as a proxy to the VP population, as well as the Ca²⁺ responses in surrounding sniffer_{VP} cells. Exposing PVN slices to the acute osmotic challenge evoked firing activity in the patched VP neurones (total mean number of evoked spikes during the stimulation: 215.0 ± 121.1; mean firing frequency: 3.6 ± 0.9 Hz; delay from stimulation to onset of firing: 30.0 ± 10.4 s; n=4 VP neurones) that subsequently evoked Ca²⁺ responses in surrounding sniffer_{VP} cells (**Fig2.B,C**) (106.5 ± 19.1 F/F0*s; n=16 sniffer cells; mean delay from stimulation: 76.8 ± 14.4 s). Importantly, sniffer_{VP} Ca²⁺ responses were almost completely blocked in slices preincubated with 1 µM TTX (0.31 ± 0.17 F/F0*s; n= 31 cells from 7 slices, p< 0.0001 vs. control, **Fig2.D**). Sniffer_{VP} responses to the osmotic challenge displayed relatively slow kinetics and were long lasting (mean Ca²⁺ duration: 178.2 ± 11.3 s), likely as a result of the osmotically-driven asynchronous release of VP (Brown & Bourque, 2006) from multiple different sources.

Clustered but not continuous firing pattern evokes somatodendritic release of VP from individual neurones

We then aimed to determine whether and how different patterns of firing activity influenced stimulus-secretion coupling of somatodendritic LDCV's from individual VP neurones. To this end, we patched eGFP-VP neurones in PVN slices containing sniffer_{VP}

cells. Patched neurones were dialysed with Alexa 488 (50 μ M) in order to better visualize their dendrites (see examples in **Fig.2A**). As shown in the representative example in **Fig.3A**, depolarizing pulses of increasing durations (0.05 s - 3 s) evoked action potential activity in a continuous firing (CF) pattern. The range of the number of action potentials evoked in this modality was 2-120, with frequencies ranging from 14.7 to 40 Hz. Yet, no Ca^{2+} responses in neighboring sniffer_{VP} cells were observed in response to these depolarizing pulses. A similar lack of sniffer_{VP} responses to CF pattern activity was observed in 4 independent experiments in which similar depolarizing protocols were applied to patched VP neurones.

We recently reported that repetitive bursting firing (RBF) pattern in VP neurones engaged a functional crosstalk with neighboring presympathetic neurones, which resulted from the diffusion of dendritically-released VP in the extracellular space (Son *et al.*, 2013). We found here that when the same VP neurone that failed to release VP in CF mode, was subjected to the RBF pattern previously used (Son *et al.*, 2013) (5 consecutive stimuli, 0.5 s duration, 0.2 s interval), a Ca^{2+} response in a neighboring sniffer_{VP} cell was observed (2.3 F/F₀*s, representative example in **Fig.3B**). Another example showing sniffer_{VP} Ca^{2+} responses following RBF pattern activity in a VP neurone (149 action potentials, 49.7 Hz) is shown in **Fig.3C,D**. A mean summary of the magnitude, duration and delay of sniffer_{VP} Ca^{2+} responses following RBF activity is shown in **Fig.3E**. The mean number of responsive sniffer_{VP} cells per eGFP-VP neurone stimulated with the RBF modality (n= 11) was 1.1 ± 0.13 .

The number of evoked action potentials and their intraburst frequency during RBF varied greatly from cell to cell. However, we found no significant correlation between the magnitude of the sniffer_{VP} cell response with either the total number of action potentials or the mean action potential frequency ($r^2 = 0.04$ for both parameters, n=31 sniffer_{VP} cells associated to 11 stimulated eGFP-VP neurones).

NMDA receptor-evoked firing potentiates somatodendritic release of VP

The results above suggest that clustered firing is more efficient in evoking somatodendritic release of VP when compared to a continuous pattern of activity. Activation of NMDARs in MNCs also evokes clustered activity (Hu & Bourque, 1992; Bains & Ferguson, 1997; Gagnon *et al.*, 2014) and we recently showed that NMDAR-evoked firing activity in VP neurones more efficiently engaged a neurosecretory-presympathetic interpopulation coupling in the PVN, compared to firing activity evoked by direct current injection (Son *et al.*, 2013). Thus, to directly assess the efficacy and kinetics of somatodendritically released VP following NMDAR activation, patched eGFP-VP neurones (n=10) in PVN slices containing sniffer_{VP} cells were focally stimulated with NMDA (picospritzer, 10 μ M). To minimize the possibility of multiple MNCs stimulation, we set our puff protocols according to control experiments we previously run to minimize this possibility (see *Methods* and more below).

As shown in the representative example of **Fig.4**, NMDA evoked a burst of action potentials in the patched VP neurone (28 spikes, 43.8 Hz), as well as a clear Ca²⁺ response in 3 surrounding sniffer_{VP} cells, which occurred with a delay of ~1s after stimulation (**Fig.4B,D**). A summary of the mean magnitude, duration and delay of Ca²⁺ responses in sniffer_{VP} cells following NMDAR-evoked firing is shown in **Fig.4E**. The mean number of responsive sniffer_{VP} cells per stimulated eGFP-VP neurone was 2.2 ± 0.45 . Similar to the responses evoked with the RBF pattern, we found no significant correlation between the magnitude of the sniffer_{VP} cell response with either the total number of action potentials or the mean action potential frequency ($r^2 = 0.01$ and 0.002 , respectively) evoked by NMDAR activation.

Similar experiments were repeated before and after bath application of a V1a receptor antagonist (V2255, 1 μ M, n=5 patched eGFP-VP neurones). As shown in the representative example of **Fig.4C,D**, V2255 almost completely blocked sniffer_{VP} Ca²⁺ responses to NMDAR

activation (mean responsive sniffer_{VP} cells: 3.20 ± 0.20 and 0.8 ± 0.58 for NMDA and NMDA+V2255 respectively; mean sniffer_{VP} Ca^{2+} area: 49.4 ± 18.4 F/F0*s and 2.9 ± 1.3 F/F0*s for NMDA and NMDA+V2255 respectively $p < 0.01$ and 0.05 respectively). These results support that sniffer_{VP} Ca^{2+} responses following NMDAR-evoked firing in VP neurones were mediated by endogenously released VP, and not by a direct effect of NMDA on the sniffer_{VP} cells themselves.

To further confirm that the sniffer_{VP} Ca^{2+} responses following focal application of NMDA resulted from stimulation of the patched eGFP-VP neurone rather than from stimulation of multiple VP neurones, we repeated a subset of experiments in which patched eGFP-VP neurones ($n = 9$) were dialysed with the Ca^{2+} chelator BAPTA (5 mM) and focally stimulated with NMDA as before. Sniffer_{VP} cell responses in this condition were almost completely blocked, with only 2 out of 68 sampled sniffer cells showing a response (mean sniffer VP responses cells: 0.25 ± 0.15 , $p < 0.001$ vs. NMDA (2.2 ± 0.45 , see above). The number of responsive sniffer cells in BAPTA was too low to statistically compared the magnitude of the evoked response to that observed in NMDA).

Finally, it is worth noting that in a subset of experiments ($n=9$) NMDAR-evoked depolarizations of eGFP-VP neurons were insufficient to evoke action potential firing. Nonetheless, Ca^{2+} responses in sniffer_{VP} cell cells were still observed in 6/9 cases (**Fig.5**). The magnitude (8.8 ± 2.2 F/F0*s) and the duration (41.4 ± 4.7 s) of these responses, however, were significantly smaller to those cases in which NMDAR activation evoked action potentials (i.e., data shown **Fig.4E**; $p < 0.05$ for both parameters, unpaired t test). No differences however in the delay were observed between the two conditions.

NMDAR-driven firing more efficiently evokes somatodendritic VP release than bursting or continuous intrinsic action potential firing within individual VP neurones.

Our results support that NMDAR-evoked firing, compared to either RBF or CF modalities, more efficiently evokes somatodendritic neuropeptide release. In order to test this more directly, a combination of 2 or 3 of these different stimulation modalities, in which the sequence was randomly varied, were evoked in individually patched VP neurones in slices containing sniffer_{VP} cells. We aimed to evoke a similar total number of action potentials with these various modalities. A representative example is shown in **Fig.6**. In this case, focal application of NMDA evoked a burst of action potentials ($n= 120$ APs, 24 Hz), and with a delay of ~ 3 s, an increase in Ca^{2+} in 3 surrounding sniffer_{VP} cell was observed (27.9 ± 8.9 F/F0*s). When the same neurone was subjected to a CF modality ($n= 174$ APs, 27 Hz) no response was observed in any sniffer_{VP} cells. Finally, when RBF activity was evoked ($n= 138$ APs, 21 Hz), a Ca^{2+} response was evoked only in 1 sniffer_{VP} cell. This response however was markedly smaller (9.3 F/F0*s) than those evoked by NMDAR activation, and occurred with a much more prolonged delay (~ 85 s).

In another representative example of a patched VP neurone (**Fig.7**), we found that neither RBF ($n= 50$ APs, 8 Hz) nor CF modalities ($n= 41$ APs, 6 Hz) evoked Ca^{2+} responses in sniffer_{VP} cells. Conversely, a smaller NMDAR-evoked burst of action potentials ($n= 16$, 1 Hz) evoked a robust Ca^{2+} response in 7 sniffer_{VP} cells (19.2 ± 2.3 F/F0*s) with a delay of ~ 1 s after the stimulation. Similarly, another patched VP neurone subjected to a combination of NMDAR-evoked firing and a CF pattern (**Fig.8**), showed that an NMDAR-mediated burst of action potentials ($n= 27$ APs, 7 Hz), evoked an increase in Ca^{2+} in 5 surrounding sniffer_{VP} cells (13.8 ± 9.1 F/F0*s, delay of ~ 3 s). Conversely, when CF activity was evoked with direct current injection ($n= 37$ APs, 12 Hz) a small response was observed in only 1 sniffer_{VP} cell (4.1 F/F0*s).

