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RESEARCH ARTICLE | *Neural Circuits*

Detection of activity-dependent vasopressin release from neuronal dendrites and axon terminals using sniffer cells

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Zaelzer C, Gizowski C, Salmon CK, Murai KK, Bourque CW. Detection of activity-dependent vasopressin release from neuronal dendrites and axon terminals using sniffer cells. *J Neurophysiol* 120: 1386–1396, 2018. First published July 5, 2018; doi:10.1152/jn.00467.2017.—Our understanding of neuropeptide function within neural networks would be improved by methods allowing dynamic detection of peptide release in living tissue. We examined the usefulness of sniffer cells as biosensors to detect endogenous vasopressin (VP) release in rat hypothalamic slices and from isolated neurohypophyses. Human embryonic kidney cells were transfected to express the human V1a VP receptor (V1aR) and the genetically encoded calcium indicator GCaMP6m. The V1aR couples to Gq_{11}, thus VP binding to this receptor causes an increase in intracellular [Ca^{2+}] that can be detected by a rise in GCaMP6 fluorescence. Dose-response analysis showed that VP sniffer cells report ambient VP levels >10 pM (EC_{50} = 2.6 nM), and this effect could be inhibited by the V1aR antagonist SR 49059. When placed over a coverslip coated with sniffer cells, electrical stimulation of the neurohypophysis provoked a reversible, reproducible, and dose-dependent increase in VP release using as few as 60 pulses delivered at 3 Hz. Suspended sniffer cells gently plated over a slice adhered to the preparation and allowed visualization of VP release in discrete regions. Electrical stimulation of VP neurons in the suprachiasmatic nucleus caused significant local release as well as VP secretion in distant target sites. Finally, action potentials evoked in a single magnocellular neurosecretory cell in the supraoptic nucleus provoked significant VP release from the somatodendritic compartment of the neuron. These results indicate that sniffer cells can be used for the study of VP secretion from various compartments of neurons in living tissue.

NEW & NOTEWORTHY The specific functional roles of neuropeptides in neuronal networks are poorly understood due to the absence of methods allowing their real-time detection in living tissue. Here, we show that cultured "sniffer cells" can be engineered to detect endogenous release of vasopressin as an increase in fluorescence.

dendritic release; neurosecretory terminals; vasopressin

INTRODUCTION

Arginine vasopressin (VP; antidiuretic hormone) is a pleiotropic neuropeptide synthesized by subsets of hypothalamic neurosecretory neurons in the supraoptic (SON) and paraventricular nuclei (PVN) and by nonneurosecretory neurons present in a small number of other brain regions including the bed nucleus of the stria terminalis, the medial amygdala, and the suprachiasmatic nucleus (SCN; Buijs et al. 1978; Meyer-Lindenberg et al. 2011; Rood and De Vries 2011). When released into the bloodstream by the neurohypophysial axon terminals of magnocellular neurosecretory cells (MNCs) of the PVN, SON, and accessory magnocellular nuclei, VP serves as a hormone that promotes water reabsorption by the kidneys and stimulates vasoconstriction to increase peripheral vascular resistance (Share 1988). VP can also be secreted into the hypophysial portal system by the axon terminals of PVN parvocellular neurosecretory neurons to stimulate adrenocorticotropic hormone release from the anterior pituitary (Ganong 1993). In addition to these well-defined humoral effects in the periphery, VP can be released within the brain by the axon terminals of nonneurosecretory neurons to regulate various behavioral and physiological parameters, including blood pressure (Martin et al. 1985), body temperature (Horowitz et al. 1992; Naylor et al. 1988), circadian rhythms (Gizowski and Bourque 2018; Gizowski et al. 2016, 2017; Kalsbeek et al. 2010; Trudel and Bourque 2010; Yamaguchi et al. 2013), and various social behaviors (Johnson and Young 2017; Neumann and Landgraf 2012; Stein 2009; Stoop et al. 2015). Moreover, VP can also be released from the dendrites of MNCs to produce local autocrine (Chevaleyre et al. 2000) and paracrine responses (Son et al. 2013).

The absence of fast postsynaptic responses (e.g., excitatory postsynaptic potentials) associated with the exocytosis of VP-containing dense-core vesicles has limited our ability to define the molecular mechanisms regulating VP release from different neuronal compartments (i.e., central axons, dendrites, and neurosecretory terminals) and to define the cellular and network mechanisms that mediate its central effects. Therefore, our understanding of these functions could be improved by the availability of techniques allowing a rapid detection of spatially resolved VP release in situ. Previous studies examining VP secretion in living animals or from tissue preparations maintained in vitro have relied primarily on immunological methods. Typically, such studies require the collection of blood samples (Robertson et al. 1970) or fluid collected via perfusion (Dreifuss et al. 1971), microdialysis (Ludwig et al. 1996), or push-pull cannulae (Myers et al. 1998) and subsequently measuring VP concentration within the samples using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). Although these approaches allow precise measurements of VP concentration, their temporal resolution is poor

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due to limitations of the rate at which fluid can be collected in quantities sufficient for peptide detection (usually on the order of minutes). Additionally, the spatial resolution of these approaches is poor because of the large tissue area from which sample is collected (typically >1 mm^2).

Previous studies have shown that heterologous cells coexpressing a Gq_{11}-coupled receptor for which activation increases intracellular [Ca^{2+}], together with a genetically encoded fluorescent Ca^{2+} indicator, can be used as "sniffer cells" to detect the release of oxytocin (OT; Piñol et al. 2014) and VP in brain slices (Gizowski et al. 2016). In this study, we define the sensitivity and selectivity of this approach and examine its potential use for the analysis of VP release from axons, dendrites, and neurosecretory terminals. Our data show that sniffer cells can be used as highly sensitive detectors of VP release from all of these compartments.

MATERIALS AND METHODS

Animals. Animals were treated in strict accordance with guidelines outlined by the Canadian Council on Animal Care (https://www.ccac.ca/), and experiments adhered to protocols approved by the Facility Animal Care Committee of McGill University (protocol no. 1190). All experiments were performed on male Long-Evans rats (80–150 g) obtained from Charles River Canada (Saint-Constant, Québec, Canada) that were housed under 12:12-h light-dark conditions and provided food and water ad libitum.

Preparation VP biosensors. All experiments were performed using sniffer cells prepared by acute transfection and were used within 24–48 h of the transfection procedure. Human embryonic kidney HEK-293 cells (gift from Dr. Salvatore Carbonetto) were grown in DMEM (Wisent, Saint-Bruno, Québec, Canada) at 37°C and 5% CO_2. Cells at 60–70% confluence were cotransfected with pGP-CMV-GCaMP6m (Addgene, Cambridge, MA) and the human V1a VP receptor (kindly provided by Dr. M. Bouvier, Université de Montréal) or the empty vector pcDNA3.1 (2.5 μg each) using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA). In some experiments, HEK-293 cells were transfected with a plasmid directing expression of mCherry (for visualization purposes only).

Image collection and fluorescence analysis. GCaMP6m fluorescence was imaged at 0.2 Hz using a BX51WI upright microscope (Olympus Canada, Richmond Hill, Ontario, Canada) and a 14-bit CoolSNAP HQ^2 camera (Photometrics, Tucson, AZ) controlled by Imaging Workbench 6.0 software (INDEC BioSystems, Los Altos, CA). Cells showing a high level of fluorescence or rhythmic spikes of fluorescence under basal conditions were not analyzed. Interestingly, such spontaneous activity was typically blocked by SR 49059, indicating that it was driven by basal VP-mediated activation. When required, tracking of cells in the images (8-bit) was performed using the Fiji TrackMate plugin (v2.8.1; Schindelin et al. 2012). Fluorescence intensity measured in regions of interest was corrected for bleaching (single exponential), and background fluorescence was subtracted from all values. Fluorescence at various time points was expressed relative to baseline. Stimulus-induced changes in fluorescence were calculated from the average of values observed during a 30-s period following the onset of the response and expressed relative to baseline (average of values collected 30 s before stimulation). For illustration, images were converted to 8-bit, and a pseudocolor look-up table was applied to facilitate visualization of the responses. The look-up table was designed to make significant midrange responses visible and gather high-intensity responses into a broad yellow area.

