



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Dendritic Peptide Release Mediates Interpopulation Crosstalk between Neurosecretory and Preautonomic Networks

Citation for published version:

Son, SJ, Filosa, JA, Potapenko, ES, Biancardi, VC, Zheng, H, Patel, KP, Tobin, VA, Ludwig, M & Stern, JE 2013, 'Dendritic Peptide Release Mediates Interpopulation Crosstalk between Neurosecretory and Preautonomic Networks' *Neuron*, vol 78, no. 6, pp. 1036-1049. DOI: 10.1016/j.neuron.2013.04.025

Digital Object Identifier (DOI):

[10.1016/j.neuron.2013.04.025](https://doi.org/10.1016/j.neuron.2013.04.025)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Neuron

Publisher Rights Statement:

This is a PDF file of an unedited manuscript that has been accepted for publication. The publisher version is available at: <http://www.sciencedirect.com/science/article/pii/S0896627313003565>

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Please cite this article as: Son, SJ, Filosa, JA, Potapenko, ES, Biancardi, VC, Zheng, H, Patel, KP, Tobin, VA, Ludwig, M & Stern, JE, 'Dendritic Peptide Release Mediates Interpopulation Crosstalk between Neurosecretory and Preautonomic Networks' Neuron 2013, <http://dx.doi.org/10.1016/j.neuron.2013.04.025>

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

**DENDRITIC PEPTIDE RELEASE MEDIATES INTER-POPULATION CROSSTALK
BETWEEN NEUROSECRETORY AND PREAUTONOMIC NETWORKS**

Son, SJ¹, Filosa JA¹, Potapenko¹, E; Biancardi¹, VC, Zheng H², Patel KP², Tobin VA³
Ludwig M³, and Stern JE¹

¹Department of Physiology, Georgia Health Sciences University, Augusta GA USA

²Department of Cellular and Integrative Physiology, University of Nebraska Medical
Center, Omaha, Nebraska

³Centre for Integrative Physiology, University of Edinburgh, Edinburgh UK

Running Title: Neurosecretory-autonomic interpopulation crosstalk

Corresponding Author

Javier E. Stern, M.D., Ph.D.

Department of Physiology

Georgia Health Sciences University

1120 15th St. Augusta GA USA

jstern@georgiahealth.edu

Phone: 706-721-2180

Fax: 706-721-7299

1 **Highlights**

2 • Proof of a novel signaling modality mediating inter-population crosstalk in the brain

3

4

5 • Dendritic VP release from neurosecretory neurons stimulates autonomic neurons

6 • Crosstalk involves Ca^{2+} -mediated activation of a CAN channel in autonomic neurons

7 • This crosstalk is involved in the generation of polymodal homeostatic responses

8

9

10

1 **SUMMARY**

2 While communication between neurons is classically considered a function of the
3 synapse, neurons can also release neurotransmitter from their dendrites. We found that
4 dendritic transmitter release coordinates activity across distinct neuronal populations to
5 generate integrative homeostatic physiological responses. We show that activity-
6 dependent release of vasopressin from hypothalamic neuroendocrine neurons in the
7 paraventricular nucleus stimulates neighboring (~100 μm soma-to-soma)
8 presympathetic neurons, resulting in a sympathoexcitatory population response. This
9 inter-population crosstalk was engaged by an NMDA-mediated increase in dendritic
10 Ca^{2+} , was influenced by vasopressin's ability to diffuse in the extracellular space, and
11 involved activation of CAN channels at the target neurons. Furthermore, we
12 demonstrate that this inter-population crosstalk plays a pivotal role in the generation of a
13 systemic, polymodal neurohumoral homeostatic response to a hyperosmotic challenge.
14 Since dendritic release is emerging as a widespread process, our results suggest that a
15 similar mechanism could mediate inter-population crosstalk in other brain systems,
16 particularly those involved in generating complex behaviors.

17

1 INTRODUCTION

2 Information processing in the CNS involves a wide array of spatiotemporal scales,
3 ranging from temporally fast and spatially precise (critical for coherent spike timing
4 between two neurons (Galarreta and Hestrin, 2001), to temporally slow and spatially
5 diffuse, a modality best-suited for the coordination of activity within or across entire
6 neuronal populations (Fuxe et al., 2007; Leng and Ludwig, 2008). Despite the
7 importance of the latter in the generation of complex behaviors (Ludwig and Leng,
8 2006), the precise signaling mechanisms underlying inter-population crosstalk in the
9 brain remain largely unknown.

10 Neuropeptides are increasingly recognized as unique signals involved in information
11 processing in the brain (Leng and Ludwig, 2008; Salio et al., 2006). They are
12 abundantly found in dendrites (Guan et al., 2005; Pow and Morris, 1989), their release
13 is generally not confined to, or targeted at synaptic/postsynaptic sites, and given their
14 relatively long half-lives (Mens et al., 1983), they can diffuse in the extracellular space
15 (ECS) to act on distant targets. Thus, unlike classical fast-acting neurotransmitters,
16 neuropeptide signaling lacks temporal and spatial precision, making it ideally suited to
17 mediate communication between populations of neurons (Fuxe et al., 2007; Landgraf
18 and Neumann, 2004; Ludwig and Leng, 2006).

19 Neuropeptides are widely used as signaling molecules in the hypothalamus,
20 particularly within the supraoptic (SON) and paraventricular (PVN) nuclei. These centers
21 are critically involved in the generation of complex polymodal homeostatic responses,
22 consisting of orchestrated activities of autonomic and neuroendocrine networks (Buijs
23 and Van Eden, 2000; Swanson and Sawchenko, 1980). During disturbances of
24 fluid/electrolyte homeostasis, activation of magnocellular neurosecretory (MNNs) and
25 presympathetic neurons in the PVN results in the concerted systemic release of the
26 hormone vasopressin (VP), along with an increase in renal sympathetic outflow,
27 respectively, acting together to restore fluid/electrolyte balance (Bourque, 2008; Toney
28 and Stocker, 2010). Importantly, an imbalanced interaction amongst these systems
29 results in maladaptive responses characteristic of disease conditions, including stress,
30 and hypertension (Ely, 1995; Esler et al., 1995). Thus, generation of homeostatic

1 responses by the PVN represents an ideal paradigm to study inter-population signaling
2 mechanisms within the brain, both under physiological and pathological conditions.

3 In addition to playing key roles in the processing and integration of synaptic inputs,
4 dendrites are recognized to be major sources of brain neuropeptides (Guan et al., 2005;
5 Pow and Morris, 1989), MNNs being one of the best-studied prototypes of dendritic
6 peptide release (Ludwig and Leng, 2006). Besides releasing their peptide content from
7 neurohypophyseal axonal terminals into the circulation, MNNs also release VP and
8 oxytocin (OT) locally from their dendrites, serving as a powerful autocrine signal by
9 which they auto-regulate their activity (Gouzenes et al., 1998; Ludwig and Leng, 1997).
10 However, whether dendritically released peptides from MNNs can act beyond their own
11 secreting population, to mediate inter-population crosstalk, has not yet being explored.
12 Using the magnocellular neurosecretory system as a unique model system, we tested
13 the hypothesis that dendritic peptide release constitutes a powerful inter-population
14 signaling modality in the brain. More specifically, we assessed whether dendritically
15 released VP mediates crosstalk between neurosecretory and presympathetic
16 hypothalamic neurons in the context of homeostatic neurohumoral responses to an
17 osmotic challenge.

18 Using a combination of *in vitro* approaches in acute hypothalamic slices, including
19 patch-clamp electrophysiology, confocal imaging and laser photolysis of caged
20 molecules, we demonstrate that dendritically-released VP from a single stimulated
21 neurosecretory neuron evoked a direct excitatory response in presympathetic neurons
22 located $\sim 100 \mu\text{m}$ away. Moreover, we found that activity-dependent dendritic VP
23 release from the whole population of neurosecretory neurons translated into a diffusible
24 pool of peptide that tonically stimulated presympathetic neuronal activity. Finally, using
25 an *in vivo* homeostatic challenge, we show that dendritic VP release is critical for the
26 recruitment of presympathetic neurons, resulting in an optimal sympathoexcitatory
27 outflow during a homeostatic challenge that requires an orchestrated neurosecretory
28 and sympathetic response.