Figure 9 summarizes and compares sniffer_{VP} cell responses among the three different firing modalities used to evoke firing in VP neurones. Overall, we found that the incidence of a successful release event (determined by a positive response in at least 1 sniffer_{VP} cell) caused by NMDAR-evoked firing was significantly higher compared to both RBF and CF patterns ($p < 0.001$, chi-square test, **Fig.9A**). Moreover, the mean number of responsive sniffer_{VP} cells within each experiment was significantly higher in NMDAR-evoked firing, compared to CF and RBF patterns ($p < 0.01$ and $p < 0.05$, respectively, one-way ANOVA, **Fig.9B**). Importantly, the magnitude of the sniffer_{VP} cell response was also significantly larger in NMDAR-evoked firing, compared to RBF and CF patterns ($p < 0.01$ and $p < 0.001$, respectively, one-way ANOVA, **Fig.9C**). Sniffer_{VP} cell responses were also larger in the RBF compared to CF pattern, although the difference did not reach statistical significance ($p = 0.07$).

Even though we attempted to evoke a similar degree of firing in each condition, the RBF modality evoked a higher number of action potentials and firing frequencies compared to NMDA and CF modes ($p < 0.0001$, one-way ANOVA, **Fig.9D,E**). However, the distribution histograms (see **Fig.9G-H**) show overlapping ranges for the three modalities, and as shown in the several individual examples shown (i.e., **Figs.6-8**), for similar number of evoked action potentials, NMDAR-evoked firing was more efficient than the other two firing modalities, and RBF more than CF, in evoking somatodendritic release of VP. This became also apparent when sniffer_{VP} cell responses were normalized by the number of evoked spikes ($p < 0.001$, one-way ANOVA, *not shown*).

Another prominent difference among the various firing modalities was the delay from the initiation of the stimulus of the VP neurone until the sniffer_{VP} Ca²⁺ response was observed. This delay was markedly shorter in NMDAR-evoked firing compared to RBF and CF patterns (**Fig.9F**). Due to the low number of responses observed in the CF group ($n = 3$), we were only able to compute statistical differences between the NMDAR-evoked and the RBF mode ($p < 0.0001$).

When all data from the different stimulation protocols were combined, we found no significant correlations between the magnitude of the sniffer_{VP} cell response with either the total number of action potentials ($r^2 = 0.04$, 0.04 and 0.07 for NMDA, RBF and CF respectively) or the mean intraburst frequency ($r^2 = 0.002$, 0.009 and 0.05 for NMDA, RBF and CF respectively) (**Fig.9G,H**). Importantly, and as also highlighted in these plots, for overlapping or even less number of action potentials and firing frequencies, NMDAR-evoked firing resulted in a more robust sniffer_{VP} cell response compared to RBF and CF patterns.

We also performed a running average of the mean sniffer_{VP} Ca^{2+} response as a function of the number of evoked action potentials (bin= 10 APs) or the evoked firing frequencies (bin= 2.5 Hz). For NMDAR-evoked firing, we observed a bell-shaped curve both for action potential number and firing frequencies (Gaussian fit, $r^2 = 0.6$ and 0.7 respectively, **Fig.9I,J**), suggesting an optimal dendritic release of VP in response to NMDAR activation at ~50 action potentials and ~15 Hz. Conversely, the RBF group only displayed a significant Gaussian distribution of sniffer_{VP} Ca^{2+} response as a function of firing frequency ($r^2 = 0.8$, **Fig.9J**), with an apparent optimal release at ~55 Hz. The low number of sniffer_{VP} Ca^{2+} responses in the CF modality prevented us from performing a similar analysis for this group.

Taken together, these results support that for similar numbers of evoked action potentials or firing frequencies, NMDAR-evoked firing more efficiently stimulates somatodendritic release of VP. Our results also demonstrate that somatodendritic release is strengthened when action potentials are clustered in repetitive bursts, rather than in a continuous irregular pattern.

NMDAR-evoked action potentials are broader, but spike broadening is similar among stimulation modalities

In isolated axonal terminals, a progressive increase in action potential duration (i.e., spike broadening) has been shown to contribute to potentiated release evoked by clustered

firing activity (Bicknell, 1988; Bourque, 1991). To determine whether NMDAR-evoked firing displayed more pronounced spike broadening compared to RBF or CF modes, we first compared the action potential waveform properties of individual spikes among groups, and then measured the mean action potential width for each of the first ten action potentials of a train evoked in representative samples from each stimulation modality. As summarized in **Table 1**, the NMDAR-evoked action potential was significantly broader than that evoked during RBF and CF modalities ($p < 0.05$ vs. CF and RBF). Other action potential properties, including peak amplitude, rise and decay times, and the hyperpolarizing after-potential (HAP) were not different among groups. As shown in **Fig.10A**, spike width progressively increased as a function of the action potential number within the train in all groups ($p < 0.0001$, two-way ANOVA). As with the single spike analysis, we found that spike width remained significantly broader in NMDA-evoked spikes during the burst, compared to RBF or CF ($p < 0.001$, two-way ANOVA). To better quantify the degree and rate of spike broadening, spike widths within each train were normalized to the duration of the first spike in the train. As shown in **Fig.10B**, we found no significant differences among groups in either spike broadening magnitude ($p = 0.5$, two-way ANOVA) or spike broadening rate (NMDAR: 0.05 ± 0.01 ms/spike; RBF: 0.05 ± 0.01 ms/spike; CF: 0.04 ± 0.01 ms/spike, $n = 6$ /group, $p = 0.8$, one-way ANOVA).

NMDAR boosting of somatodendritic release of VP is independent of the properties of evoked somatic firing activity

To determine if the more efficient somatodendritic release following NMDAR activation was dependent on a particular action potential waveform and/or firing pattern evoked, NMDA was focally applied to a subset of eGFP-VP neurones ($n = 5$), and the burst of evoked action potentials was then used as a voltage command waveform (VCW) to evoke the same firing response in the same neurone, but in the absence of NMDAR activation. The Ca^{2+} response

in sniffer_{VP} cells was assessed in response to both stimuli. As shown in **Fig.11**, in this subset of experiments NMDAR activation evoked bursts of action potentials (67.0 ± 15.9 action potentials; 12.7 ± 3.5 Hz) that consistently evoked a Ca^{2+} response in sniffer_{VP} cells (21.3 ± 6.7 F/F₀*s; mean delay: 5.2 ± 1.9 s; n= 10 sniffer_{VP} cells). Surprisingly however, when the same action potential firing activity was evoked using the VCW, no Ca^{2+} responses were observed in sniffer_{VP} cells.

NMDAR boosting of somatodendritic release of VP correlates with a larger dendritic Ca^{2+} signal in VP neurones

Somatodendritic release of neuropeptides involves Ca^{2+} -dependent exocytosis of LDCVs (Pow & Morris, 1989; Kennedy & Ehlers, 2011). Thus, we next assessed whether NMDAR-evoked firing resulted in larger changes in dendritic Ca^{2+} levels compared to RBF and CF modalities. To this end, we performed simultaneous patch-clamp and confocal Ca^{2+} imaging in eGFP-VP neurones loaded with the Ca^{2+} -sensitive dye Fluo5-F (50 μM), and neurones were stimulated with the 3 different firing modalities as used above (NMDA, RBF and CF). The mean number of evoked action potentials among the different firing modalities was not significantly different (NMDA: 71.1 ± 9.1 APs; RBF: 73.3 ± 7.6 APs; CF: 78.3 ± 8.9 APs, p= 0.3, one-way ANOVA repeated measures, n= 8). The Ca^{2+} dendritic measurements were obtained at a mean distance of 21.7 ± 2.3 μm from the soma, and were normalized by the corresponding total number of APs evoked in each stimulus. Changes in dendritic Ca^{2+} levels occurred almost instantaneously with the evoked firing activity, and no evident differences in the delay from stimulation to the evoked ΔCa^{2+} were observed. As shown in **Fig.12**, we found that the increase in dendritic Ca^{2+} evoked by the CF and RBF patterns were significantly smaller compared to the corresponding response evoked by NMDAR activation (p< 0.002, one-way ANOVA repeated measures).

We also compared changes in dendritic Ca^{2+} levels in neurones in which firing was evoked by NMDAR activation followed by the voltage command waveform (VCW), as described above. The increase in dendritic Ca^{2+} evoked by the VCW was also significantly smaller compared to the same firing evoked by NMDAR activation (NMDAR: 0.2 ± 0.04 $\text{F}/\text{F}_0^*/\text{s}/\text{APs}$; VCW: 0.1 ± 0.02 $\text{F}/\text{F}_0^*/\text{s}/\text{APs}$ $p < 0.05$ paired t test; $n = 10$).

DISCUSSION

The precise mechanisms that couple firing activity and neuropeptide release, particularly from dendrites, remain poorly understood. Given that neuropeptides do not evoke fast postsynaptic currents, their release cannot be readily detected with conventional electrophysiological approaches. Thus, microdialysis has been extensively used to measure brain neuropeptides (Ludwig *et al.*, 1996; Landgraf & Neumann, 2004), albeit with poor temporal (tens of minutes) and spatial (1 mm^2) resolutions.

To overcome this limitation, we used “sniffer” biosensor cells by genetically engineering Chinese ovarian hamster (CHO) cells to express VP or OT receptors with a genetically encoded fluorescent Ca^{2+} indicator. Here, we show that sniffer_{VP} responses were dose-dependent, with a threshold detection level of 0.5 nM and an EC₅₀ of 7.2 nM for VP (which are similar to the previously reported binding affinity for V1a receptors (K_i of 4.2 nM)) (Mouillac *et al.*, 1995), being thus an efficient tool to detect physiological levels of the neuropeptide. This was unequivocally shown by robust sniffer_{VP} Ca^{2+} responses following activation of the VP neuronal population following an osmotic challenge, or by activation of a single VP neurone. A similar approach was recently used to detect axonal neuropeptide release (Pinol *et al.*, 2014; Gizowski *et al.*, 2016), and at the time of submission of this manuscript, a work was published that also demonstrated the efficient use of HEK cells transfected with GCaMP6 to detect both axonal and dendritic release of VP (Zaelzer *et al.*, 2018). Thus, together with this present work, these studies strongly support sniffer cells as

highly sensitive biosensors to quantitatively measure activity-dependent somatodendritic release of neuropeptides. Still, none of these previous studies addressed the role of different degrees and patterns of firing activity in regulating the efficacy of somatodendritic release of VP, which was a major goal of the present study.