Dose-response curves and pharmacology. Coverslips containing HEK-293 cells expressing V1a receptor (V1aR) and GCaMP6m or GCaMP6m alone were placed in a warmed (32°C) recording chamber and perfused (2–3 ml/min) with carbogenated (95% O_2-5% CO_2) artificial cerebral spinal fluid (ACSF) comprising, in mM, 120 NaCl, 3 KCl, 1.23 $NaHPO_4$, 1.48 $MgCl_2$, 2 $CaCl_2$, 29.95 $NaHCO_3$, and 10 D-glucose (all from Sigma-Aldrich Canada, Oakville, Ontario, Canada). Frozen stocks of VP or OT in water (0.1 mM; Cedarlane, Burlington, Ontario, Canada) were diluted in ACSF as required. Application of the peptides was as follows: 3 min of baseline followed by 3 min of peptide at the desired concentration and 6 min of recovery (wash). The V1aR antagonist, SR 49059 (Sigma-Aldrich; diluted in DMSO), was used at a concentration of 10 μM. The protocol for drug application was as follows: 3 min of baseline (ACSF + 10 μM SR 49059) followed by 3-min application of VP or OT (100 nM) in ACSF + 10 μM SR 49059 and a 6-min wash with ACSF (no SR 49059). GCaMP6m fluorescence was imaged at 0.2 Hz using a $\times$10 objective (numerical aperture = 0.4) and a CoolSNAP HQ_2 camera. Fluorescence was evoked with a BDX light-emitting diode system (450–495 nm; 60% power, 0.2-s exposure; X-Cite XLED1 system, Lumen Dynamics Group, Mississauga, Ontario, Canada) and the EN GFP 41017 filter cube (excitation: HQ470/40x, beam splitter: Q495lp, emission: HQ525/50m; Chroma Technology, Bellows Falls, VT).

For the dose-response analysis, changes in fluorescence measured in all cells (ΔF) were fitted to the equation $\Delta F = F_0 + \{a / [1 + (x/x_0)^b]\}$ using SigmaPlot 12.0 (Systat Software, San Jose, CA), where F_0 and a are the minima and maxima, b is a slope factor, x is the concentration of VP tested, and x_0 is the half-maximal concentration.

Posterior pituitary stimulation. Rats were killed by decapitation, and the neurointermediate lobe of the pituitary was extracted as previously described (Bourque 1990). Following incubation in ACSF at 32°C for 60 min, the tissue was placed dorsal side up on a coverslip covered with HEK-293 cells expressing the V1aR and GCaMP6m in a recording chamber perfused with ACSF (32°C) at 2–3 ml/min. A bipolar electrode (twin 65-μm-outer-diameter platinum wires) was inserted into the posterior pituitary, and electrical pulses (50 V, 0.4 ms) were delivered via an isolated stimulator triggered by a programmable digital timer. GCaMP6m fluorescence in HEK-293 cells was detected using a Lambda DG-4 lamp (330–650 nm; Sutter Instrument, Novato, CA), an EN GFP 41017 filter cube (excitation: HQ470/40x, beam splitter: Q495lp, emission: HQ525/50m; Chroma Technology), a water-immersion XLUMPlan FI $\times$20/0.95 W/0 objective, and a CoolSNAP CF^2 camera. Images were collected at 0.2 Hz with a 2-s exposure. The stimulation protocol was as follows: 3 min of baseline followed by 20 s of stimulation at 3 Hz and 10 min of recovery.

VP detection with HEK-293 biosensors in brain slices. Angled horizontal hypothalamic slices were obtained as previously described (Stachniak et al. 2014; Trudel and Bourque 2010) and placed in a beaker containing carbogenated ACSF at 32°C. Transfected HEK-293 cells were briefly treated with trypsin, lifted, resuspended in culture medium, and then gently plated directly over the slice that was resting in the ACSF-filled beaker. Preparations were allowed to rest for 2 h, allowing cells to attach to the slice before starting the experiment. Slices were then transferred to a recording chamber where they were perfused at 2–3 ml/min. As required, a bipolar stimulating electrode (twin 65-μm-outer-diameter platinum wire) was placed in the SCN where electrical pulses (20–80 μA, 0.1–0.5 ms; 10 Hz, 40 s) were delivered as explained above. Fluorescence of GCaMP6m in HEK-293 cells was observed using the EN GFP 41017 filter cube. In one data set, the images were collected at 0.2 Hz with an exposure of 200 ms. In experiments examining dendritic release, a faster acquisition rate of 5 Hz was used, with exposures of ~100 ms.

Detection of somatodendritic release from MNCs. Whole cell recordings done as previously described (Stachniak et al. 2014; Trudel and Bourque 2010) were obtained from SON MNCs in slices covered with HEK sniffer cells. Patch pipettes prepared from glass capillary tubes (1.2-mm outer diameter; A-M Systems, Carlsborg, WA) were filled with a solution containing, in mM: 140 K^+-gluconate, 2 $MgCl_2$, 10 HEPES, 2 ATP(Na_2), and 0.4 GTP(Na_2), pH adjusted to 7.25 with NaOH. Pipette resistance was 3.5–5.5 MΩ, and series resistance was 10–30 MΩ. After 3 min of stable baseline recording, a current step of

100 pA was applied to elicit action potential firing to cause activity-dependent dendritic VP release.

Western blot. HEK-293 cells expressing GCaMP6m, V1aR, and/or empty pcDNA3.1 were lysed on ice with RIPA buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, and protease inhibitors) and sonicated. Protein lysate was separated from cellular debris by centrifugation at 14,000 rpm for 10 min at 4°C. Sample buffer with a final concentration of 5.3% 2-mercaptoethanol was added, and lysates were running without heating using standard SDS-PAGE techniques. Polyvinylidene difluoride membranes were immunoblotted with anti-GAPDH (1:300,000 in 3% BSA; MAB374; EMD Millipore, Billerica, MA), anti-GFP (1:10,000 in 4% fat-free dehydrated milk; 632381; Clontech, Mountain View, CA), and anti-V1aR (1:1,000 in 3% BSA; AVR-010; Alomone, Jerusalem, Israel) and exposed with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Mississauga, Ontario, Canada).

Immunocytochemistry. Cells were fixed with 4% formaldehyde in 0.1 M PBS 24 h after transfection. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked for 2 h in 10% normal donkey serum (Jackson). Coverslips were incubated at 4°C overnight with primary antibodies, washed three times, and finally incubated at room temperature for 1 h with secondary antibodies (Invitrogen). Primary antibodies used were anti-GFP (1:1,000; ab13970; Abcam) and anti-V1aR (1:1,000; AVR-010; Alomone). The specificity of the anti-V1aR antibody is documented on the company Web site at https://www.alomone.com/p/anti-vasopressin-v1a-receptor/AVR-010?go=coa. Nuclei were stained with TO-PRO-3 Iodide in PBS (1:10,000; T3605; Invitrogen) in the second of three washes following staining with secondary antibodies.

Experimental design and statistical analysis. All group data are reported or displayed as means ± SE, and the exact sample size is provided for each experimental group or condition either in the main text or as indicated within or below bar graphs. Differences between groups were assessed using SigmaPlot 12.0. The software first assessed normality of the data distribution. In all cases where the normality test failed, a suitable nonparametric test was performed. All tests used for comparisons are specified in the main text.