29

30 **RESULTS**

1 **Intimate dendro-somatic and dendro-dendritic interrelationships between**
2 **presympathetic and magnocellular neurosecretory PVN neurons.**

3 It is well-documented that neurosecretory and presympathetic neuronal somata in
4 the PVN are anatomically compartmentalized within specific subnuclei (Swanson and
5 Kuypers, 1980; Swanson and Sawchenko, 1980). Using a combination of retrograde
6 tract tracing and immunohistochemistry to identify presympathetic PVN neurons that
7 innervate the rostroventrolateral medulla (RVLM; PVN-RVLM neurons) and VP MNNs,
8 respectively, we verified this early observation (**Fig.1A**). However, a more detailed
9 analysis revealed that thick and varicose dendritic processes from VP MNNs extended
10 beyond their own neuronal compartment, coming in close proximity to somatodendritic
11 elements of presympathetic neurons in the ventromedial, dorsal cap and posterior
12 parvocellular subnuclei (Swanson and Kuypers, 1980) (**Fig.1B-G**). The identity of these
13 processes as dendrites was confirmed by MAP2 immunoreactivity (**Fig.1I**), and by being
14 abutted by numerous dopamine β hydroxylase (DBH)-immunoreactive presynaptic
15 boutons (**Fig.1J**). Conversely, VP axons ran laterally out of the PVN boundaries, then
16 turning ventrally and caudally towards the median eminence (**Fig.1H**) (Swanson and
17 Kuypers, 1980). These studies support thus a distinctive anatomical microenvironment
18 that would enable dendro-dendritic/somatic communication from neurosecretory to
19 presympathetic neurons, possibly via dendritically released VP.

20 **Activation of V1a receptors in presympathetic PVN neurons increases their firing**
21 **activity via a Ca²⁺-dependent activation of TRPM4/5 channels.**

22 To determine if presympathetic neurons sense dendritically-released VP from
23 MNNs, we first assessed for the expression of V1a receptors (the most common type of
24 VP receptor found in the brain (Zingg, 1996)) in retrogradely-labeled PVN-RVLM
25 neurons. As shown in **Fig.2A-D**, we found a dense V1a receptor immunoreactivity in
26 somato-dendritic regions of presympathetic neurons. Similar results were found with an
27 alternative V1a antibody (**Fig.S1**), recently shown to label V1a receptors in olfactory
28 bulb neurons (Tobin et al., 2010). The resolution of the light microscopic approach
29 however, does not readily distinguish V1a clusters located near the surface membrane
30 of PVN-RVLM neurons from ones potentially located at presynaptic terminals. Further

1 supporting the expression of V1a receptors by PVN-RVLM neurons, however, we report
2 expression of V1a receptor mRNA in these neuronal population (**Fig.2E**).

3 Focal application of VP onto presympathetic PVN neurons resulted in direct
4 membrane depolarization and increased firing discharge (n=16, P<0.001, **Fig.2F-I**). VP
5 effects were almost completely blocked by a selective V1a receptor antagonist (β -
6 Mercapto- β , β -cyclopentamethylenepropionyl¹, O-me-Tyr², Arg⁸]-Vasopressin, 1 μ M, P<
7 0.01, n=8, **Fig.2H**), but persisted in the presence of the ionotropic glutamate and
8 GABA_A receptor antagonists kynurenate (1 mM) and bicuculline (20 μ M)(basal: 0.30
9 \pm 0.13 Hz; VP: 2.75 \pm 0.53 Hz, P<0.01, n=6), or in the presence of a low Ca²⁺ synaptic
10 block media (basal: 0.58 \pm 0.38 Hz; VP: 3.85 \pm 0.38 Hz, P< 0.02).

11 The VP-mediated increase in firing activity in presympathetic neurons was preceded
12 (3.1 \pm 0.8 s) by an increase in [Ca²⁺]_i (P< 0.01, n=8, **Fig.3A-C**), and was abolished by
13 chelation of intracellular Ca²⁺ with BAPTA (10 mM) (n=8, **Fig.3D**). In voltage-clamp
14 mode, VP evoked an outwardly rectifying current with an apparent reversal potential of
15 \sim -15 mV (**Fig.3E**). Taken together, these results support the involvement of a Ca²⁺-
16 activated non-selective cation current (CAN) (Petersen, 2002). We found PVN-RVLM
17 neurons to express dense immunoreactivity (**Fig.S2A-D**) and mRNA (**Fig.S2E**) for
18 TRPM4 channels, a major CAN channel member of the transient receptor potential
19 (TRP) family (Ullrich et al., 2005), and previously reported in the hypothalamus
20 (Ghamari-Langroudi and Bourque, 2002; Teruyama and Armstrong, 2007; Teruyama et
21 al., 2011). We also found that VP excitatory effects on PVN-RVLM neurons were
22 blocked by flufenamic acid (FFA, 200 μ M) (n=9, **Fig.S2F**), a relatively specific blocker of
23 TRPM4/5 channels (Ullrich et al., 2005). In the presence of FFA, PVN-RVLM neurons
24 were still capable of displaying a burst of action potential in response to a puff of 20 μ M
25 NMDA (n=3, *not shown*), indicating that FFA effects were not due to non-specific effects
26 on overall neuronal function, or due to changes in PVN-RVLM responsiveness to
27 NMDA. Further studies however, are needed to precisely identify the molecular identity
28 of the CAN channel underlying VP actions in presympathetic neurons.

29 **Dendritic release of vasopressin mediates cell-cell communication between**
30 **neighboring neurosecretory and presympathetic neurons.**

1 To directly probe for a crosstalk between MNNs and presympathetic neurons, we
2 developed an approach using transgenic eGFP-VP rats (Ueta et al., 2005) that received
3 an injection of a fluorescent retrograde tracer in the RVLM (**Fig.S3**). Our approach
4 consisted on selectively activating individual VP neurons using laser photolysis of
5 caged-NMDA, while simultaneously monitoring the electrical activity of neighboring
6 presympathetic neurons in acute hypothalamic slices. To validate this approach, we
7 show that laser photolysis of caged-NMDA onto restricted somatodendritic regions of
8 patched eGFP-VP neurosecretory neurons induced reproducible inward currents along
9 with a concurrent high-frequency burst of action potentials (**Fig.4A**), previously shown to
10 efficiently evoke dendritic release of peptides from MNNs in brain slices (Kombian et al.,
11 1997). Moreover, photolysis of caged-NMDA in the somata of Fluo-5F loaded eGFP-VP
12 neurons increased $[Ca^{2+}]_i$ levels, which rapidly propagated into dendritic compartments
13 (**Fig.S4**).

14 To test the hypothesis that dendritic VP release acts as a crosstalk signal between
15 neurosecretory and presympathetic neurons, we then obtained patch recordings from
16 PVN-RVLM neurons, and assessed their responses to photolysis of caged-NMDA in
17 neighboring eGFP-VP neurons. On average, 3 different eGFP-VP neurons were
18 photoactivated *per* patched PVN-RVLM neuron. The mean distance between the
19 somata of presympathetic and the photoactivated VP neurons was $111.6 \pm 7.9 \mu\text{m}$.
20 Photolysis of caged-NMDA at the somata of individual eGFP-VP neurons consistently
21 evoked an excitatory response in neighboring PVN-RVLM neurons, characterized by a
22 burst of activity, which was underlain by a membrane depolarization (n=38 eGFP-VP
23 neurons/11 PVN-RVLM neurons, $P < 0.001$, **Fig.4B**). Responses in presympathetic
24 neurons occurred with a mean latency of 3.5 ± 1.0 sec following photolysis in
25 neighboring eGFP-VP neurons. In a few cases (n=4), stimulation of an eGFP-VP
26 neuron failed to evoke a response in PVN-RVLM neurons, which were however
27 responsive to other eGFP-VP neurons in the same preparation.