An important limitation of the “sniffer” cell approach that needs to be acknowledged is that it does not determine the precise location of the release event within the stimulated neurone. We attempted to improve this limitation by intracellularly loading the recorded VP neurone to better label their dendritic arbors. However, even in this condition, we rarely observed that responses in sniffer cells followed a specific spatial pattern in relation to the stimulated neurone. Whether this was due to technical limitations including incomplete dendritic staining and/or uneven distribution of sniffer cells around the patched neuron, or alternatively, whether this represents random pattern by which released neuropeptides diffuse in the extracellular space, acting in a volume transmission manner (Ludwig & Leng, 2006; Son *et al.*, 2013) is at present unknown. Irrespectively, we did not attempt to make any conclusions regarding the specific site of release and/or diffusion patterns of released VP in the extracellular space. This limitation however did not compromise our ability to assess differences in the overall magnitude of somatodendritic release of VP, particularly when comparing responses to the different stimulation patterns within a single individual neurone.

Impact of different spiking properties and modalities on somatodendritic release of VP

Classical studies in isolated neurohypophysial terminals showed that axonal release of VP displays frequency-dependent facilitation (between 4-20 Hz), whereas spike clustering elicited more VP release than continuous firing (Dutton & Dyball, 1979; Cazalis *et al.*, 1985; Bicknell, 1988; Bourque, 1991). Clustering and bursting firing may induce facilitation of axonal release via increased Ca^{2+} uptake at terminals (Cazalis *et al.*, 1985), a phenomenon

possibly associated to spike broadening, another frequency- dependent property of MNC axonal terminals (Bicknell, 1988; Bourque, 1991). Another beneficial aspect to clustering/bursting firing is that it minimizes secretory fatigue at axonal terminals (Cazalis *et al.*, 1985; Bicknell, 1988). Thus, it is generally accepted that the typical clustered/phasic mode displayed by VP neurons in response to an osmotic challenge is best suited to optimize hormonal release at neurohypophysial axonal terminals.

While somatodendritic release of VP and OT is also activity-dependent (Ludwig *et al.*, 2005), to what extent this process is influenced by the magnitude and/or pattern of somatic electrical activity, as summarized above for axonal terminals, is completely unknown. To address this fundamental question, we patched identified VP neurones in PVN slices containing sniffer_{VP} cells. In quite contrast to previous reports on axonal release (Dutton & Dyball, 1979; Cazalis *et al.*, 1985; Bicknell, 1988), we found that continuous spiking activity over a broad range of spike numbers and frequencies failed to evoke any detectable somatodendritic release. Conversely, clustered firing activity evoked release in ~60% of the cases, even from the same neurones that failed to release during continuous firing. No correlation between sniffer_{VP} cell responses and either the total number or frequency of evoked action potentials in clustered mode was observed. However, we found an optimal dendritic release of VP to occur with bursts of ~55 Hz, which is considerably higher than that reported for axonal terminals (peaking at ~15 Hz and displaying release fatigue at ~50 Hz) (Dutton & Dyball, 1979; Bicknell, 1988). Our results clearly show that action potential clustering enhanced neuropeptide release from somatodendritic compartments. However, a limitation of our approach was that we used a single RBF protocol (i.e., 5 bursts), which was chosen based on our previous results showing that this pattern efficiently evoked an interpopulation crosstalk mediated by somatodendritic release of VP (Son *et al.*, 2013). And while we were still able to evoke a wide range of total number of action potentials and firing frequencies with this protocol, it still remains to be determined more precisely, whether the number of bursts *per se* influences the efficacy of somatodendritic release.

The present results also highlight the fact that neuropeptide stimulus-secretion coupling is less efficient in somatodendritic compartments compared to axons. To what extent these differences reflect limited ability of somatic action potentials to back-propagate into dendritic segments, is at present unknown. MNCs express high levels of voltage-dependent, A-type K^+ channels (Luther *et al.*, 2002), which were suggested to be more predominantly expressed in dendrites of MNCs (Widmer *et al.*, 1997). Indeed, we found in a previous study that blockade of A-type K^+ channels in PVN neurons facilitated back-propagation of Ca^{2+} signals into dendritic compartments following somatic spikes (Sonner *et al.*, 2010). Thus, future studies are also warranted to investigate the role of A-type K^+ channels in regulating stimulus-secretion coupling at dendritic compartments.

NMDAR-evoked firing boosts somatodendritic release of VP

NMDARs in MNCs promote clustered firing (Hu & Bourque, 1992; Bains & Ferguson, 1997; Gagnon *et al.*, 2014) and evoke dendritic release of VP and OT (de Kock *et al.*, 2004; Son *et al.*, 2013). A major finding of our study was that NMDAR-mediated spiking significantly boosted dendritic release of VP, as shown by an increase in the percentage of positive release events observed (~80% cases), a significantly larger sniff_{VP} Ca^{2+} response, and a prominently shorter latency for the release event to occur, when compared to the other firing modalities. This potentiation was not due to more spikes and/or higher spike frequency elicited by NMDARs. In fact, a more robust NMDAR-evoked somatodendritic release was even observed at lower number of spikes or firing frequencies compared to the other modalities. We cannot completely rule out that the boosting effect of NMDA was due to activation of multiple VP neurones by the puff stimulation. This however was unlikely due to the fact that NMDA-evoked release was almost completely blocked when the patched neurone onto which the puff was focally directed was dialyzed with the Ca^{2+} chelator BAPTA. Moreover, these results are in line with a recent study in which we found a similar boosting

effect using a more precise, laser-evoked uncaging of NMDA, when compared to RBF firing (Son *et al.*, 2013).

Activity-dependent somatodendritic release of VP occurred with a long latency after stimulation, which was significantly shortened when firing was evoked by NMDAR activation. Similar long latencies for neuropeptide release (up to 1 minute) were reported in primary hippocampal cultures (Xia *et al.*, 2009) and in endocrine cells (Chow *et al.*, 1996; Elhamdani *et al.*, 1998). The long latency reported here was unlikely due to a slow response of the sniffer cells, since they displayed a rapid response onset when directly activated by the agonist. Moreover, since Ca^{2+} changes in dendrites exhibited almost no latency, and peaked within ~5 seconds following somatic firing, it is also unlikely that a slow dendritic invasion of electrical signals and/or Ca^{2+} was a contributing factor. Thus, similar to endocrine cells, long latencies for dendritic release could reflect lack of spatial localization between Ca^{2+} channels and dendritic LDCV.

NMDAR activation is necessary to evoke somatodendritic release of VP at physiological firing rates.

Previous work elegantly demonstrated that axonal and dendritic release of neuropeptides from MNCs can occur in a compartmentalized manner and be regulated independently (Ludwig & Leng, 2006). Still, the mechanisms that contribute to this compartmentalized regulation remain unknown. VP neurones typically exhibit a phasic firing pattern, with firing rates of 8-15 Hz, a frequency known to optimize axonal release of VP (Dutton & Dyball, 1979; Brown & Bourque, 2006). We found that in response to NMDAR-evoked firing, an optimal dendritic release of VP occurred at ~15 Hz, which was markedly lower than that observed with the RBF modality, indicating that activation of NMDARs was necessary for dendritic release to occur at these physiological firing frequencies. Thus, depending on whether NMDARs are engaged or not, a particular firing discharge in VP

neurones could result in somatodendritic and/or axonal release, respectively. Based on these results, we propose that NMDARs may act as a “gating” mechanism, contributing to the differential regulation of neuropeptide release from these two neuronal compartments in MNCs.

Possible mechanisms underlying the potentiation of somatodendritic release of VP by NMDAR-evoked firing

We found that NMDAR-evoked action potentials were significantly broader compared to those evoked by direct current injection either in continuous or bursting modes. Broader spikes in MNCs reflect more Ca^{2+} influx *per* spike (Bourque & Renaud, 1985), a result that was also in line with the a larger increase in dendritic Ca^{2+} levels observed following NMDAR-evoked firing compared to RBF and CF modalities. Spike broadening however, known to facilitate axonal release of neuropeptides from MNCs (Andrew & Dudek, 1985; Bourque & Renaud, 1985), was similar among the different firing modalities. Thus, to directly determine if differences in spike waveform and/or firing pattern evoked by NMDAR activation contributed to strengthening dendritic release of VP, we stimulated the same VP neurone with NMDA, and subsequently used the evoked burst of action potentials as a voltage-command waveform (VCW) to evoke the same action potential/firing properties but in the absence of NMDAR activation. We reasoned that if the NMDAR-evoked single spike waveform and/or the firing pattern *per se* were sufficient to boost dendritic release of VP, the VCW should evoke a similar boosting effect. Surprisingly however, we found that the VCW failed to evoke dendritic release of VP, suggesting that differences in spike width, spike broadening, or a particular firing pattern evoked by NMDAR activation were not critical factors contributing to their boosting effect. Similarly, since no differences in spike width (or action-potential evoked dendritic Ca^{2+} levels) were observed between RBF and CV

modalities, these factors could not account for the enhanced somatodendritic release evoked by the former.

An important caveat to consider is that action potential invasion of dendrites could have been prevented during the VCW stimulation. However, the fact that rapid changes in dendritic Ca^{2+} levels, although of smaller magnitude, were still observed using the VCW, argues against this possibility.

The smaller dendritic Ca^{2+} levels evoked with the VCW however, suggest that sources other than action potentials themselves contributed to the overall enhanced dendritic Ca^{2+} signal following NMDAR activation. One likely additional source is Ca^{2+} influx through the NMDARs themselves, which was previously shown to contribute to the generation of dendritic Ca^{2+} spikes (Bains & Ferguson, 1999). In fact, our results showing that in some cases a subthreshold depolarization evoked by NMDAR activation was sufficient to evoke dendritic release of VP, although to a significantly smaller degree to that observed in the presence of spikes, support an important contribution for NMDAR-mediated Ca^{2+} influx to the boosting effect on somatodendritic release mediated by NMDAR activation. Still, whether NMDAR-mediated dendritic Ca^{2+} influx results in a particular spatio-temporal profile of dendritic Ca^{2+} that leads to a faster and more efficient dendritic release of VP, possibly by mobilizing a readily releasable pool of LDCVs, will remain to be determined in future studies.