RESULTS

Sniffer cells detect VP with high sensitivity. VP-sensitive sniffer cells were prepared by cotransfecting HEK-293 cells using plasmids driving expression of the human VP V1a receptor (V1aR) and the fluorescent calcium indicator GCaMP6m (Fig. 1*A*). On binding to VP, V1aRs activate endogenous phospholipase C and produce inositol (1,4,5)-triphosphate to cause Ca^{2+} release from intracellular stores (Birnbaumer 2000). The VP-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is reported as an increase in the fluorescence of GCaMP6m (Fig. 1*B*). Because OT and VP peptides can both bind to V1aRs (Peter et al. 1995), we first assessed the sensitivity and specificity of the system by examining the effects of various concentrations of OT and VP on sniffer cells 24–48 h posttransfection, a time window when both proteins are expressed (Figs. 1*C* and 2). As illustrated in Fig. 3*A*, bath application of VP caused a dose-dependent increase in GCaMP6m fluorescence (ΔF; percentage change relative to baseline), with an apparent threshold at 10 pM. A similar effect could be caused by higher concentrations of OT (≥1 nM; Fig. 3*B*). Fitting the dose-response data with a logistic equation confirmed that the EC_{50} for the effect of VP on fluorescence (half-maximum at 2.6 ± 1.0 nM; $r = 0.6436$) was ~100 times lower than the effect of OT (Fig. 3). Therefore, sniffer cells are highly sensitive to VP and respond selectively to this peptide in the picomolar to low-nanomolar range.

To address the specificity of the effects for the V1aR, we compared the effects of applying 10 or 100 nM VP and OT on cells cotransfected with V1aR and GCaMP6m or with GCaMP6m alone. In agreement with the observation that untransfected HEK-293 cells express V1aR under basal conditions (Fig. 1*C*), we found that both peptides could cause a tiny but significant increase in $[Ca^{2+}]_i$ in cells transfected with GCaMP6m alone (Fig. 4, *A* and *B*). However, responses evoked in cells overex-

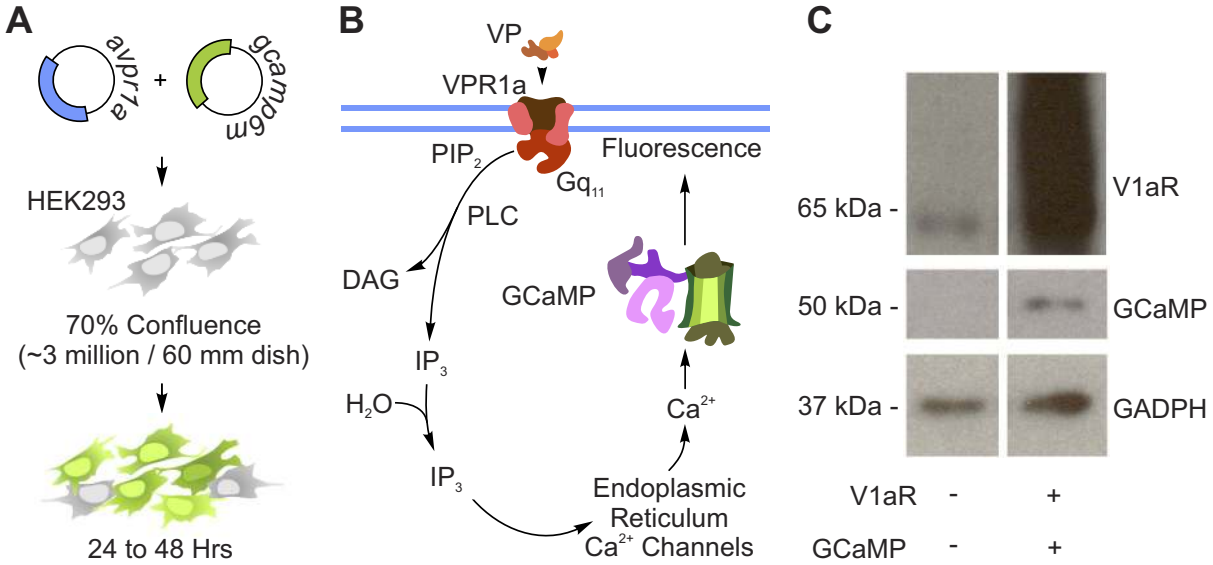

Fig. 1. Sniffer cells for detection of extracellular vasopressin (VP). *A*: schematic representation of sniffer cell preparation. Human embryonic kidney HEK-293 cells are grown in a 60-mm petri dish and transfected with plasmids driving expression of the human V1a VP receptor (V1aR; *Avpr1a*) and the calcium indicator protein GCaMP6m and used 24–48 h later. *B*: principle of sniffer cell mechanism. VP binding to V1aR (VPR1a) activates phospholipase C (PLC), which converts phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3), which triggers release of Ca^{2+} from intracellular stores. Binding of Ca^{2+} to GCaMP6m (GCaMP) increases cell fluorescence. *C*: Western blot showing expression of V1aR and GCaMP6m in transfected and untransfected cells. Note the slight expression of V1aR in untransfected cells (band near 62 kDa), whereas HEK-293 cells overexpressing the receptor display a broader band of staining between 62 and 76 kDa, as found in other studies (Innamorati et al. 1998).

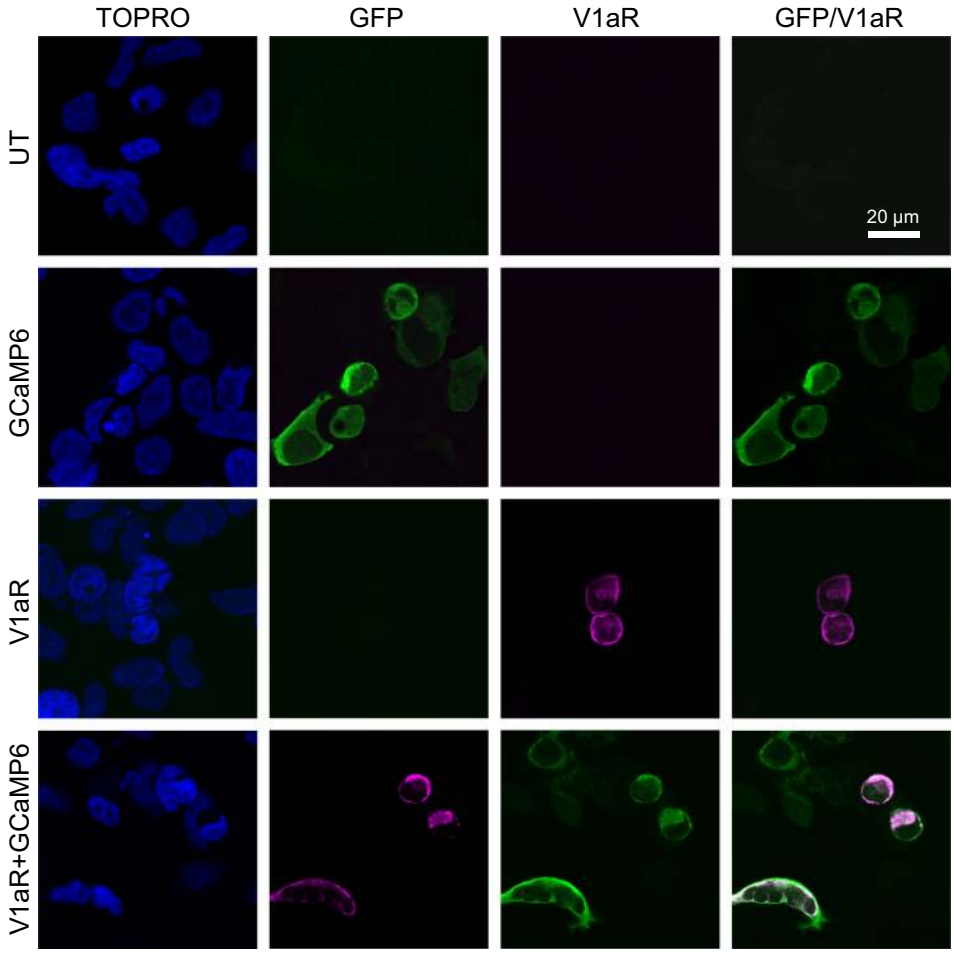

Fig. 2. Immunocytochemical detection of calcium indicator protein GCaMP6m and V1a vasopressin receptor (V1aR) in sniffer cells. Panels show human embryonic kidney HEK-293 cells stained for the nuclear marker TO-PRO or immunolabeled with antibodies against green fluorescent protein (GFP), V1aR, or both (GFP/V1aR). Cells were either untransfected (UT) or transfected with the plasmids indicated to the left of each row.