28 Direct photolysis of caged-NMDA onto the recorded neurons (eGFP-VP or
29 presympathetic) resulted in an almost instantaneous effect ($P < 0.05$ n=11, **Fig.4**).
30 Importantly, presympathetic responses to photoactivation of eGFP-VP neurons were
31 almost completely blocked following bath application of the V1a receptor antagonist

1 **Fig.4C**). No relationship between the uncaging distance and magnitude or delay of the
2 evoked response was observed.

3 While action potentials are necessary for axonal VP release, dendritic release can
4 occur in Ca^{2+} -dependent, but action potential-independent manner (Ludwig et al., 2002).
5 Thus, to rule out a potential, but unlikely contribution of VP released from an axon
6 collateral within the PVN (Hatton et al., 1985; Ludwig, 1998), experiments were
7 repeated in the presence of 1 μM TTX. Under these conditions, photolysis of NMDA
8 onto eGFP-VP neurons still evoked a membrane depolarization in neighboring PVN-
9 RVLM neurons (n=18), effects that were blocked by the V1a receptor antagonist ($P <$
10 0.01, n=14, **Fig.5**). Conversely, following activation of eGFP-VP neurons,
11 presympathetic responses were prevented by a 0 Ca^{2+} / 3 mM EGTA aCSF ($\Delta 1.2 \pm 0.2$
12 mV, n=10, $P > 0.3$), without altering PVN-RVLM responses to direct uncaging of NMDA
13 ($\Delta 5.5 \pm 0.5$ mV, $P < 0.05$ n=4). These results support that Ca^{2+} -dependent dendritic
14 release of VP (Ludwig et al., 2002) stimulates the activity of neighboring presympathetic
15 PVN neurons via activation of V1a receptors.

16 To rule out the possibility that PVN-RVLM neuronal responses were due to diffusion
17 of caged-NMDA beyond the photoactivated region in the eGFP-VP neurons, a subset of
18 recorded PVN-RVLM neurons were dialysed with the NMDAR blocker MK-801 (1 mM).
19 While intracellular MK801 significantly blocked the effect of direct photolysis of caged-
20 NMDA onto the recorded neurons ($\Delta 1.3 \pm 0.9$ mV, $P > 0.2$, n=4), photolysis onto eGFP-
21 VP neurons still evoked a depolarizing response from the same PVN-RVLM neurons
22 ($\Delta 5.2 \pm 1.4$ mV, $P < 0.05$, n=4) (**Fig.S5**). Additional controls included laser stimulation
23 without the presence of caged-NMDA, and bath application of caged-NMDA alone, both
24 of which failed to evoke neuronal responses (*not shown*). Finally, NMDA uncaging onto
25 eGFP-VP neurons (n=33) failed to evoke changes in $[\text{Ca}^{2+}]_i$ in the vast majority of
26 Rhod-2 loaded astrocytes (~87%). In the few responsive astrocytes, increases in $[\text{Ca}^{2+}]_i$
27 occurred with a long delay (> 40 s) (**Fig.S6**).

28 To further prove the neurosecretory-presympathetic neuronal crosstalk, we
29 performed dual-patch recordings from identified eGFP-VP and PVN-RVLM neurons. In
30 13/15 pairs tested, we found that evoked bursting firing in VP neurons resulted in a
31 significant membrane depolarization ($P < 0.001$, n=13) and increase firing discharge ($P <$

1 0.02, n=13) in the neighboring presympathetic neurons (**Fig.6B,F**). These effects were
2 largely blocked following bath application of the V1a antagonist (n=7) or in pairs in
3 which eGFP-VP neurons were dialyzed with BAPTA (n=5) (**Fig.6C,F**). No responses
4 were observed in the remaining two pairs. We found that the latency for the evoked
5 PVN-RVLM depolarization was significantly longer when a prominent after-
6 hyperpolarizing potential (AHP) following the evoked bursts of action potentials was
7 observed in the paired eGFP-VP neuron (n=9, **Fig.6B1**), compared to neurons in which
8 AHPs were absent (n=6, **Fig.6B2**), or those in which a depolarizing afterpotential (DAP)
9 was observed instead (n=3; **Fig.6D**) ($P < 0.001$, **Fig.6E**). Moreover, a significant
10 correlation between the eGFP-VP AHP duration and the PVN-RVLM latency was found
11 (Pearson $r = 0.89$, $P < 0.0001$). The mean latency in paired recordings in which AHPs in
12 eGFP-VP neurons were absent was similar to that observed following photolysis of
13 caged NMDA ($P > 0.3$, see above), in which AHPs were not observed. In contrast to the
14 effect on latency, the magnitude of the PVN-RVLM response was independent of the
15 presence or duration of an AHP in the stimulated eGFP-VP neurons (not shown).

16 Finally, to determine whether astrocytes participate as intermediaries in the
17 neurosecretory-presympathetic crosstalk, experiments were repeated following
18 functional ablation of astrocytes with the selective gliotoxin L-aminoadipic acid (L-AAA,
19 $250 \mu\text{M}$, 30-60 min)(McBean, 1994; Xu et al., 2008). Under this condition, stimulation of
20 eGFP-VP neurons still efficiently evoked an excitatory response in PVN-RVLM neurons
21 ($P < 0.001$, n=5, (**Fig.6D,F**).

22 In a few cases (n=4) in which both neurons were intracellularly labeled with
23 fluorescent dyes, segments of dendrites from the paired neurons were found in close
24 proximity ($12.5 \pm 3.1 \mu\text{m}$) (**Fig.6G1-G3**).

25 **An activity-dependent diffusible VP pool tonically stimulates presympathetic** 26 **neuronal activity.**

27 Our results demonstrate that evoked dendritic peptide release from an individual VP
28 neuron can diffuse locally to affect the activity of a neighboring presympathetic neuron.
29 We then tested whether the basal average activity of the neurosecretory VP population
30 as a whole was sufficient to generate a tonic diffusing peptide pool, to continuously
31 modulate presympathetic neuronal activity. Blockade of V1a receptors *per se* resulted in

1 membrane hyperpolarization and inhibition of firing activity in presympathetic neurons
2 ($P < 0.001$ and $P < 0.01$, respectively, $n=14$, **Fig.7A,B**), unveiling the presence of a
3 diffusible, tonic pool of VP. Conversely, the firing activity of eGFP-VP neurons was not
4 affected (baseline: 2.2 ± 0.6 Hz; V1a antagonist: 2.2 ± 0.7 Hz, $n=5$). To test whether the
5 strength of the diffusible pool was dependent on the degree of activity of the VP
6 population, we performed manipulations that either increased or decreased VP neuronal
7 activity. The VP tone was enhanced by increasing extracellular K^+ concentration (8.0
8 mM K^+), as indicated by a more pronounced effect of the V1a antagonist in this
9 condition, compared to normal K^+ ACSF ($P < 0.01$, **Fig. 7D**). Conversely, in the presence
10 of the κ opioid receptor agonist U-50488 (1 μ M), known to strongly inhibit VP neuronal
11 activity ($P < 0.01$, **Fig.S7A**, see also (Brown et al., 1998)), the V1a antagonist effect on
12 presympathetic neuronal activity was significantly blunted ($P < 0.05$ vs. control,
13 **Fig.7C,D**). In the presence of the V1a antagonist, however, U-50488 failed to affect the
14 firing activity of presympathetic neurons ($P > 0.6$, $n=4$, **Fig.S7B**), arguing against a direct
15 effect of U50488 on the latter. No correlation between basal PVN-RVLM firing activity
16 and the magnitude of the V1a antagonist effect was found in any of these different
17 conditions (Pearson r value: -0.02, $P > 0.5$). Dialysis of BAPTA into the recorded PVN-
18 RVLM neurons prevented the effects of the V1a antagonist (baseline: 0.7 ± 0.1 Hz; V1a
19 antagonist: 0.6 ± 0.1 Hz, $P > 0.3$, $n=6$).