Finally, we recently showed that in addition to synaptically-located NMDARs, MNCs also express functional extrasynaptic NMDARs that robustly influence MNCs excitability and firing activity (Fleming *et al.*, 2011), and that these receptors are coupled to distinct signaling cascades than their synaptic counterparts (Potapenko *et al.*, 2012; Naskar & Stern, 2014). Extrasynaptic NMDARs are activated by ambient extracellular glutamate whose levels are independent of the degree of synaptic activity, but tightly controlled by astroglial glutamate transporters (Fleming *et al.*, 2011; Potapenko *et al.*, 2012). Importantly, the degree of neuronal coverage by glial processes in the magnocellular system (Perlmutter *et al.*, 1985;

Oliet *et al.*, 2001), and thus the ability of glial glutamate transporters to restrict activation of extrasynaptic NMDARs by ambient glutamate (Fleming *et al.*, 2011; Joe *et al.*, 2014), changes in a state-dependent manner (i.e. during dehydration or lactation). Thus, it will be important in future studies to determine the relative contribution of synaptic and extrasynaptic NMDARs to dendritic release of VP under basal and physiologically challenging conditions.

In summary, our studies support sniffer cells as highly efficient biosensors to quantitatively monitor central release of neuropeptides. Moreover, we provide novel information regarding precise mechanisms that control somatodendritic release of neuropeptides known to be involved in critical physiological and behavioral functions.

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ADDITIONAL INFORMATION

COMPETING INTERESTS

All authors acknowledge that they there are no conflict of interests to disclose in accordance with the Journal policy.

AUTHOR CONTRIBUTIONS

S.P. M.Z. and E.C conducted experiments.

S.P and J.E.S. designed the experiments and wrote the manuscript.

FUNDING

This work was supported by a National Institute of Neurological Disorders and Stroke Grant NIH NS09640 (Stern JE).

ACKNOWLEDGEMENTS

We would like to thank Dr. Yoichi Ueta, University of Occupational and Environmental Health, Japan, for the kind donation of the transgenic eGFP-VP rats.

Table 1. Properties of action potentials evoked by the different stimulation modalities

Stimulation modality	<i>n</i>	AP amplitude (mV)	AP half-width (ms)	AP rise (ms)	AP decay (ms)	HAP peak (mV)
NMDA	6	77.0 ± 4.3	1.20 ± 0.10	0.37 ± 0.04	1.26 ± 0.13	-26.3 ± 2.9
RBF	6	77.1 ± 3.8	0.83 ± 0.02**	0.39 ± 0.03	1.14 ± 0.16	-23.5 ± 2.9
CF	6	81.2 ± 33.6	0.93 ± 0.03*	0.35 ± 0.03	1.15 ± 0.14	-26.7 ± 2.8

Abbreviations: RBF, repetitive bursting firing; CF, continuous firing; AP, action potential; HAP, hyperpolarizing after potential. Values are means ± S.E.M. * $p < 0.05$ and ** $p < 0.01$ vs NMDA, one-way ANOVA.

FIGURE LEGENDS

Figure 1. Validation of sniffer_{VP} cells as VP biosensors. Pseudocolor images showing that sniffer_{VP} basal intracellular Ca²⁺ concentration (**A1**) was increased in 4 sniffer_{VP} cells (arrows) following exogenous VP application (50 nM, bolus, **A2**). A plot of these sniffer_{VP} Ca²⁺ responses (F/F₀) over time is shown in **A3**. The arrowhead indicates the time of VP application, and the asterisks correspond to the time points of the images shown in **A1-2**. Note that in some cases (i.e., sniffer_{VP} #1) basal fluorescence was almost indistinguishable from the background. Note also that since results are expressed as F/F₀, the brightest response (i.e., sniffer_{VP} #4) does not correspond with the largest F/F₀ peak, since its basal fluorescence was also relatively high. The sniffer_{VP} Ca²⁺ response was reproducible showing little decrement in response to repeated VP applications (2 min intervals, **B1**). Summary data showing that sniffer_{VP} responses to VP were dose- (**B2**, n= 8) and distance-dependent (**B3**, n= 5, bin= 50 μm). Changes in distance were accomplished by moving the puff pipette containing VP closer or farther away from the sniffer_{VP} cells. Scales: 15 μm.

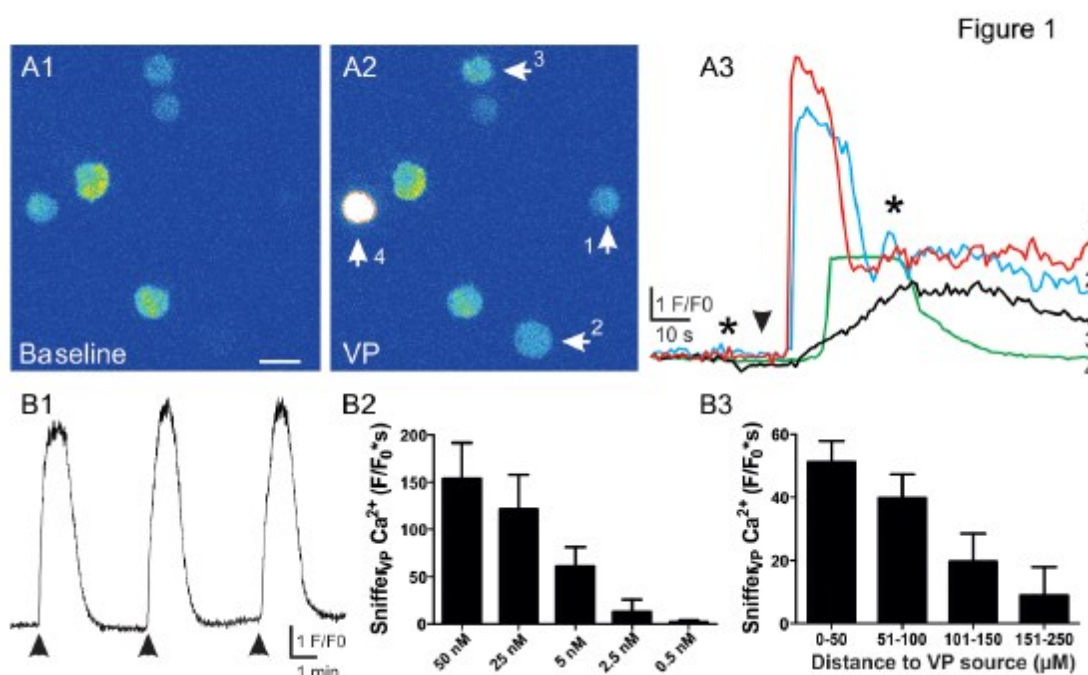


Figure 2. Sniffer_{VP} cells detect endogenous somatodendritic release of VP evoked by an osmotic challenge. **A1-3**, Samples of patched eGFP-VP neurones loaded with Alexa 488 (green) surrounded by sniffer_{VP} cells (red). Pseudocolor images showing baseline (**B1**) and increased Ca^{2+} levels in 3 different sniffer_{VP} cells (arrows, **B2-3**) after application of mannitol (bolus, +10 mOsm). **C**, Plot of changes in Ca^{2+} levels over time in the 3 sniffer_{VP} cells shown in B in response to the osmotic stimulation (arrowhead). Asterisks correspond to the time points of the images shown in B1-3. *Inset*, sample of the increased firing discharge in a patched eGFP-VP neurone in response to the osmotic stimulation (arrowhead). **D**, Summary data of the mean area, duration and delay of the sniffer_{VP} cell responses to mannitol in control ACSF (n= 16 obtained from 4 slices) and in the presence of TTX (n= 31 cells from 7 slices). *** p< 0.0001 vs. mannitol. Scale bars: 15 μm .