pressing both V1aR and GCaMP6m were significantly greater than those observed in cells expressing GCaMP6m alone ($P < 0.001$, Mann-Whitney rank sum test; Fig. 4*C*).

Further evidence that the responses evoked in cotransfected HEK-293 cells are mediated by V1aR was obtained using a pharmacological approach. As illustrated in Fig. 4*D*, bath application of the competitive V1aR antagonist SR 49059 (Tahara et al. 1998; 10 μM) significantly reduced $[Ca^{2+}]_i$ responses evoked by 10 nM VP and OT. Thus sniffer cells display significant V1aR-mediated increases in GCaMP6m fluorescence when local VP levels rise above ~10 pM. Finally, we examined the stability of responses induced by consecutive applications of a saturating dose of VP (100 nM; 30-min interval) in the same group of cells. As shown in Fig. 4*E*, changes in fluorescence induced by VP were not significantly different ($P > 0.05$, Wilcoxon signed rank test; $n = 13$).

Detection of VP release from neurosecretory terminals. MNCs in the SON and PVN release VP into the bloodstream through neurosecretory axon terminals in the posterior pituitary (Bourque 1991), where secretion occurs in proportion to the electrical activity of these neurons (Dreifuss et al. 1971). To determine whether sniffer cells can detect activity-dependent VP release from neurosecretory terminals, we examined the effects of electrically stimulating the isolated rat neurointermediate lobe in vitro. Because the neurointermediate lobe consists only of the isolated posterior pituitary and nonvasopressinergic cells of the intermediate lobe, we refer to this simply as the isolated posterior pituitary (PP). The isolated PP was placed on a coverslip containing HEK-293 cells cotransfected with GCaMP6m and V1aR, and a bipolar electrode was inserted into the tissue (Fig. 5*A*). As illustrated in Fig. 5*B*, electrical stimulation of the PP (3 Hz) caused a reversible increase in the fluorescence of sniffer cells, and this effect could be reproduced by application of a second stimulus (Fig. 5*C*). Compared with the effect of the first test ($\Delta F = 120 \pm 16\%$), the mean response induced by the second stimulus ($\Delta F = 186 \pm 25\%$) displayed a small but significant degree of potentiation ($P = 0.0076$, paired *t*-test; $n = 61$; Fig. 5*D*).

We next examined the effects of stimulus intensity on the responses of sniffer cells. As illustrated in Fig. 5*E*, the average increase in ΔF induced by stimulation increased in proportion to stimulus intensity and was statistically significant at intensities of 10–30 V. To ensure that these responses were not caused by a direct response of the sniffer cells to the electrical stimulus, we examined the effects of electrical stimulation on HEK cells located at the same distance of the stimulation electrode but in the absence of a PP. As shown in Fig. 5*E*, no responses could be detected under such conditions except with stimulus intensity of 30 V, where a small but significant increase was observed ($\Delta F = 27.8 \pm 8.7\%$; $n = 221$).

Detection of VP release from central axons. We next examined VP release in angled horizontal slices of rat hypothalamus, which preserve nonneurosecretory VP neurons in the

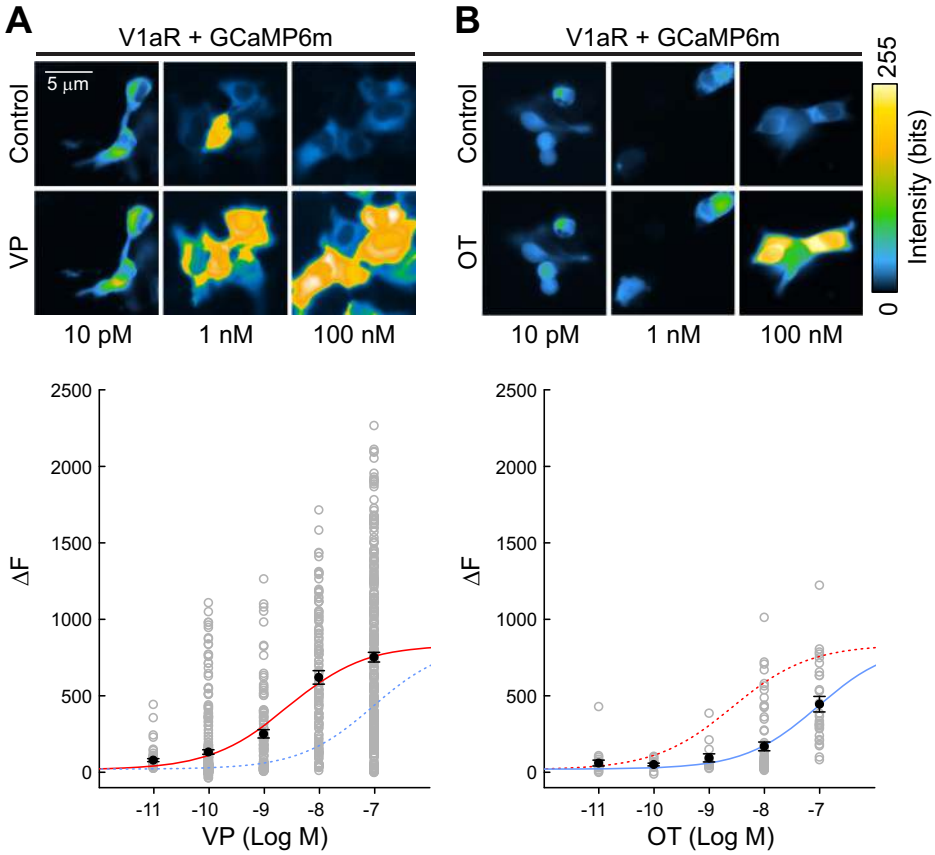

Fig. 3. Sensitivity of sniffer cells to exogenous vasopressin (VP) and oxytocin (OT). *A* and *B*: *top* shows images of sniffer cell fluorescence in the absence (Control) and presence of different concentrations of VP or OT. *Bottom* shows the changes in fluorescence (ΔF) induced by different concentrations of VP (10^{-11}, $n = 58$; 10^{-10}, $n = 250$; 10^{-9}, $n = 103$; 10^{-8}, $n = 126$; 10^{-7}, $n = 292$) and OT (10^{-11}, $n = 21$; 10^{-10}, $n = 14$; 10^{-9}, $n = 14$; 10^{-8}, $n = 62$; 10^{-7}, $n = 28$) expressed as percentage change relative to baseline. Open circles show values in all individual cells, and closed symbols are means ± SE. Solid curves are fits of the logistic equation to the entire data set (red, VP; blue, OT; see MATERIALS AND METHODS for details). Dashed lines repeat the corresponding fits on the opposite data set. GCaMP6m, calcium indicator protein; V1aR, V1a VP receptor.