20 A diffusible signal in the ECS could be influenced both by its half-life and the ECS
21 tortuosity. Blockade of tissue aminopeptidase activity (amastatin 10 μ M), increased the
22 firing activity of presympathetic neurons ($P < 0.01$, $n=8$, **Fig.7E**). The amastatin effect
23 was not only blocked, but actually turned into an inhibitory effect in the presence of the
24 V1a receptor blocker ($P < 0.01$ vs. amastatin control, $n=7$, **Fig.7E**). These results
25 indicate that aminopeptidase blockade increased not only the availability and excitatory
26 actions of endogenous VP, but also of an unknown inhibitory signal, which was only
27 unmasked when the VP excitatory effect was blocked. The identity of this inhibitory
28 peptide signal was not further investigated in this study.

29 Reducing the coefficient of diffusion in the ECS with 5% dextran (40 kDA) (Min et al.,
30 1998; Piet et al., 2004) also blocked the V1a antagonist effect on presympathetic firing
31 discharge ($-6.5 \pm 8.8\%$, $P > 0.6$, $n=4$). Taken together, these results indicate that

1 tonically released VP within the PVN serves as a neurosecretory population signal,
2 which acting in a diffusible manner, increased the activity of the presympathetic PVN
3 neuronal population.

4
5 **Dendritic release of VP within the PVN contributes to the recruitment of**
6 **sympathetic neurons during an osmotic challenge.**

7 We finally assessed whether dendritic release of VP serves as an inter-population
8 signal by which the integrated sympathoexcitatory output from the entire presympathetic
9 neuronal population was modulated. To this end, we performed *in vivo* studies to
10 directly monitor sympathoexcitatory outflow from the PVN. We found that direct
11 microinjection of VP (8-32 pmol) onto the PVN elicited a dose-dependent
12 sympathoexcitatory response, reflected by an increase in renal sympathetic nerve
13 activity (RSNA, $P < 0.02$, $n=9$, **Fig.8A,B**). These results indicate that the VP excitatory
14 effect observed on presympathetic neurons *in vitro* translated into a systemic,
15 population sympathoexcitatory response.

16 It is well-documented that a central osmotic challenge results in a robust PVN
17 homeostatic response that involves an orchestrated activation of VP MNNs and
18 presympathetic neurons, leading to increased plasma VP levels along with a
19 concomitant increase in sympathetic outflow (Bourque, 2008; Toney and Stocker,
20 2010), respectively. Thus, we used this paradigm to assess the functional relevance of
21 the neurosecretory-presympathetic crosstalk in the context of a homeostatic challenge.
22 Intra-carotid infusions of graded concentrations of NaCl (0.3, 0.9 and 2.1 osmol/l)
23 induced a significant and dose-dependent increase in RSNA ($P < 0.0001$, $n=7$,
24 **Fig.8C,D**). This osmotically-driven sympathoexcitatory response was significantly
25 attenuated (~50%) by a previous bilateral microinjection of the V1a antagonist within the
26 PVN (2 nmol in 100 nl, $P < 0.001$ vs. control, **Fig.8C,D**). As shown in **Fig.8E**, the
27 intracarotid osmotic stimulation evoked a significant increase in VP release within the
28 SON ($P < 0.001$, one way ANOVA repeated measures, $n=7$). These results indicate that
29 osmotically-driven dendritic VP release participates in the recruitment of presympathetic
30 neurons during a homeostatic challenge requiring an orchestrated neurosecretory and
31 sympathetic response.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31

DISCUSSION

The two modalities by which the PVN commands the generation of complex homeostatic responses are represented in distinct neuronal populations, including neurosecretory neurons projecting to the median eminence or the posterior pituitary, and presympathetic neurons innervating the spinal cord and/or brainstem nuclei, including the rostroventrolateral medulla (RVLM) (Swanson and Sawchenko, 1980). Given its diverse, though well-characterized anatomical and functional organization, the PVN stands as an ideal brain region to study inter-population signaling modalities in the brain. Despite its highly integrative function, it is well-documented that neurosecretory and presympathetic PVN neuronal populations are anatomically compartmentalized, displaying minimal or complete lack of hard-wired interconnections (Hatton et al., 1985; Swanson and Kuypers, 1980; Swanson et al., 1980). This has led to the notion that polymodal homeostatic control by the PVN involves parallel processing of neuroendocrine and autonomic information. In this study, we challenged this prevailing idea by testing the novel hypothesis that dendritic release of peptides serves as an inter-population signal mediating crosstalk between neurosecretory and presympathetic PVN neuronal populations. Along with nigrostriatal dopaminergic neurons (Cheramy et al., 1981), hypothalamic MNNs are one the best-characterized prototypes of dendritic neurotransmitter release (Ludwig and Leng, 2006). Dendritic release of VP and OT from MNNs acts as powerful feedback signals by which MNNs auto-regulate their own activity, to optimize systemic hormone release in response to physiologically relevant challenges (Kombian et al., 1997; Ludwig and Leng, 1997). Results from the present study demonstrate that in addition to its autocrine actions, VP acts as a diffusible signal to bridge information across neurosecretory and presympathetic neuronal populations. This novel crosstalk involves Ca^{2+} -dependent dendritic release of VP, diffusion in the extracellular space, and activation of V1a VP receptors coupled to a CAN channel in presympathetic neurons. Lastly, we demonstrate this crosstalk modality to be critical for an optimal homeostatic sympathetic response to a central osmotic challenge (Bourque, 2008; Toney and Stocker, 2010).

1 **Dendritically released VP mediates a neurosecretory-to-presympathetic neuronal**
2 **crosstalk.**

3 We found that selective activation of a single neurosecretory VP neuron (either by
4 photolytic NMDA uncaging or direct current injection in dual-patch recordings) evoked a
5 V1a receptor-mediated depolarization and firing discharge in neighboring
6 presympathetic (PVN-RVLM) neurons. Several lines of evidence support that the
7 underlying mechanism involved activation of a Ca^{2+} -activated non-selective cation
8 (CAN) channel (Petersen, 2002), including: (a) VP induced in PVN-RVLM neurons an
9 increase in $[\text{Ca}^{2+}]_i$ that preceded the enhanced membrane excitability; (b) intracellular
10 Ca^{2+} dependency of the VP effect, (c) voltage-dependent properties of I_{VP} , including an
11 outwardly rectifying I/V curve and (d) the I_{VP} reversal potential, characteristic of a mixed-
12 cationic conductance. While our pharmacological (flufenamic acid),
13 immunohistochemical and single-cell RTPCR experiments (**Fig.S2**) suggest the
14 involvement of TRPM4 channels, recently reported to be present in MNNs (Teruyama et
15 al., 2011), future studies are needed to identify the precise molecular identity of the
16 underlying CAN channel.

17 Following bursts of action potentials, VP neurons express two types of opposing
18 post-spike membrane potentials: AHPs and DAPs. These potentials temporally overlap,
19 competing with each other to either inhibit or increase post-firing membrane excitability,
20 respectively (Armstrong et al., 2010). We found that when prominent AHPs were
21 observed in the stimulated VP neurons, the response latency of the presympathetic
22 neurons was prolonged. This suggests that AHPs act as a “braking” system that
23 efficiently regulate activity-dependent dendritic VP release, and consequently, the timing
24 of the inter-neuronal crosstalk in the PVN.

25 MNNs project almost exclusively to the posterior pituitary (Swanson and Kuypers,
26 1980), with only a few reported cases of scarce axon collaterals, arising from, but
27 terminating outside the PVN (Hatton et al., 1985). Thus, the topographical segregation
28 between the dendritic and axonal terminal fields of MNNs minimized the possibility that
29 photolytically-evoked VP released within the PVN originated from an axon terminal,
30 rather than a dendritic source. This is further supported by our results showing that the
31 evoked neurosecretory-presympathetic crosstalk persisted in the presence of TTX,

1 which prevents axonal but not somatodendritic release of neuropeptides from MNNs.
2 Similar to dorsal raphe serotonin neurons (de Kock et al., 2006), NMDA was shown to
3 evoke dendritic release from MNNs in the absence of action potentials (de Kock et al.,
4 2004), and dendritic VP release involves a Ca^{2+} -dependent exocytotic event (Ludwig et
5 al., 2002). Accordingly, we found that photolysis of caged-NMDA in the somata of VP
6 neurons resulted in a rise in $[\text{Ca}^{2+}]_i$ both in neuronal somata and dendrites. This in turn
7 was followed by an excitatory response in presympathetic neurons, which was
8 prevented by a synaptic block media. Finally, intracellular Ca^{2+} chelation in VP neurons
9 prevented the neurosecretory-presympathetic coupling observed in dual-patch
10 recordings.