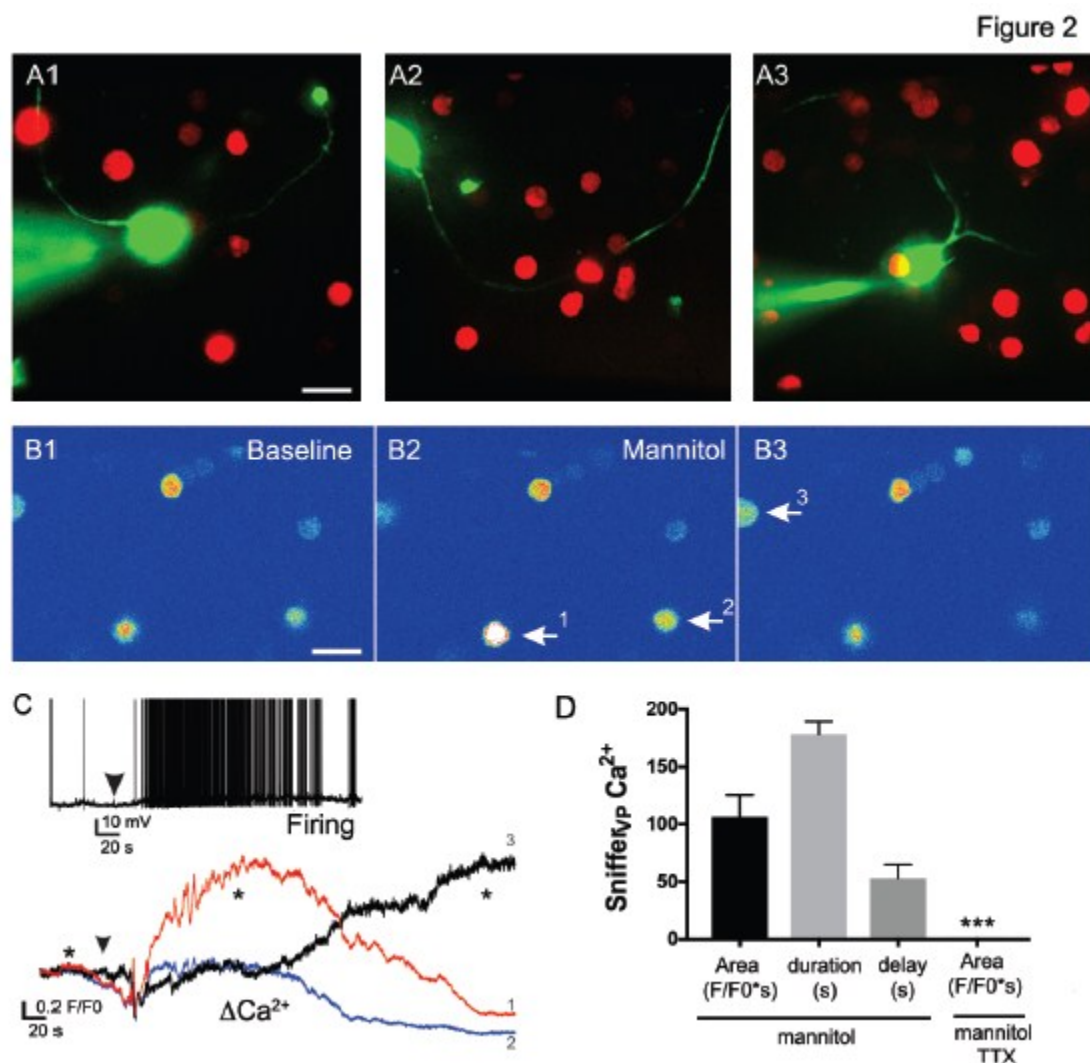


Figure 3. Clustered but not continuous firing pattern evokes somatodendritic release of VP from individual neurones. Pseudocolor image showing a patched eGFP-VP neurone surrounded by sniffer_{VP} cells (arrows, **A1**). Note the lack of sniffer_{VP} cell responses (**A2-A3**) when continuous firing activity (CF) was evoked in the patched neurone (arrowhead) using depolarizing pulses of increasing duration (**A3, inset**). **B1-2**, When the same neurone from A was then stimulated to evoke a repetitive bursting firing pattern (RBF, *inset B3*), a robust Ca^{2+} increase was observed in one of the sniffer_{VP} cells (**B3**, red arrow in B2) approximately 30 s after stimulation (arrowhead). **C1**, Fluorescence image of a different patched eGFP-VP neurone loaded with Alexa 488 (green) surrounded by sniffer_{VP} cells (red). **C2-3**, Corresponding pseudocolor images showing baseline (**C2**) and sniffer_{VP} Ca^{2+} responses (**C3**, arrows) when the patched neurone was stimulated to generate a RBF pattern (**D, inset**). A plot of the sniffer_{VP} Ca^{2+} response over time to the RBF stimulation (arrowhead) is shown in **D**. Asterisks correspond to the time points of the images shown in **C2-3**. **E**, Summary data of the mean area, duration and delay of sniffer_{VP} responses to RBF stimulation ($n = 31$ sniffer_{VP} cells). Scale bars: 15 μm .

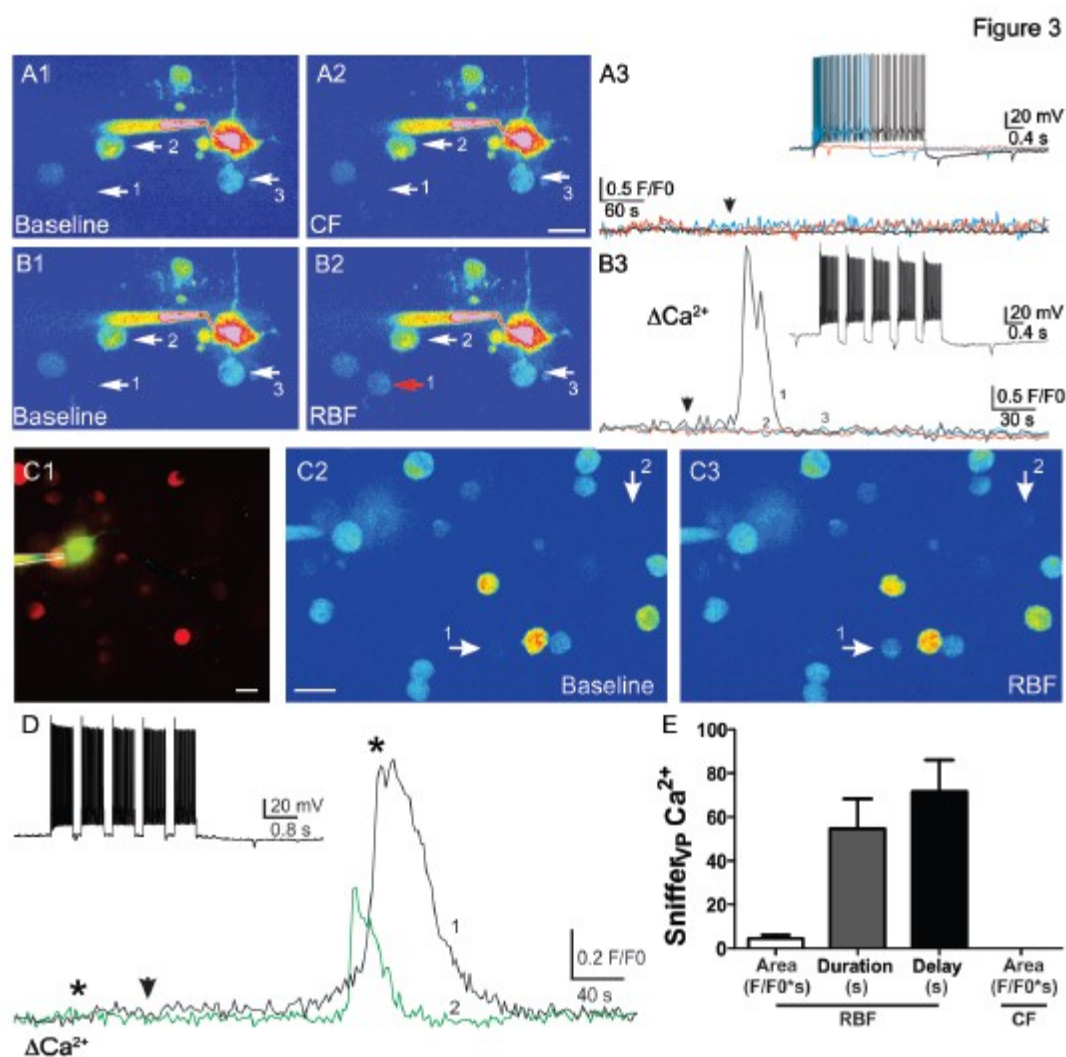


Figure 4. NMDA receptor activation evokes robust somatodendritic release of VP. **A**, Fluorescence images of sniffer_{VP} cells (red, **A1**) surrounding a patched eGFP-VP neurone loaded with Alexa 488 (green, **A2**). Both images are superimposed in **A3**. **B**, Corresponding pseudocolor images showing sniffer_{VP} Ca²⁺ baseline (**B1**) and responses in 4 cells (**B2-3**, arrows) following focal application of NMDA (10 μ M) to the patched neurone. **C1-3**, NMDA stimulation to the same neurone failed to evoke sniffer_{VP} cell responses in the presence of the V1aR antagonist V2255 (1 μ M). **D**, Plots of sniffer_{VP} Ca²⁺ changes over time in the 4 cells shown in **B-C**, in the absence (upper panel) and presence (lower panel) of V2255. Arrowheads indicate the time of the NMDA stimulation, and asterisks correspond to the time points of the images shown in **B1-3** and **C1-3**. The *inset* shows the firing discharge of the patched neurone in response to NMDA stimulation. **E**, Summary data of the mean area, duration and delay of sniffer_{VP} responses to NMDAR-evoked firing in control ACSF (n= 21, from 10 patched eGFP-VP neurones) Scale bar: 15 μ m.