SCN together with some of their efferent axons (Trudel and Bourque 2010; 2003). For this purpose, a dense carpet of HEK-293 cells coexpressing V1aR and GCaMP6m was laid over the slice (Fig. 5, *A* and *B*), and a bipolar stimulating electrode was inserted into the SCN (Fig. 5*C*). We recently showed that this approach can be used to detect VP released by the axon terminals of SCN neurons in the organum vasculosum lamina terminalis (OVLT) and SCN but not over other sites, including the nucleus accumbens (NAc) and insular cortex (InsC; Gizowski et al. 2016). Indeed, electrical stimulation caused a rapid and reversible increase of ΔF in sniffer cells located over both the SCN and OVLT but had no effect on the fluorescence of sniffer cells located over the NAc and InsC. Stimulation of the SCN caused a significant increase in ΔF over the OVLT (71.53 ± 23.34%; $n = 40$ from 3 animals; $P < 0.002$, paired *t*-test) and SCN (78.6 ± 26.97%; $n = 54$ from 4 animals; $P < 0.001$) but had no effect on ΔF of sniffer cells overlying the InsC (1.59 ± 1.99%; $n = 73$ from 3 animals; $P = 0.961$) or NAc (1.38 ± 1.44%; $n = 94$ from 3 animals; $P = 0.258$, paired *t*-test; Fig. 5*D*). The absence of response in the latter 2 regions indicates that the activation of sniffer cells over the OVLT and SCN resulted from detection of local release rather than diffusion from a distant site. Moreover, as previously shown for release in the OVLT (Gizowski et al. 2016), the increase in ΔF observed in sniffer cells located above the SCN could be blocked by SR 49059, confirming that the response specifically reflected release of VP (Fig. 6, *E* and *F*).

Detection of dendritic VP release. Previous studies have shown that the dendrites of MNCs contain a high density of VP-filled dense-core vesicles that can undergo exocytosis (Pow and Morris 1989) and mediate activity-dependent VP release within the SON (Ludwig and Leng 2006). To determine whether sniffer cells are capable of detecting activity-dependent VP release from a single MNC, we performed whole cell current-clamp recordings from MNCs in rat hypothalamic slices overlaid with sniffer cells (Fig. 7, *A* and *B*). As shown in Fig. 6, *C–E*, inducing a train of action potentials with a 100-pA current pulse caused a significant increase in the fluorescence of local sniffer cells (130 ± 33.21%; $n = 39$, slices = 5; $P = 0.018$, paired *t*-test; Fig. 6*E*). Although the minimum number of action potentials required for release was not specifically examined due to the slow image acquisition rate used in our experiments (200-ms interval), significant increases in sniffer cell responses could be observed following the generation of as few as 11 action potentials (data not shown). The delay between the onset of action potential firing and the onset of increases in ΔF in responsive sniffer cells was quite slow and variable, averaging 4.25 ± 0.5 s (Fig. 7*F*). Nonetheless, responses evoked in sniffer cells were specific to V1aRs because they could be abolished by SR 49059 (Fig. 7*G*).

DISCUSSION

Activity-dependent VP release from the axon terminals of SCN neurons was recently shown to cause a slow depolarization and excitation of OVLT neurons to promote water intake before sleep (Gizowski et al. 2016). Similarly, VP release from the dendrites of MNCs in the PVN has been shown to cause a slow depolarization of local preautonomic neurons to drive an increase in sympathetic tone during osmotic stress (Son et al. 2013). Other than for these exceptions, the cellular and network mechanisms by which

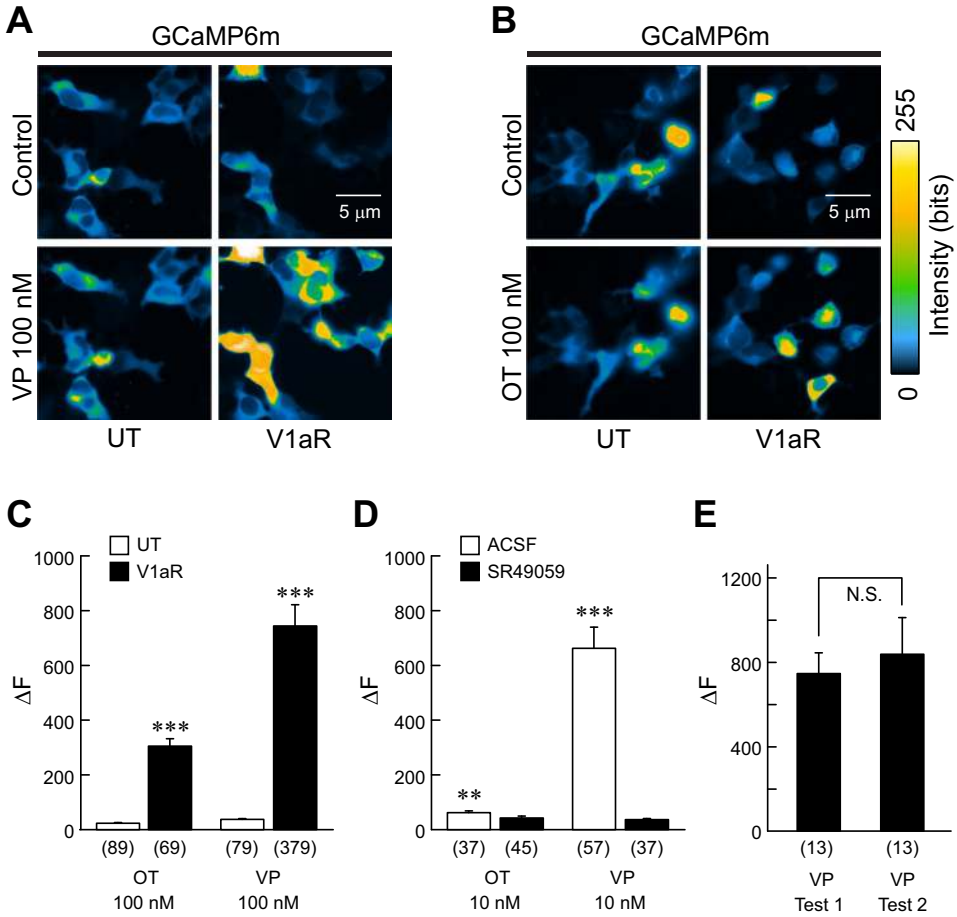

Fig. 4. Specificity of sniffer cell responses for V1a vasopressin (VP) receptor (V1aR). *A* and *B*: images of calcium indicator protein GCaMP6m fluorescence in the absence (Control) and presence of 100 nM VP (*A*) or oxytocin (OT; *B*) in GCaMP6m-expressing cells that were either transfected with V1aR or untransfected (UT). *C*: bar graphs show means ± SE. Changes in fluorescence (ΔF) were induced by 10 and 100 nM in OT and VP (*n* indicated below each bar). Note that responses are significantly greater in cells transfected with V1aR (***$P < 0.001$, Mann-Whitney rank sum test). *D*: bar graphs show means ± SE. ΔF induced by 10 nM OT and VP in cells expressing both V1aR and GCaMP6m in absence (artificial cerebral spinal fluid, ACSF) or presence of SR 49059 (*n* indicated below each bar). Note that responses induced by VP are greater than OT but that both are significantly reduced by SR 49059 (**$P < 0.05$, ***$P < 0.001$, Mann-Whitney rank sum test). *E*: bar graphs show means ± SE. ΔF induced by consecutive applications of 100 nM VP (*n* indicated below bars). N.S., not significant.

VP pathways regulate brain function remain poorly understood. In fact, mechanistic details concerning the specific contributions of most central neuropeptides are scarce because detecting their release in the vicinity of target postsynaptic neurons has been impracticable.