11 While the combined anatomical, imaging and electrophysiological data reported here
12 strongly support a direct communication between neurosecretory and presympathetic
13 neurons, we cannot conclusively rule out the participation of other intermediaries. For
14 example, the evoked VP release from a single MNN could act in a recurrent positive
15 feedback manner to recruit additional VP neurons to release further amounts of VP
16 (Kombian et al., 1997; Ludwig and Leng, 1997). Moreover, dendritically released VP
17 could also act on nearby astrocytes to evoke release of a potential gliotransmitters.
18 However, our data showing that stimulation of VP neurons failed to consistently activate
19 nearby astrocytes, and the fact that the neurosecretory-presympathetic coupling
20 persisted following ablation of astrocyte function, would argue against this possibility.

21 22 **Dendritically released VP acts as a diffusible inter-population signal contributing** 23 **to homeostatic neurohumoral responses**

24 Given its long half-life (~20 min in the brain (Mens et al., 1983)), VP is ideally suited
25 to act as a diffusible signal, potentially affecting multiple neurons at relatively distant
26 locations. We found the firing activity of presympathetic PVN neurons to be tonically
27 stimulated by an endogenous VP “tone”, whose strength was enhanced either by
28 increasing the activity of VP neurons, or by prolonging VP lifetime in the extracellular
29 space (aminopeptidase block) (Chen and Pittman, 1999). Conversely, the strength of
30 the VP tone was diminished when VP neuronal activity was inhibited (κ opioid
31 agonist)(Brown et al., 1998), or when the coefficient of diffusion of molecules in the ECS

1 was lessened (5% dextran) (Piet et al., 2004). Thus, our findings support the ability of
2 dendritically released VP from the neurosecretory population to act in a diffusible
3 manner to modulate the activity of neighboring presympathetic neurons.

4 A central hyperosmotic challenge triggers a coordinated systemic release of VP,
5 along with an increased renal sympathetic nerve activity (RSNA) (Bourque, 2008; Toney
6 and Stocker, 2010). These responses are largely mediated by activation of
7 neurosecretory and presympathetic SON/PVN neuronal populations, respectively
8 (Antunes et al., 2006; Chen and Toney, 2001; Leng et al., 2001; Oliet and Bourque,
9 1993). In addition to systemic release, osmotic stimuli also evoke local dendritic release
10 of VP (Leng and Ludwig, 2008), which serves as a “population feedback” signal by
11 which VP neurons auto-regulate their own activity to optimize hormone secretion from
12 their axonal terminals (Gouzenes et al., 1998). In this study, we found that a
13 hyperosmotic-induced increase in RSNA was largely attenuated when V1a receptors
14 within the PVN were locally blocked.

15 Given that VP MNNs are intrinsically osmosensitive (Oliet and Bourque, 1993), it is
16 reasonable to speculate that osmotically-driven dendritic VP release effectively
17 augmented the endogenous neurosecretory VP tone, directly stimulating in turn the
18 neighboring presympathetic neuronal population. Alternatively, dendritically released VP
19 could also act by increasing presympathetic neuronal responsiveness to forebrain
20 glutamatergic afferent inputs, known to contribute to osmotically-driven sympathetic
21 responses by the PVN (Antunes et al., 2006; Shi et al., 2007). This could occur either by
22 strengthening osmosensitive glutamatergic afferents (i.e., pre- or post-synaptically), or
23 simply by depolarizing the presympathetic resting membrane potential closer to spike
24 threshold. We found that VP excitatory effects on presympathetic PVN neurons
25 persisted in the presence of ionotropic glutamate receptor blockade, suggesting that a
26 direct VP excitatory signal *per se* is sufficiently strong to evoke firing discharge and
27 increase sympathetic outflow from the presympathetic neuronal population.

28 The extent to which other PVN neuronal populations are also targeted by
29 dendritically released VP is at present unknown. Clearly, recruitment specificity is a
30 critical factor for the generation of a physiologically relevant homeostatic responses,

1 which is likely achieved by the selective expression of V1a receptors in the relevant
2 neuronal populations.

3 Collectively, our findings provide, to the best of our knowledge, the first
4 demonstration that activity-dependent dendritic release of peptides constitutes an
5 efficient inter-population signaling modality in the brain. More specifically, they support
6 our hypothesis that a local crosstalk between hypothalamic neurosecretory and
7 presympathetic neuronal populations plays an important role in the generation of central
8 integrative homeostatic responses (Pittman et al., 1982). Finally, given that
9 neurohumoral activation (a process involving elevated neurosecretory and sympathetic
10 outflows) is a hallmark in prevalent diseases such as hypertension and heart failure
11 (Cohn et al., 1984; Esler et al., 1995; Pliquett et al., 2004), our studies provide insights
12 into potentially novel pathophysiological mechanism contributing to morbidity and
13 mortality in these prevalent diseases.

14

15 **EXPERIMENTAL PROCEDURES**

16

17 **Animals.** Male Wistar rats (160-220 g) were purchased from Harlan laboratories
18 (Indianapolis, IN), and housed in a 12 hr:12 hr light-dark cycle with access to food and
19 water ad libitum. In a subset of experiments, we also used male heterozygous
20 transgenic VP-eGFP Wistar rats (5-6 weeks old), in which VP neurons are
21 endogenously fluorescent (Ueta et al., 2005). All procedures were carried out in
22 agreement with the University of Georgia Health Sciences and the University of
23 Nebraska Medical Center Institutional Animal Care and Use Committee guidelines, and
24 were approved by the respective committees.

25 **Retrograde tracing.** To identify presympathetic RVLM-projecting PVN neurons (PVN-
26 RVLM), rats were anesthetized (ketamine-xylazine mixture, 90 and 50 mg kg⁻¹,
27 respectively, i.p.) and a stereotaxic apparatus was used to pressure inject 500 nl of
28 rhodamine-labeled microspheres (Lumaflo, Naples, FL, USA) or cholera toxin B (CTB)
29 (1%, List Biological Laboratories) into the RVLM (starting from Bregma: 12 mm caudal
30 along the lamina, 2 mm medial lateral, and 8 mm ventral). Injection sites were contained
31 within the caudal pole of the facial nucleus to ~ 1 mm more caudal, and were ventrally

1 located with respect to the nucleus ambiguous. The location of the tracer was verified
2 histologically (Sonner et al., 2011). Injections located either more rostral or lateral to the
3 targeted area did not result in PVN labeling, and these animals were discarded from the
4 study. Animals were used 3-4 days after surgery.

5 **Slice preparation.** Rats were anesthetized with pentobarbital (50 mg kg⁻¹) and
6 perfused through the heart with a cold sucrose solution (containing in mM: 200 sucrose,
7 2.5 KCl, 3 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, 20 D-glucose, 0.4 ascorbic acid, 1
8 CaCl₂ and 2 pyruvic acid (290-310 mosmol l⁻¹). Rats were decapitated, brains dissected
9 out, and coronal slices (200 μm) of the hypothalamus containing the PVN were cut in an
10 oxygenated ice cold ACSF (containing in mM: 119 NaCl, 2.5 KCl, 1 MgSO₄, 26
11 NaHCO₃, 1.25 NaH₂PO₄, 20 D-glucose, 0.4 ascorbic acid, 2 CaCl₂ and 2 pyruvic acid;
12 pH 7.4; 290-310 mosmol l⁻¹), using a vibroslicer (D.S.K. Microslicer, Ted Pella, Redding,
13 CA). For synaptic blockade, a 0.2 mM CaCl₂ and 400 μM CdCl₂ ACSF was used. In
14 other cases, a 0 mM CaCl₂ / 3 mM EGTA solution was used, as indicated. Slices were
15 kept in a holding chamber containing ACSF (room temperature) until used.