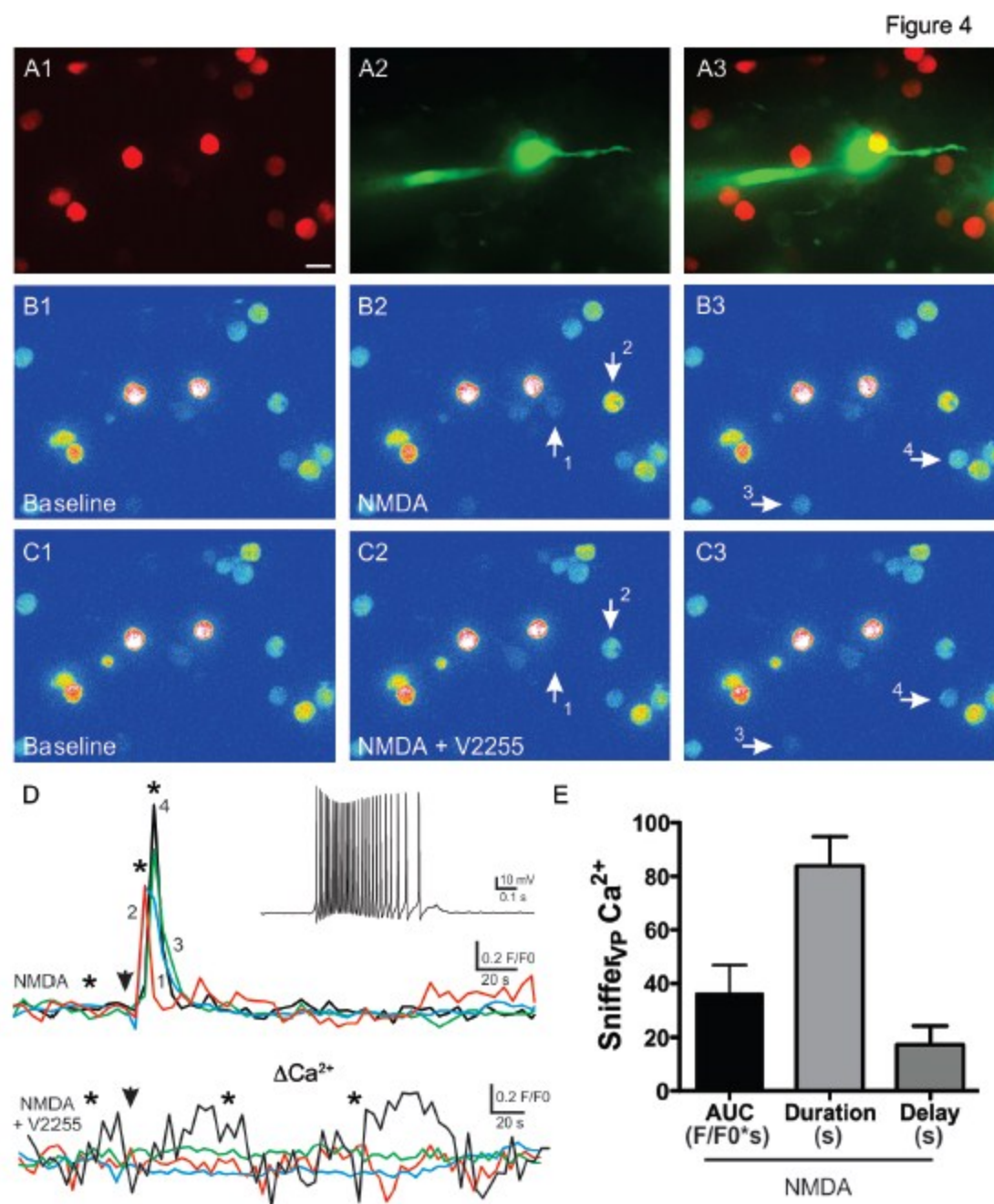


Figure 5. NMDAR activation evokes dendritic release of VP in the absence of action potentials. NMDA application (10 μ M) caused a Ca^{2+} increase in 2 sniffer_{VP} cells (arrows in A2). This stimulus evoked a clear depolarization in the patched eGFP-VP neuron, which was insufficient however to evoke action potentials (B *inset*). Traces in B show the changes in sniffer_{VP} Ca^{2+} levels in both cells shown in A2. Arrowheads show the time of NMDA application, and asterisks correspond to the time points of the images shown in A. C, summary data of mean area, duration and delay in response to NMDA without triggering action potentials (n= 16, from 6 patched eGFP-VP neurones) Scale bar: 15 μ m.

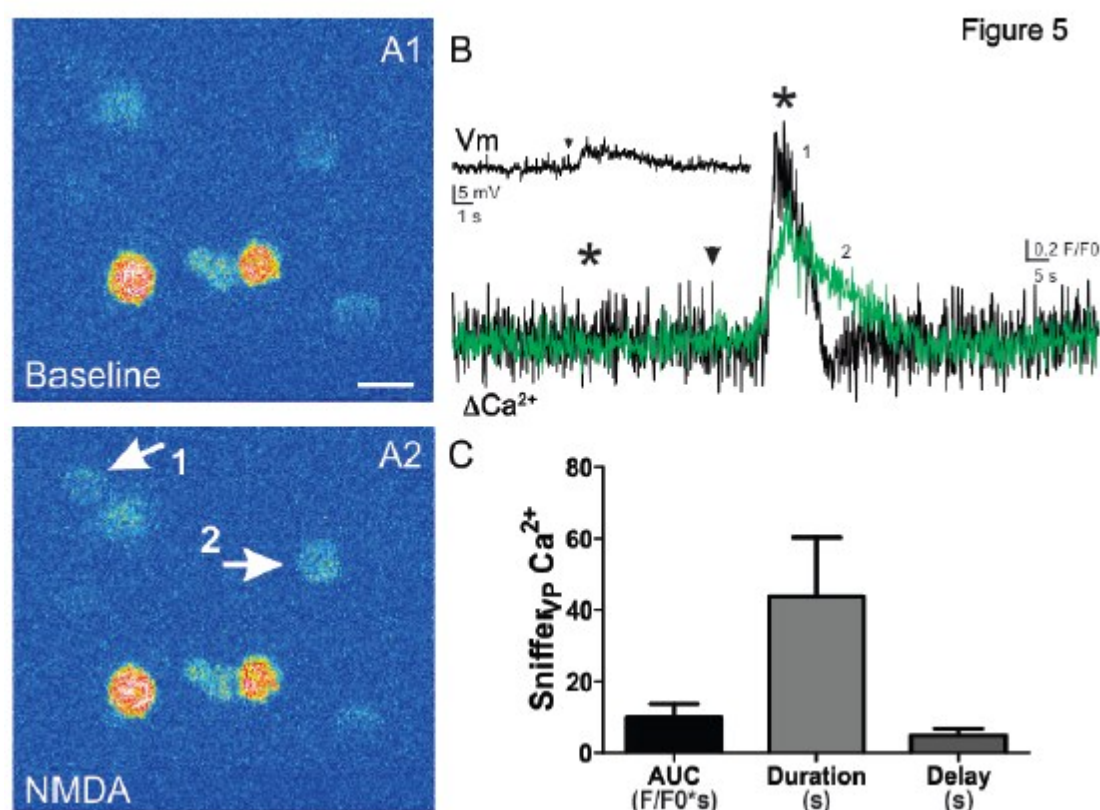


Figure 6. Somatodendritic release of VP is potentiated by NMDAR-evoked firing when compared to the other firing modalities elicited in the same VP neuron . **A**, NMDAR-evoked firing in a patched eGFP-VP neurone (NMDA 10 μ M, **A1**) resulted in positive Ca^{2+} responses in 3 sniffer_{VP} cells (**A2-A3**, arrows). The corresponding Ca^{2+} plots are shown in **A4**. **B**, A continuous firing discharge (CF) evoked in the same eGFP-VP neurone (40 pA, single depolarizing pulse, **B1**) failed to generate sniffer_{VP} responses (**B2-B4**). **C**, When the same eGFP-VP neurone was stimulated to evoke repetitive bursting firing (RBF, 80 pA, 5 depolarizing pulses, 0.5 s each, 1 s interval, **C1**), a positive Ca^{2+} response was observed in only 1 sniffer_{VP} cell (**C2-4**). The magnitude and delay of this response was evidently smaller and longer compared to the response observed following NMDAR-evoked firing. Arrowheads in panels **A4-C4** show the time of the stimulation, and asterisks correspond to the time points of the images shown in panels 2 and 3.

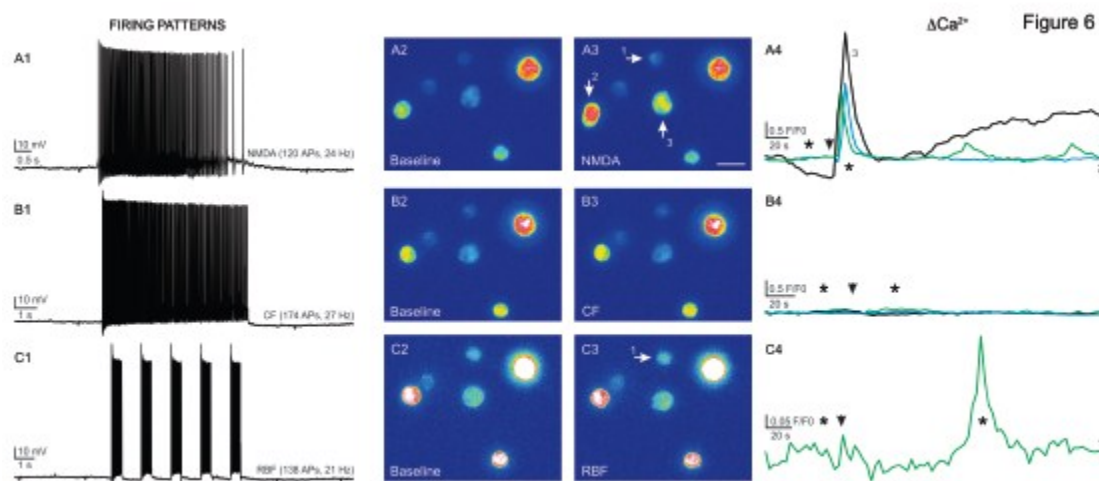


Figure 7. Representative example showing a more robust somatodendritic release of VP evoked by NMDAR-mediated firing when compared to continuous or bursting firing in the same eGFP-VP neurone. A, Fluorescence images of sniffer_{VP} cells (red, A1) in the vicinity of a patched eGFP-VP neurone loaded with Alexa 488 (green, A2). Both images are superimposed in A3. Corresponding pseudocolor images showing basal (B1- D1) and sniffer_{VP} Ca²⁺ responses when the patched neurone was stimulated to generate a RBF pattern (B2), a CF pattern (C2) or when firing was evoked by focal application of NMDA (10 μ M, D2), in the sequence displayed. Plots of sniffer_{VP} Ca²⁺ responses to each stimulation protocols are shown in B3-D3 respectively. Note that in this eGFP-VP neurone, only NMDAR-evoked firing resulted in robust Ca²⁺ responses in 7 different sniffer_{VP} cells (arrows, D2). Arrowheads in B3-D3 indicate the stimulation time, and asterisks correspond to the time points of the images shown in panels 1 and 2. Scale bar: 15 μ m.