Release of neurotransmitters that activate ionotropic receptors can be readily detected by patch-clamp recording of the large- and short-latency (<1 ms) postsynaptic currents generated on exocytosis. However, the activation of neuropeptide receptors commonly fails to cause overt postsynaptic effects. Rather, such receptors either modulate presynaptic release of other transmitters or alter the properties of voltage-gated ion channels that are inactive at resting potential (Marder and Thirumalai 2002). Even when neuropeptide receptors affect the ongoing activity of postsynaptic channels, the low amplitude and slow kinetics of endogenous postsynaptic responses make it difficult to ascertain the magnitude and kinetics of peptide release with electrophysiological methods. Consequently, most studies on VP release have relied on post hoc immunoassay of fluid samples collected by microdialysis or push-pull perfusion (Landgraf and Neumann 2004; Myers et al. 1998). Although capable of providing information concerning context-dependent VP release within broad regions, these methods lack the spatial and temporal resolution required for online detection of VP release in areas that contain only a small number of target neurons.

Previous studies have shown that cultured cells expressing Gq_{11}-coupled receptors and a genetically encoded calcium indicator can be used as sniffer cells to detect the release of OT and VP in brain slices (Gizowski et al. 2016; Piñol et al. 2014). This approach relies on the principle that Gq_{11}-coupled receptors initiate a phospholipase C-dependent signaling cascade that causes release of Ca^{2+} from intracellular stores (Koshimizu et al. 2012) and thus an increase in the fluorescence of the calcium indicator (Chen et al. 2013).

Our experiments showed that sniffer cells produced by transient expression of human V1aRs and GCaMP6m in HEK-293 can report increases in ambient VP levels above ~10 pM. However, because expression is not constitutive, different HEK-293 cells at different phases of their cycle will express different amounts of V1aR and GCaMP6. Moreover, cells prepared on different occasions will also express different protein levels due to changes in the quality of transfection. Therefore, the responses of individual cells prepared in this way cannot be used for quantification, and the use of a stable, immortalized cell line might reduce these problems and the variability of responses evoked by fixed VP concentrations. In our study, full dose-response curves were derived from experiments where large populations of cells were tested on the same day. This approach confirmed clear differences in the global sensitivity to OT and VP. However, due to differences in the responsiveness of individual cells, it is not possible to quantify peptide levels using fluorescence measurements where only a few sparse cells are being examined, for example, within a small region over a brain slice. In these instances, therefore, the method is mainly useful for all-or-none determination of whether peptide was released.

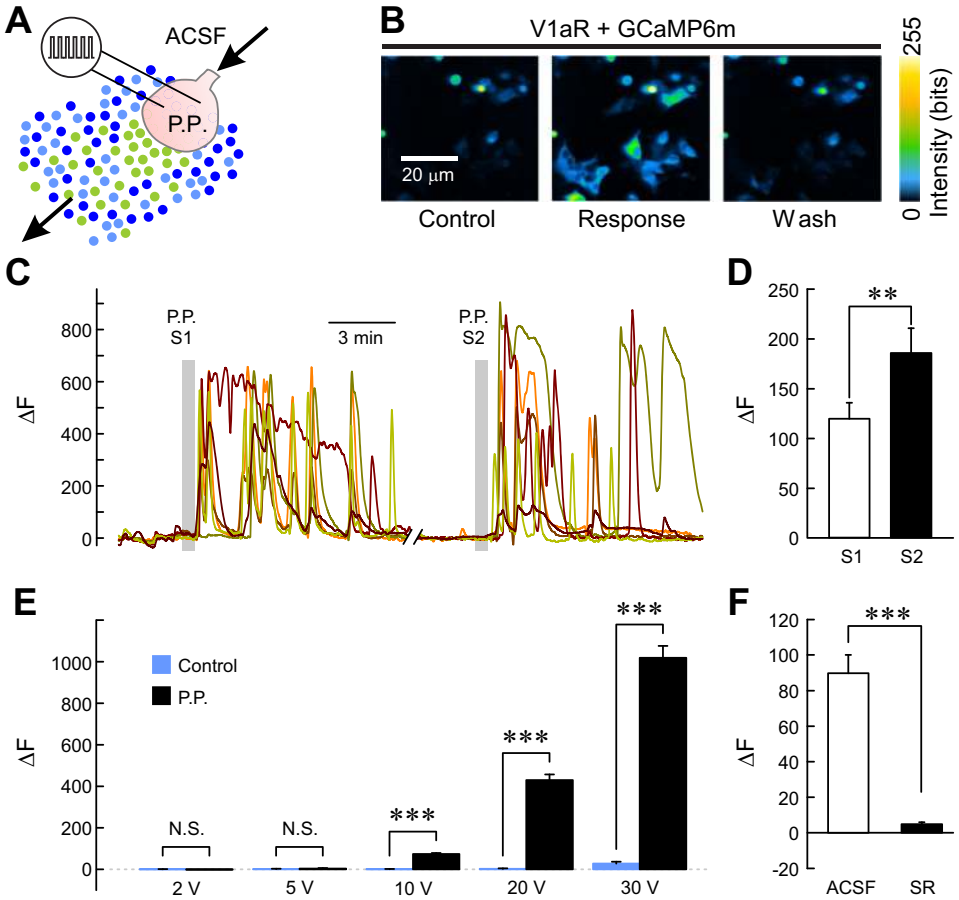

Fig. 5. Detection of vasopressin release from the isolated posterior pituitary. *A*: schematic of the experiment. Isolated rat posterior pituitary (P.P.) was placed onto a coverslip plated with sniffer cells, and a bipolar stimulating electrode was inserted into the tissue. Images were captured from adjacent sniffer cells located downstream of the P.P. ACSF, artificial cerebral spinal fluid. *B*: images of sniffer cell fluorescence obtained before (Control), during the response to P.P. stimulation (Response), and after recovery (Wash). GCaMP6m, calcium indicator protein; V1aR, V1a vasopressin receptor. *C*: graphs plot changes in fluorescence (ΔF) in individual sniffer cells induced by 2 identical stimuli (S1 and S2; shaded area). *D*: bar graphs show means ± SE. ΔF was observed during S1 and S2 in all cells analyzed ($n = 61$; $^{**}P < 0.01$, paired *t*-test). *E*: bar graphs show means ± SE. Shown are ΔF in sniffer cells induced by electrical stimulation of the P.P. (5 Hz) at different stimulus intensities (2 V, $n = 17$; 5 V, $n = 10$; 10 V, $n = 11$; 20 V, $n = 22$; 30 V, $n = 19$) or ΔF observed after delivery of stimuli with the electrode placed at the same distance but without a P.P. attached (Control, blue; 2 V, $n = 25$; 5 V, $n = 28$; 10 V, $n = 25$; 20 V, $n = 14$; 30 V, $n = 221$). Note that responses to P.P. stimulation are significantly greater than controls at 10, 20, and 30 V ($^{***}P < 0.001$, 1-way analysis of variance on ranks followed by Dunn multiple comparison; N.S., not significant). *F*: bar graphs show means ± SE. ΔF was observed in sniffer cells stimulated in the absence (ACSF) or presence or SR 49059 (SR; $^{***}P < 0.001$, Mann-Whitney rank sum test; $n = 42$).

As mentioned above, sniffer cells showed a high degree of sensitivity (~10 pM to VP) and greater degree of sensitivity for VP (~100 times greater than for OT). Although this level of sensitivity makes it possible to detect endogenous VP release in situ, the selectivity of the method does not allow a user to infer whether VP or OT was released unless other information indicates that only one of these peptides could be released in a particular circumstance. For example, the SCN does not contain OT neurons, so responses evoked by SCN stimulation must reflect VP release.