16 **Electrophysiology.** Hypothalamic slices were transferred to a recording chamber and
17 superfused with ACSF (30–32 °C) at a flow rate of ~3.0 ml min⁻¹. Conventional whole-
18 cell patch clamp recordings were obtained as previously described (Sonner et al.,
19 2011). Patch pipettes (4-7 MΩ) composed of thin walled (1.5 mm outer diameter, 1.17
20 mm inner diameter) borosilicate glass (GC150T-7.5, Clark, Reading, UK), were pulled
21 on a horizontal electrode puller (P-97, Sutter Instruments, Novato, CA). The internal
22 solution contained (mM): 135 potassium gluconate, 10 EGTA, 10 HEPES, 10 KCl, 0.9
23 MgATP, 0.3 NaGTP and 20 phosphocreatine (Na⁺); pH 7.2-7.3. When noted, neurons
24 were intracellularly labeled with Alexa Fluor 555 (100 μM) or biocytin (1%). Recordings
25 were obtained with a Multiclamp 700A amplifier (Axon Instruments, Foster City, Ca)
26 from fluorescently-labeled PVN-RVLM neurons located in the parvocellular ventromedial
27 or dorsal cap subnuclei, or from eGFP-VP neurons, located in the lateral magnocellular
28 subnucleus. Neurons were visualized with a combination of fluorescence illumination
29 and infrared differential interference contrast (IR-DIC) videomicroscopy. The voltage-
30 output was digitized at 16-bit resolution, 10 kHz (Digidata 1320A, Axon Instruments),

1 and saved on a computer for offline analysis. For current-clamp recordings, all protocols
2 were run using an output gain of 5 and a Bessel filter of 2 kHz. Firing activity and
3 membrane potential were recorded in continuous mode before and during application of
4 drugs. For bath-applied drugs, mean values were calculated from a 2-min period before
5 drug application, and in a 2-min period around the peak effect. For briefer applications
6 (picospritzer) and uncaging, values were calculated from a 1-min period before, and 10-
7 20 sec period around the peak effect, using Clampfit (Axon Instruments, Foster City,
8 Ca) or miniAnalysis (Synaptosoft Inc., NJ) software. DC current injection was used in an
9 attempt to maintain neurons at a similar low level of basal firing activity, while holding
10 them at an approximate membrane potential of -45 to -55 mV. The mean series
11 resistance was monitored throughout the recording ($< 20 \text{ M}\Omega$) and data discarded if
12 changes $>20\%$ were observed).

13 **Confocal calcium imaging.** Magnocellular neurosecretory neurons were loaded
14 through the patch pipette with Fluo-5F pentapotassium salt (100 μM ; Molecular Probes,
15 Carlsbad, CA), as previously described (Filosa et al., 2006; Sonner et al., 2011). Once in
16 the whole-cell mode, the dye was allowed to dialyze into the cell for at least 20 minutes
17 before the initiation of the recordings, in order to allow complete dialysis of the dye. For
18 astrocyte Ca^{2+} measurements, slices were incubated at room temperature (RT) in ACSF
19 containing Rhod2-AM and pluronic acid (2.5 $\mu\text{g/ml}$). Imaging was conducted using the
20 Yokogawa real time live cell laser confocal system combined with a highly-sensitive
21 EMCCD camera (iXON+885, Andor Technology, South Windsor, CT). Fluorescence
22 images were obtained using diode-pumped solid-state laser (Melles Griot, Carlsbad,
23 CA) at 488 nm and emitted light at >495 (Fluo4) or 561 nm and emitted light >607 nm
24 (Rhod2). Images were acquired at a rate of 4 Hz. The fractional fluorescence (F/F_0) was
25 determined by dividing the fluorescence intensity (F) within a region of interest (ROI; 6 x
26 6 pixels $\approx 4.8 \times 4.8 \mu\text{m}$) by a baseline fluorescence value (F_0) determined from 30
27 images before photolysis of caged-NMDA. Data was analyzed using Andor IQ software
28 (Andor Technology).

29
30 **Photolysis of caged-compounds.** Uncaging was performed using the Andor
31 Technology Revolution system (iXON EMCCD camera with the Yokogawa CSU 10,

1 confocal scanning unit). Slices were perfused with MNI-caged-NMDA (50 μ M). A UV
2 laser excitation (405 nm) was directed to a region of interest drawn on the image of
3 identified PVN-RVLM or eGFP-VP somata through a 40x or 63x microscope objective.
4 Based on the pixel dwell time (200-800 μ s) and the ROI area scanned (\sim 40 μ m²), the
5 uncaging protocol lasted 180 – 720 ms. The confocality of the system allows for the
6 photolysis beam to occur at the focal plane of choice, with minimal diffusion beyond the
7 activated plane. Uncaging was done while simultaneously recording neuronal firing
8 activity or Δ [Ca²⁺]_i in the slice (see **Figs.S4-S5**). For these experiments, an ACSF
9 containing 20 μ M Mg²⁺ was used to facilitate detection of evoked NMDA responses,
10 although similar but smaller responses were also observed in control ACSF (*not*
11 *shown*).

12 **PVN microinjections and in vivo renal sympathetic nerve activity (RSNA)**
13 **measurements.** On the day of the experiment, rats were anesthetized with urethane
14 (0.75g/kg i.p.) and α -chloralose (70mg/kg i.p.) and the left femoral artery was
15 cannulated and connected to a computer-driven data recording and analyzing system
16 (PowerLab, ADI instruments, Colorado springs, CO, USA) via a pressure transducer
17 (Gould P23 1D) for recording arterial blood pressure and heart rate. The anesthetized
18 rat was placed in a stereotaxic apparatus (Davis Kopf Instruments, Tujunga, CA, USA).
19 A longitudinal incision was made on the head and the bregma was exposed. The
20 coordinates for the PVN were determined from the Paxinos and Watson Atlas. They
21 were 1.5mm posterior to the bregma, 0.4mm lateral to the midline, and 7.8mm ventral to
22 the dura. A small burr hole was made in the skull. For the microinjections, a thin needle
23 (0.5mm OD and 0.1mm ID) connected to a micro syringe (0.5ml; model 7000.5
24 Hamilton micro syringe) was lowered into the PVN. To record RSNA, the left kidney was
25 exposed through a retroperitoneal flank incision. A branch of the renal nerve was
26 isolated from the fat and connective tissue and was placed on a pair of thin bipolar
27 platinum electrodes. The nerve-electrode junction was insulated electrically from the
28 surrounding tissue with a silicone gel (Wacker Sil-Gel, 604 A B). The electrical signal
29 was amplified (10000 times) with a Grass amplifier (P55) with a high- and low-frequency
30 cutoff of 1000 and 100Hz, respectively. The output signal from the Grass amplifier was
31 directed to a computer-run data acquisition system (PowerLab, ADI instruments,

1 Colorado springs, CO, USA) to record and integrate the raw nerve discharge. The
2 signal recorded at the end of the experiment (after the rat was dead) was deemed as
3 background noise. The basal value of the nerve activity was defined by subtracting the
4 background noise from the actual nerve activity value before the administration of drugs
5 into the PVN. The peak response of RSNA to the administration of drugs into the PVN
6 during the experiment (averaged over a period of 20-30s) was subsequently expressed
7 as a percent change from baseline (Biancardi et al., 2011).

8
9 **Central hyperosmotic stimulation and VP microdialysis.** Central hyperosmotic
10 stimulation was achieved by delivering graded concentrations of NaCl (0.3, 0.9, and 2.1
11 osmol/l) through an internal carotid artery (ICA) catheter. The ICA is the main artery
12 supplying limbic areas, including the hypothalamus, and this approach was previously
13 shown to elicit efficiently and reproducibly central, but not peripheral hyperosmolarity
14 (Chen and Toney, 2001). Injections were delivered in a volume of 300 μ l over a period
15 of 10-15 seconds. For microdialysis, rats were anaesthetized with urethane (ethyl
16 carbamate, intraperitoneally 1.2 g/kg, Sigma Chemical Co., UK). In-house designed
17 microdialysis probes (molecular weight cut-off of 6 kDa, Fleaker® Hollow Fibre,
18 Spectrum® Med. Inc., USA) were stereotaxically implanted with the U-shaped tip
19 located within or adjacent to the right SON (1.0 mm posterior to bregma; 1.7 mm lateral
20 to midline; 9.3 mm below the surface of the skull, as previously described (Ludwig et al.,
21 2002). After an equilibration period of at least 1 h, consecutive 30-min dialysis samples
22 were collected at a flow rate of 3 μ l/min. After two 30-min baseline periods, rats were
23 stimulated osmotically as described above, and a further two consecutive dialysate
24 samples were collected, frozen and stored at -20°C until assay for vasopressin. The
25 vasopressin content in the microdialysates was measured by a highly sensitive and
26 selective radioimmunoassay (detection limit: 0.1 pg/sample; cross-reactivity less than
27 0.7%) as previously described (Landgraf et al., 1995).