Figure 7

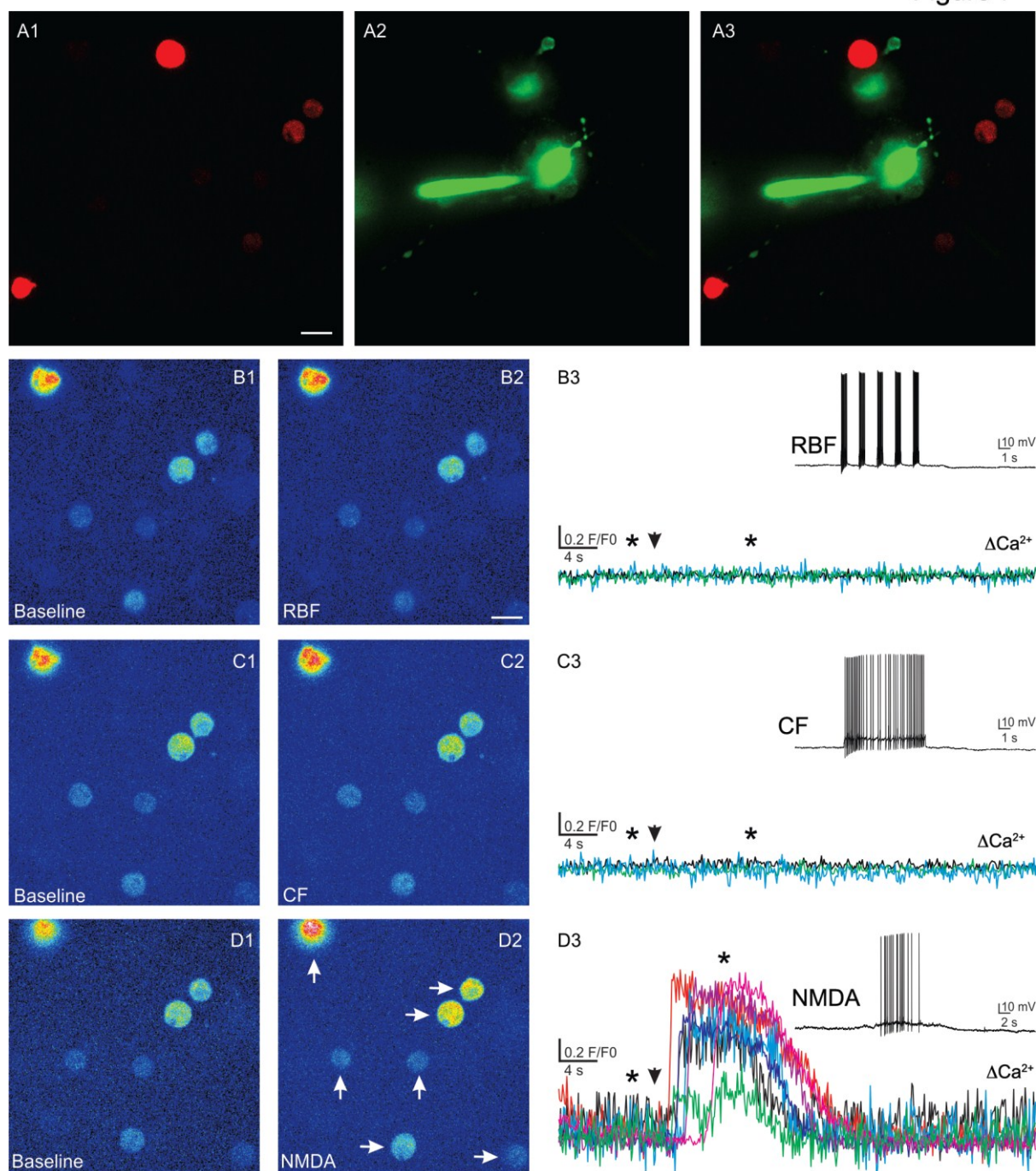


Figure 8. Another representative example showing a more robust somatodendritic release of VP evoked by NMDAR-mediated firing when compared to continuous firing mode in the same eGFP-VP neurone Pseudocolor images showing a patched eGFP-VP neurone surrounded by sniffer_{VP} cells (**A1-2**). Focally-applied NMDA (10 μ M) to the patched neurone evoked a burst of action potentials (27 APs, 7 Hz, **B1**) that triggered a Ca^{2+} increase in 5 sniffer_{VP} cells (arrows in **A2**, **B2**). A continuous firing pattern evoked by direct current injection (CF, 37 APs, 12 Hz, **C1**) caused a Ca^{2+} response in only 1 sniffer_{VP} cell (**C2**). Arrowheads indicate the time of the stimulation and asterisks correspond to the time points of the images shown in **A**. Scale bar: 15 μ m.

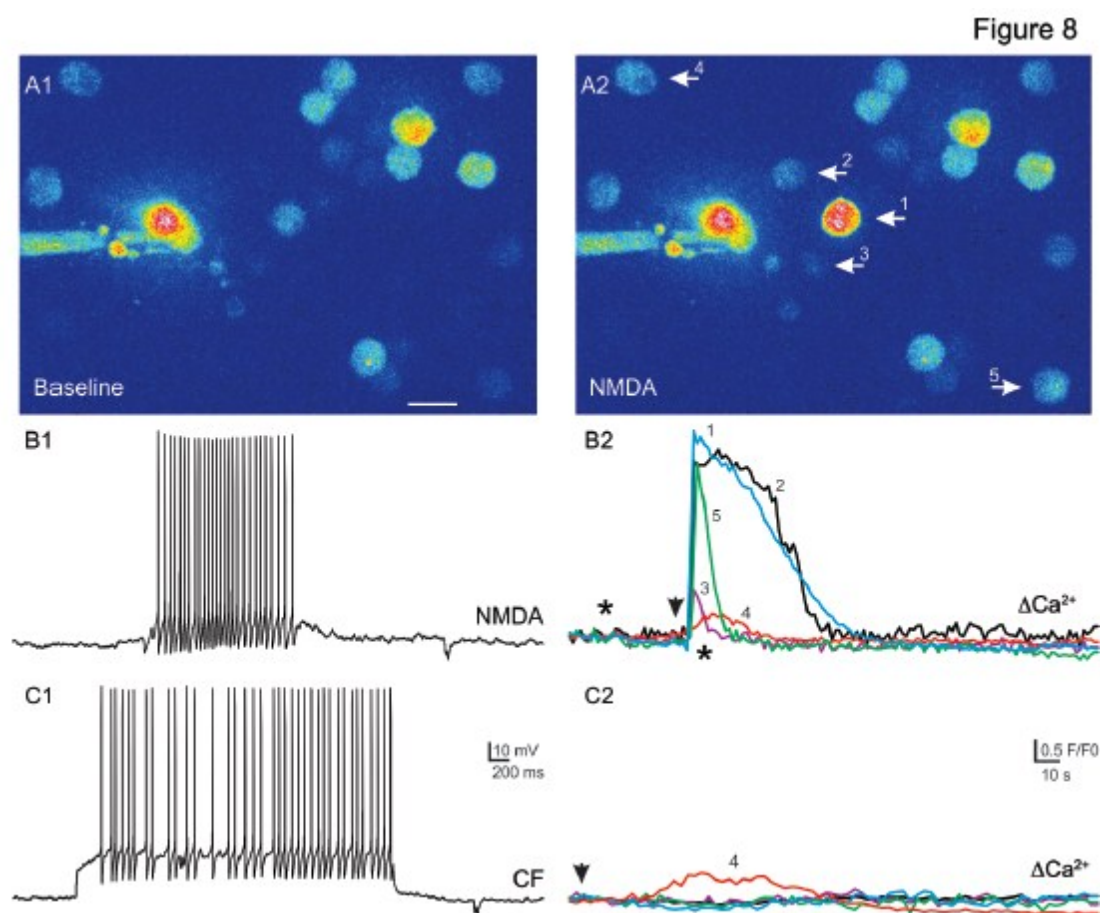


Figure 9- Summary of relationship between somatodendritic release of VP and the number and frequency of action potentials evoked with the three different firing modalities. A, The incidence of successful somatodendritic release events was significantly higher in NMDAR-evoked firing when compared to RBF or CF. A summary of the mean number of sniffer_{VP} responsive cells and sniffer_{VP} Ca²⁺ magnitude (area) in response to the three stimulation modalities are shown in B and C, respectively. A summary of the mean number of action potentials and mean firing frequency evoked by the three stimulation modalities in patched eGFP-VP neurons are shown in D and E, respectively. F, Summary of the mean sniffer_{VP} Ca²⁺ response delay following stimulation of eGFP-VP neurones with the three different firing modalities. G, Plots of sniffer_{VP} Ca²⁺ responses (area) as a function of the number of APs or action potential frequency (H). Note the lack of correlations observed in the three different stimulation modalities (# APs: $r^2 = 0.04$, 0.04 and 0.07 for NMDA, RBF and CF respectively; Frequency: $r^2 = 0.002$, 0.009 and 0.05 for NMDA, RBF and CF respectively). I, Plot showing a running average of the mean sniffer_{VP} Ca²⁺ response as a function of the number of evoked APs (bin= 10 APs). The NMDAR-evoked, but not the RBF modality was fit with a Gaussian function ($r^2 = 0.6$). J, Plot showing a running average of the mean sniffer_{VP} Ca²⁺ response as a function of the firing frequency (bin= 2.5 Hz). Both, the NMDAR-evoked and the RBF modalities were fit with a Gaussian function ($r^2 = 0.7$ and 0.8 respectively). Note that the apparent optimal frequency for dendritic release in the case NMDAR-evoked firing was shifted towards lower firing frequencies compared to the RBF modality. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ vs NMDA (B, C, E, F) or vs. RBF (D), one-way ANOVA. For A, D, and E: n (stimulated VP neurones)= 45, 46 and 30 for NMDA, RBF and CF respectively. For B, n (individual sniffer_{VP} cells)= 67, 54 and 15 for NMDA, RBF and CF respectively. For F, n (individual sniffer_{VP} cells with a positive response)= 47, 29 and 3 for NMDA, RBF and CF respectively.

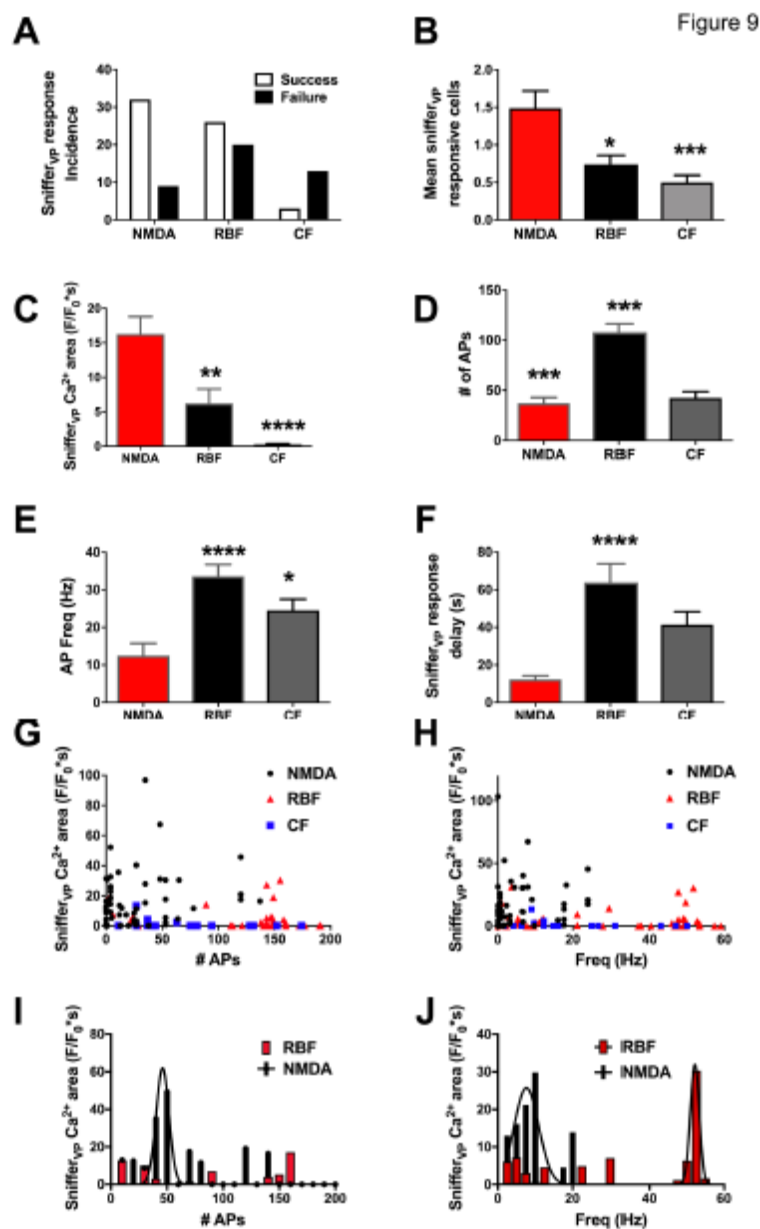


Figure 10. Spike broadening during repetitive firing was similar among the different stimulation modalities. A, Plot of the mean spike width as a function of the action potential number within the train in all groups ($n= 6/\text{group}$). **B,** Plot of the mean spike width normalized to the duration of the first spike of the train ($n= 6/\text{group}$).