In principle, using a more selective Gq_{11}-coupled VP receptor could circumvent this issue. For example, the human form of the Gq_{11}-coupled V1b receptor displays an affinity for VP that is 1,600 times greater than for OT (Koshimizu et al. 2012). Whether use of this receptor would significantly improve the selective sensitivity of VP sniffer cells remains to be determined. It is important to note that untransfected HEK cells expressed basal levels of V1aRs that were sufficient to mediate tiny but significant increases in Ca^{2+} on exposure to VP. Therefore, it would be important to suppress this constitutive expression genetically or use a cell line lacking V1aR to optimize the potential benefit provided by transfection of the V1b or other receptor. Another factor to consider is that HEK sniffer cells are likely to express basal levels of other types of Gq_{11}-coupled receptors. Such receptors might be activated by substances other than VP that are coreleased with the peptide or even secreted by another cell in response to postsynaptic activation. It is, therefore, important to hold knowledge concerning the anatomic organization of VP neurons and fibers in the area being studied and to validate the involvement of the desired sniffer receptor (e.g., V1aR) using a selective antagonist during experiments aiming to ascertain the release of endogenous VP.

Unlike postsynaptic currents induced by ionotropic receptors, which occur <1 ms following arrival of an action potential into the nerve terminal, sniffer-cell-mediated detection of VP secretion occurred with a significant delay (average 4.2 s). The reason for this delay is likely to be the time taken for VP to diffuse from its site of release within the parenchyma to reach the overlying sniffer cells because cells exposed to

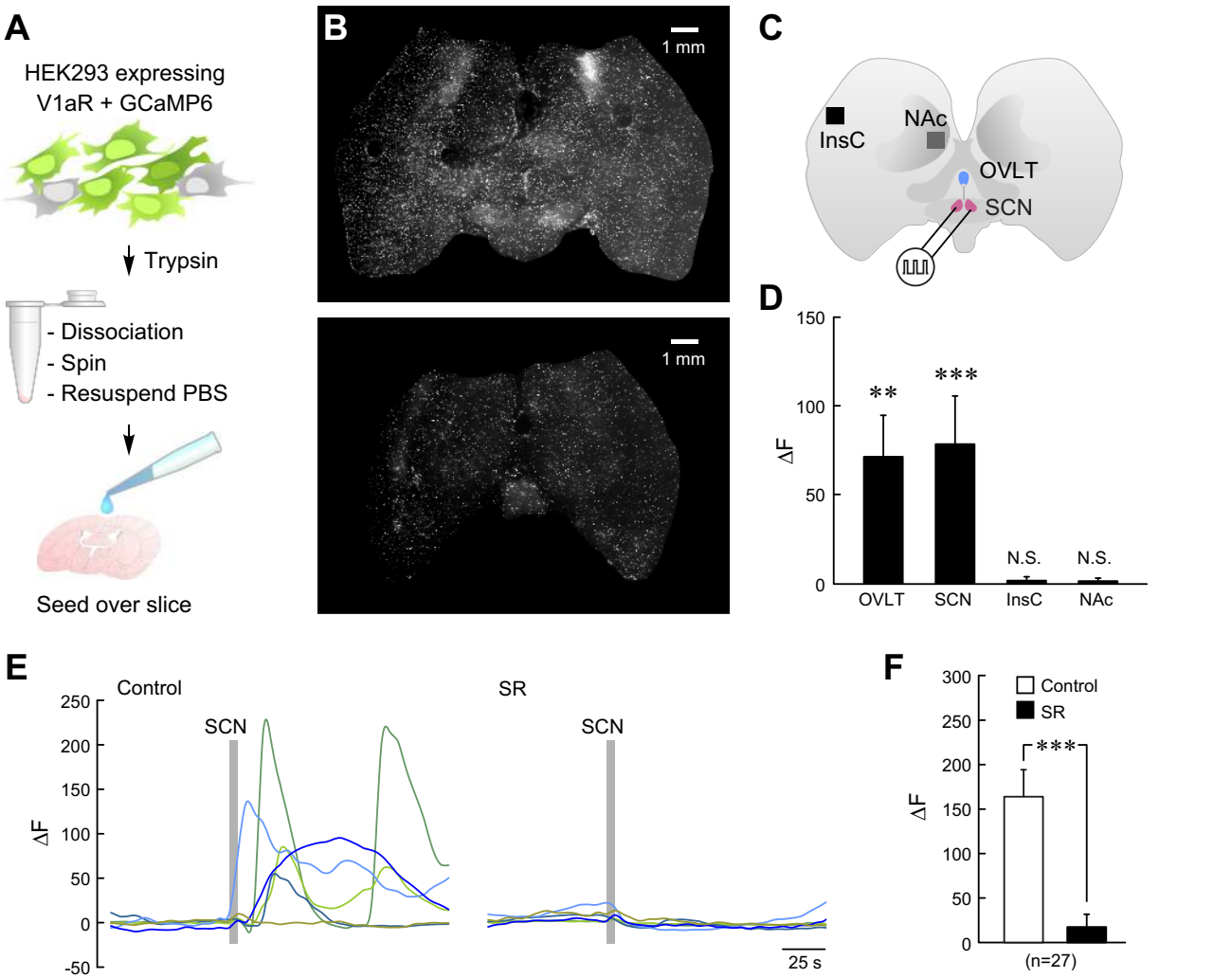

Fig. 6. Detection of vasopressin release in brain slices. *A*: schema of the preparation. Sniffer cells were detached from the culture dish, spun to remove the media, resuspended in 1 ml of saline, and gently seeded over a slice lying at the bottom of a beaker containing carbogenated artificial cerebral spinal fluid (ACSF). After >60 min, the slice was placed in the imaging chamber. GCaMP6m, calcium indicator protein; V1aR, V1a vasopressin receptor. *B*: images show slices covered with human embryonic kidney cells (HEK293) expressing mCherry (white dots) plated at full concentration (*top*) or diluted 50% (*bottom*). *C*: schematic of the imaging experiment. Bipolar stimulating electrode was inserted into the suprachiasmatic nucleus (SCN) to deliver electrical pulses, and sniffer cells were imaged in 4 different areas: insular cortex (InsC; $n = 73$), nucleus accumbens (NAc; $n = 94$), organum vasculosum laminae terminalis (OVLT; $n = 40$), and SCN ($n = 54$). *D*: bar graphs plot means ± SE. Shown are changes in fluorescence (ΔF) in sniffer cells overlying the areas shown in *C* (**$P < 0.01$, ***$P < 0.001$; N.S., not significant; paired *t*-test). *E*: graphs plot ΔF in sniffer cells overlying the SCN when subjected to SCN stimulation (gray bars) in the absence (Control) or presence of SR 49059 (SR). Plots are from the same cells in both conditions. *F*: bar graphs plot means ± SE values of ΔF induced by SCN stimulation in sniffer cells imaged over the SCN in Control and SR (***$P < 0.001$, Wilcoxon signed rank test; $n = 27$).

exogenous VP responded within 1 s following application of exogenous VP with a puffer pipette. Therefore, the temporal resolution of this method is suitable for the analysis of VP release that occurs in response to brief trains of action potentials but is not likely to provide a means to detect VP release on an impulse-to-impulse basis. Nonetheless, the approach improves on the kinetics and spatial resolution offered by push-pull approaches and indicates that sniffer cells can be used to detect activity-dependent VP release from 3 distinct neuronal compartments: neurosecretory axon terminals, central axons, and soma-dendrites.