28
29 **Immunohistochemistry.** Rats were anesthetized with pentobarbital (50 mg kg⁻¹) and
30 perfused transcardially in 4% paraformaldehyde in 0.01 M phosphate buffer saline
31 (PBS). Brains were then removed, postfixed for 2-4 hours, cryoprotected in 30%

1 sucrose in 0.01 M PBS (4 °C, 3 days), and then stored at -80 °C until further use.
2 Coronal slices (30 µm) containing the PVN were cut and collected in 0.01 M PBS.
3 Slices were then incubated in 0.01 M PBS with 0.1% Triton X-100, 0.04% NaN₃
4 (PBS_{TX}NaN₃), and 5% normal horse serum for 1 hour at room temperature. Slices were
5 then rinsed thoroughly with 0.01 M PBS, followed by incubation with one or a
6 combination of the following primary antibodies rabbit (1:100; Millipore) or goat (1:50;
7 Santa Cruz) anti-V1a receptor; goat anti-CTB (1:2500; List Biological Laboratories);
8 rabbit anti-TRPM4 (1:2000, kindly donated by Dr. Teruyama, LHSU, Baton Rouge);
9 rabbit anti-microtubule-associated protein 2 (MAP2, 1:500, Sigma); Mouse anti-
10 dopamine β hydroxylase (DBH, 1:20,000, Millipore). Incubation in primary antibodies
11 (overnight at 4 °C in PBS_{TX}NaN₃) where followed by specific fluorescently labeled
12 secondary antibodies (1:250; Jackson ImmunoResearch Laboratories, West Grove,
13 Pennsylvania, USA) for 4 hours at 4 °C in PBS_{TX}NaN₃. Slices were then rinsed
14 thoroughly, mounted, and visualized using confocal microscopy (Carl Zeiss
15 MicroImaging, Inc., Thornwood, NY, USA; 63x oil immersion, zoomed x2; single optical
16 plane = 0.5 µm thick) (Biancardi et al., 2010). Negative controls included incubations in
17 the absence of primary antibodies and preabsorption with the respective antigen
18 peptide, as previously done (Biancardi et al., 2010). Both of these control approaches
19 resulted in absence of staining (see for example **Fig.S1**).

20
21 **Single cell RTPCR.** Single cell RT-PCR was carried out as previously described with
22 minor modification (Sonner et al., 2011). The cytoplasm of the patched neuron, taking
23 care not to contain the nucleus, was pulled into a patch pipette containing 2 µl DEPC-
24 treated water, and then mixed with 1 µl of RNase inhibitor (Applied Biosystems, Foster
25 city, CA, USA) and immediately frozen in liquid nitrogen, and kept at -80 C until
26 processed. RT for cDNA was carried out with iScript cDNA Synthesis Kit (BIO-RAD, CA,
27 USA) according to manufacturer's manual. PCR amplification was performed with a
28 fraction of cDNA as a template. The mixture of PCR reaction contained (in µl): 1 of
29 10µM each primer, 10 of 2× master mix buffer (Go Taq Green Master Mix, Promega),
30 and 8 of the cDNA template. The annealing temperature in the thermal cycler was 60 °C
31 and 50 cycles were performed. A nested approach was used to quantify V1a receptor

1 mRNA. The primers used included: First nested PCR: 5'-cgaggtgaacaatggcactaaaac-3'
2 and 5'-tgtgatggaagggtttctgaatc-3'; Second nested PCR 5'-tcatctgctaccacatctggcg-3' and
3 5'-gtgtaacccaaaagccccttatgaaag-3'; Primers for TRPM4: 5'-cctgcaggcccaggttagaga-3' and
4 5'-ttcagcagagcgtccatgag-3'. GAPDH primers: 5'-ttcaacggcacagtcaagg-3' and 5'-
5 tggttcacacccatcacaaa-3'. All primers were synthesized by Intergrated DNA technologies
6 (Iowa, USA). Final PCR products were electrophoresed on a 2% agarose gels in TAE
7 buffer (40mM Tris-acetate, 1mM EDTA, pH 8) containing 0.5 µg/ml ethidium bromide,
8 observed UV light, and photographed. Negative controls were performed by replacing
9 cDNA with water.

10 **Statistical Analysis.** All values are expressed as means ± S.E.M. In most cases,
11 unpaired or paired t tests were used, as indicated. One or two-way ANOVA tests with
12 Bonferroni posthoc tests were used as needed. Pearson's correlation test was used to
13 determine if correlations existed between two parameters. Differences were considered
14 significant at P< 0.05. All statistical analyses were conducted using GraphPad Prism
15 (GraphPad Software, San Diego, CA).

16
17 **Drugs.** All drugs with the exception of MNI-caged-NMDA (Tocris, Ellisville, MO USA),
18 were purchased from Sigma-Aldrich (St. Louis, MO, USA). For simplicity, the selective
19 V1a antagonist β-Mercapto-β,β-cyclopentamethylenepropionyl¹, O-me-Tyr², Arg⁸]-
20 Vasopressin (Sigma V2255), is referred to as "V1a antagonist" throughout the
21 manuscript.

22

1 **FIGURE LEGENDS**

2

3 **Figure 1. Dendro-somatic and dendro-dendritic interrelationships between**
4 **presympathetic and magnocellular neurosecretory PVN neurons.** (A) Confocal
5 image showing the topographical segregation between immunoreactive magnocellular
6 neurosecretory vasopressin (VP, green) and retrogradely-labeled presympathetic PVN-
7 RVLM (red) neuronal somata. In (B) and (C), the squared regions are shown at
8 progressively higher magnification, to better depict the presence of thick and varicose
9 VP dendrites within the presympathetic neuronal compartment. Additional examples of
10 VP dendrites extending from the lateral magnocellular subnucleus into the three main
11 presympathetic PVN subnuclei, including the dorsal cap (D), parvocellular ventromedial
12 (E) and parvocellular posterior (F) are shown. (G) PVN-RVLM neurons surrounded by
13 VP dendrites are shown at high magnification. (H) Low magnification image of the PVN
14 showing that the vast majority of immunoreactive vasopressin (green) and oxytocin
15 (red) axons emerge from the PVN, running laterally and ventrally towards the median
16 eminence. (I) Image showing thick, immunoreactive VP processes (green), which are
17 also MAP2 immunoreactive (white, arrows) nearby a PVN-RVLM neuron (red, asterisk).
18 Note the abundance of MAP2 processes that are negative for VP. (J) Image showing
19 DBH immunoreactive boutons (white, arrows) abutting VP-immunoreactive processes
20 (green) near a PVN-RVLM neuron (red, asterisk). Vertical and horizontal arrows point
21 dorsally and ventrally, respectively. 3V: third ventricle. Scale bars in A-G= 20 μm ; H=
22 200 μm ; I, J= 5 μm .