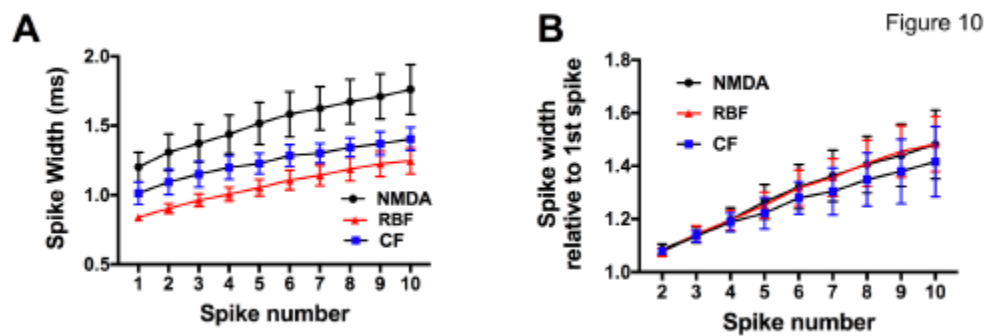


Figure 11. NMDAR facilitation of somatodendritic release of VP is not dependent of the properties of the evoked somatic firing activity. **A1**, Plots of Ca^{2+} changes over time recorded from 3 sniffer_{VP} cells following focal application of NMDA (10 μM) to a recorded eGFP-VP neurone. The *inset* shows the firing response evoked in the patched neurone (31 APs, 3 Hz). **A2**, The evoked activity in the patched neurone was then used as a voltage command waveform (VCW, *inset*) applied to the same neurone. The lower traces represent Ca^{2+} changes over time recorded from the same 3 sniffer_{VP} cells as in A1 following the VCW stimulation. Note the lack of sniffer_{VP} Ca^{2+} responses. **B**, Summary data of the mean area, duration and delay in response to NMDA and VCW (n= 10 sniffer_{VP} cells from 5 patched eGFP-VP neurons).

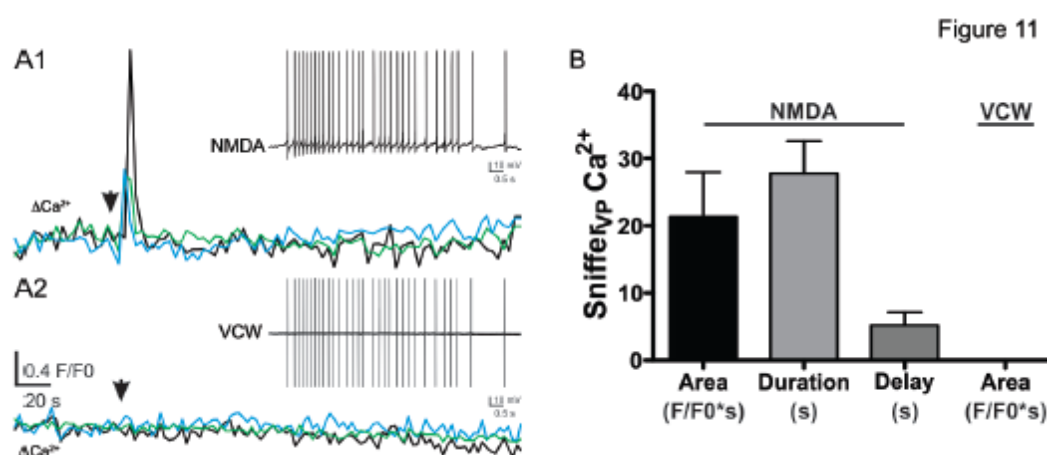
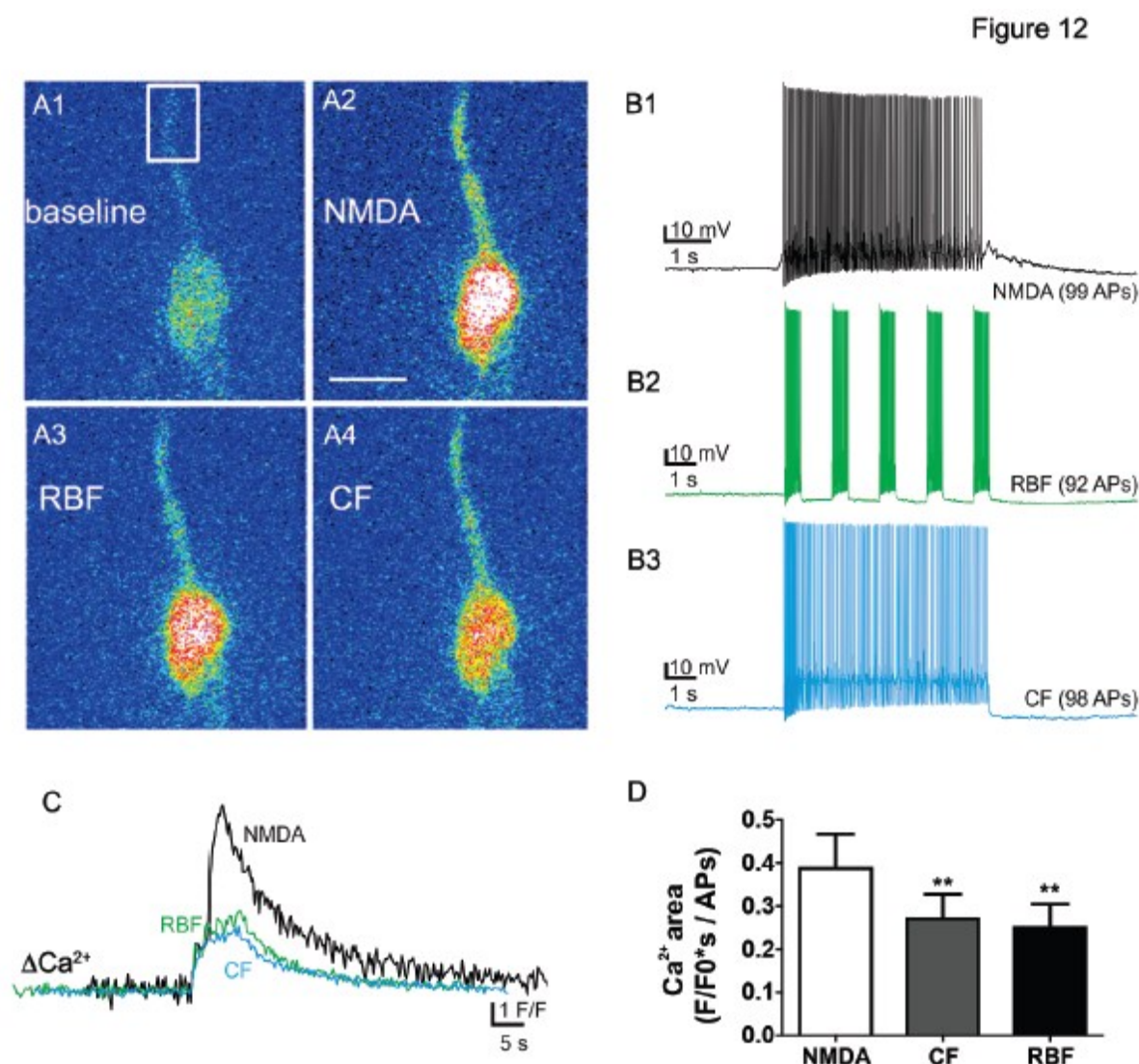


Figure 12- NMDAR-evoked firing results in larger increases in dendritic Ca^{2+} levels compared to continuous or repetitive bursting firing. A, Representative pseudocolor confocal Ca^{2+} images of a patched eGFP-VP neurone dialyzed with Fluo-5F showing somatodendritic Ca^{2+} changes in response to NMDAR-evoked firing (A2), or direct current injection in repetitive bursting mode (RBF, A3) and continuous mode (CF, A4). B, Electrophysiological recordings showing the firing activity evoked in this eGFP-VP neurone in response to NMDA (50 μM , B1), RBF (B2) and CF (B3) modalities. In all cases we attempted to evoke approximately the same number of action potentials in each condition. C, Plot of dendritic Ca^{2+} changes as a function of time in this eGFP-VP neurone in response to each firing modality. The Ca^{2+} signal was analyzed in the region shown in A1. Note the larger Ca^{2+} response to NMDAR activation compared to RBF and CF. D, Summary data showing mean dendritic Ca^{2+} responses normalized by the number of action potentials evoked in each case ($n = 8$). ** $p < 0.01$ vs. NMDA. Scale bar: 15 μm .



Soledad Pitra completed her PhD under the co-mentorship of Dr. Javier E. Stern and Dr. Laura M Vivas, at the National University of Cordoba, Argentina. Dr. Pitra's research interest includes the understanding of hypothalamic neurobiological mechanisms involved in the CNS control of cardiometabolic functions both in health and disease states.