A highly significant degree of VP release could be evoked by electrical stimulation of the isolated neurohypophysis using as few as 60 pulses applied at 3 Hz (Fig. 4*E*). This approach, therefore, provides a powerful tool for future studies on excitation-secretion coupling that could expand previous knowledge gained from classic radioimmunoassay experiments on neurohypophysial axon terminals (Bicknell 1988). For example, conditions demanding a high degree of sustained VP secretion have been shown to induce a phasic pattern of firing in VP MNCs, which consists of alternating periods of activity and silence lasting tens of seconds each (Brown and Bourque 2006; Brown et al. 2004), and this pattern enhances the amount of peptide released per impulse compared with tonic firing (Dutton and Dyball 1979). Interestingly, previous studies have shown that a more intense and briefer form of rhythmicity can also be induced by the activation of *N*-methyl-D-aspartate receptors in MNCs (Gagnon et al. 2014; Hu and Bourque 1992). The sniffer cell approach could, therefore, be used to establish whether this type of activity can further enhance VP secretion compared with phasic firing. Similarly, the possibility that different patterns of activity may elicit the release of

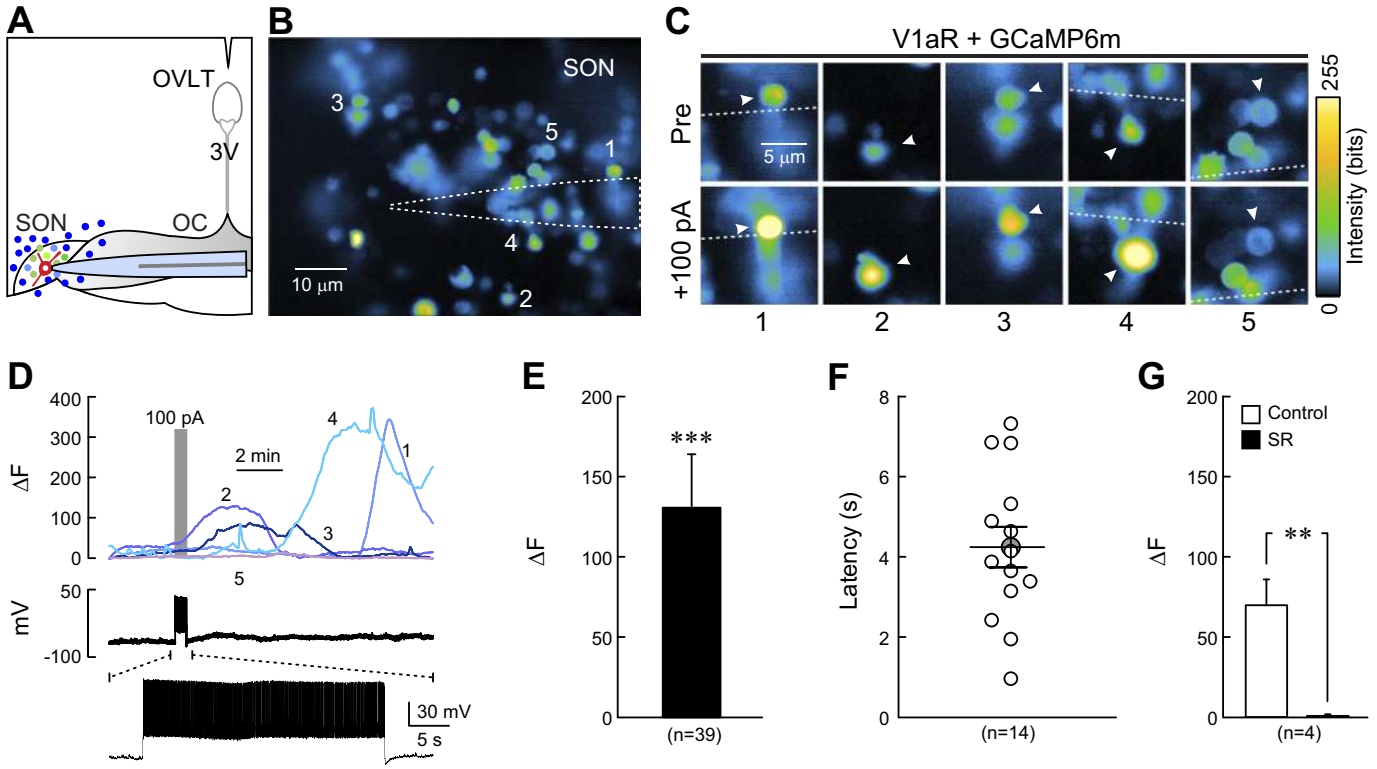

Fig. 7. Detection of somatodendritic vasopressin release from single magnocellular neurosecretory cells (MNCs). *A*: schema of the experiment. MNCs in the supraoptic nucleus (SON) adjacent to the optic chiasma (OC) were recorded using whole cell current-clamp in slices covered with sniffer cells. Images of fluorescence in the area surrounding the recorded cell were captured every 200 ms. OVLT, organum vasculosum lamina terminalis. *B*: field of view showing sniffer cells located above an MNC (not visible) recorded with a patch electrode (outlined with dashed lines). Numbers identify 5 specific sniffer cells analyzed in *C* and *D*. *C*: arrowheads in images indicate each of the 5 cells identified in *B* before (Pre) and after application of a depolarizing current pulse (+100 pA). GCaMP6m, calcium indicator protein; V1aR, V1a vasopressin receptor. *D*: *top* graph plots changes in fluorescence (ΔF) relative to Control for the cells identified in *C* and *D*. *Bottom* traces shows action potential activity evoked by the depolarizing pulse (shaded area) on the same time scale as ΔF (*top* trace) as well as an expanded trace (lower) showing action potentials. *E*: bar graph shows the means ± SE. ΔF was observed in 39 responsive sniffer cells during tests performed on 6 neurons (***$P < 0.001$ vs. Pre, Wilcoxon signed rank test). *F*: open circles plot latencies to response onset in sniffer cells analyzed during tests performed on 14 separate neurons (gray symbol shows means ± SE). *G*: bar graphs plot means ± SE. Shown is ΔF in 4 responsive sniffer cells tested in the absence (Control) or presence of SR 49059 (SR) in a single neuron (**$P < 0.01$, paired *t*-test).

different cotransmitters can potentially be explored using this method.

Sniffer cells could also detect VP release from the axons of SCN neurons. Notably, release detected within the OVLT following electrical stimulation of the SCN must have originated from the axons of SCN VP neurons because the OVLT does not contain VP-releasing cells and the SCN serves as the exclusive source of VP fibers that innervate this structure (Gizowski et al. 2016). The sniffer cell approach, therefore, provides a unique opportunity to investigate the network- and activity-dependent release of endogenous VP from central pathways hypothesized to mediate behavior.

Finally, our experiments provide a striking demonstration that action potential firing can also cause VP release from the soma-dendritic compartment of MNCs (Ludwig and Leng 2006; Ma and Morris 2002; Pow and Morris 1989). Although we cannot fully exclude the possibility that spiking could have induced VP release from axon collaterals located in the SON, previous studies have shown that somato-dendritic release can provide feedback autocontrol of electrical activity in these cells (Chevaleyre et al. 2000). Moreover, as indicated earlier, dendritic VP release from PVN MNCs has been shown to cause a paracrine excitation of local nonneurosecretory neurons (Son et al. 2013). The activity- and state-dependent control of dendritic release likely plays a key role in many central peptidergic systems. The availability of sniffer cells to investigate these processes opens the way for a new understanding of neuropeptide signaling in central networks.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.Z., K.K.M., and C.W.B. conceived and designed research; C.Z., C.G., and C.K.S. performed experiments; C.Z., C.G., and C.K.S. analyzed data; C.Z.,

K.K.M., and C.W.B. interpreted results of experiments; C.Z., C.K.S., and C.W.B. prepared figures; C.Z. drafted manuscript; C.G. and C.W.B. edited and revised manuscript; C.Z., C.G., C.K.S., K.K.M., and C.W.B. approved final version of manuscript.

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