23

24 **Figure 2. Presympathetic PVN neurons express functional V1a receptors.** (A)
25 Sample confocal image at low magnification showing CTB-retrogradely-labeled PVN-
26 RVLM neurons (blue), and V1a receptor immunoreactivity (green, Millipore primary
27 antibody) in the PVN. In (B) and (C), examples of two PVN-RVLM neurons showing
28 dense V1a immunoreactive clusters, respectively, are depicted. In (D), the squared area
29 in (B) is shown at an expanded scale, to better show V1a immunoreactive clusters
30 (arrows) located at the surface of the shown dendrite. (E) Single-cell V1a mRNA
31 expression in identified PVN-RVLM and eGFP-VP neurons. A non-template negative

1 control is shown in the right lane, and a small piece of a DNA ladder is shown in the left
2 lane. (F) A puff of VP (1 μ M, arrowheads) delivered directly onto a presympathetic PVN-
3 RVLM neuron evokes a high-frequency burst of action potentials. (G) Sample trace
4 showing that in the presence of tetrodotoxin (TTX 0.5 μ M), puffs of VP of decremental
5 durations depolarized a presympathetic PVN neuron in a proportionally decremental
6 manner. Note the presence of Ca^{2+} spikes (arrow). (H) Summary data of VP effects on
7 firing activity and (I) membrane potential in presympathetic neurons (n=16). Scale bars
8 in A, B= 20 μ m; D= 2.5 μ m.

9

10 **Figure 3. VP effects on presympathetic neurons involves an increase in $[\text{Ca}^{2+}]_i$**
11 **and activation of a CAN channel. (A)**, Sample traces showing simultaneous $\Delta[\text{Ca}^{2+}]_i$
12 (lower) and membrane potential (upper) measurements in a PVN-RVLM neuron in
13 response to bath-applied VP (1 μ M, line). Note that the increase in $\Delta[\text{Ca}^{2+}]_i$ preceded
14 the increase in firing discharge. (B), Representative pseudocolor (upper) and black and
15 white (lower) images of VP-evoked Ca^{2+} changes in the PVN-RVLM neuron shown in A.
16 Image numbers correspond to the time points shown in A. (C), Summary data showing
17 mean VP-evoked Ca^{2+} changes (F/F_0) in presympathetic PVN neurons (n=8). (D),
18 Sample trace and summary data showing that chelation of intracellular Ca^{2+} with
19 BAPTA (10 mM) in the patch pipette prevented VP excitatory effect (puff, arrowhead) in
20 presympathetic PVN neurons (n=8). (E) Sample trace in voltage-clamp mode showing a
21 VP-mediated (puff, arrowhead) inward current (I_{VP}) in a presympathetic PVN neuron.
22 The right panel shows a mean I/V plot of I_{VP} evoked at various holding potentials (n=5).
23 **P< 0.01.

24

25 **Figure 4. Photo-activation of neurosecretory VP neurons evokes a V1a receptor-**
26 **mediated excitatory response in neighboring presympathetic neurons. (A1)**
27 Inward currents evoked in a patched eGFP-VP neuron following photolysis of caged-
28 NMDA onto various somatodendritic regions of interest (ROI) (1-3). Note that photolysis
29 in an ROI a few microns away from the recorded neuron (4) failed to evoke a response.
30 (A2), Repetitive laser photolysis of caged NMDA onto the same eGFP-VP neuron
31 evoked reproducible inward currents (upper trace), membrane depolarizations (lower

1 trace), and bursts of action potentials (right traces). **(B1)** Slice containing a patched
2 presympathetic PVN-RVLM neuron (red, asterisk) and neighboring eGFP-VP neurons
3 (green, 1-4) that were activated with photolysis of caged-NMDA. **(B2)** Direct uncaging of
4 NMDA onto the patched presympathetic neuron (asterisk, yellow flashes) evoked a
5 burst of action potentials. **(B3)** Burst of action potentials recorded in the patched
6 presympathetic neuron in response to uncaging of NMDA onto the eGFP-VP neurons
7 shown in **B1** (1-4, red flashes). Note the delayed response in the PVN-RVLM neurons
8 compared to B2. **(C1)** Combined DIC and fluorescence image of a slice containing a
9 patched presympathetic neuron (asterisk) and neighboring eGFP-VP neurons (green).
10 **(C2)** Presympathetic neuronal responses to uncaging of NMDA onto the recorded PVN-
11 RVLM neuron neuron (upper traces) or onto an eGFP-VP neuron (arrow in **C1**, and
12 lower traces), in control ACSF (left) and in the presence of a V1a receptor antagonist
13 (right). **(C3)** Summary data of mean changes in the number of action potentials, action
14 potential frequency, and membrane potential in presympathetic PVN-RVLM neurons,
15 evoked by photolysis of caged-NMDA onto the presympathetic neurons themselves
16 (n=11, left) or onto eGFP-VP neurons in the absence (n=38, middle) or presence (n=7,
17 right) of the V1a antagonist. *P< 0.01 and **P< 0.001 vs. respective ACSF.

18

19 **Figure 5. The NMDA-mediated crosstalk between neurosecretory VP and**
20 **presympathetic PVN-RVLM neurons is not dependent on action potential firing.**

21 **(A1)** Sample of a slice containing a patched presympathetic PVN-RVLM neuron that
22 was loaded with alexa fluor 555 (red, asterisk) and neighboring eGFP-VP neurons
23 (green) that were photoactivated with caged-NMDA. **(A2)** Representative traces
24 obtained from the recorded PVN-RVLM neuron following 5 repetitive photolysis of
25 caged-NMDA directly onto the recorded cell (upper trace, yellow flashes), or in
26 response to simultaneous photolysis onto the three eGFP-VP neurons (1-3, red flashes)
27 shown in A1, in control ACSF + 1 μ M TTX (middle trace), and following bath application
28 of the V1a receptor antagonist + TTX (lower trace). **(B)** Summary data of mean changes
29 in PVN-RVLM membrane potential evoked in the presence of TTX following photolysis
30 of caged-NMDA onto the presympathetic neurons themselves (n=6, blue bar), or onto
31 eGFP-VP neurons (red bars) in the absence (n=18, middle bar) or presence (n=14, right

1 bar) of the V1a receptor antagonist. **(C1)** Another example showing a recorded
2 presympathetic PVN-RVLM neuron (alexa fluor 555, red) and a photoactivated eGFP-
3 VP neuron (green, arrow). **(C2)** Sample traces obtained from the PVN-RVLM neuron
4 from C1, following photolysis of caged-NMDA in the presence of 1 μ M TTX directly onto
5 the PVN-RVLM neuron itself (asterisk, upper trace, yellow flash), or onto an eGFP-VP
6 neuron (red flashes), in control ACSF (middle trace) and following bath application of
7 the V1a receptor antagonist (lower trace), in the presence of TTX. *P< 0.01 vs.
8 respective ACSF. Scale bars: 15 μ m.

9

10 **Figure 6. Neurosecretory-presympathetic crosstalk unveiled during dual-patch**
11 **recordings from eGFP-VP and PVN-RVLM neurons.** **(A1)** Sample pair of
12 intracellularly labeled (Alexa 633, blue, arrows) PVN neurons during simultaneous dual-
13 patch recordings. The identity of the patched neurons as eGFP-VP (cyan, single arrow)
14 and retrogradely-labeled PVN-RVLM (purple, double arrow) is shown in **A2**. **(B1)**
15 Electrically evoked bursting activity in the eGFP-VP neuron resulted in a delayed
16 membrane depolarization and increased firing discharge in the neighboring PVN-RVLM
17 neuron. Note the pronounced AHP following action potential firing in the eGFP-VP
18 neuron (filled arrowhead). **(B2)** Another sample of paired recordings showing a similar
19 response. Note however the lack of AHP in the eGFP-VP neuron (empty arrowhead),
20 and the shorter latency of the evoked PVN-RVLM response. **(C)** Sample of a paired
21 recording in which the eGFP-VP neuron was dialysed with BAPTA. Note the lack of
22 response in the PVN-RVLM neuron. **(D)** Sample of a paired recording in the presence of
23 L-aminoadipic acid (L-AAA, 250 μ M, 30 min), showing an increase in PVN-RVLM firing
24 activity following stimulation of the eGFP-VP neuron. Note the presence of a DAP in the
25 eGFP-VP neuron (double filled arrowheads), and the brief latency for the evoked PVN-
26 RVLM response. **(E)** Comparison of the mean inter-neuronal coupling latency in eGFP-
27 VP neurons displaying or not an AHP following the evoked burst of action potentials
28 (n=10 and 8, respectively). **(F)** Summary data of mean changes in PVN-RVLM
29 membrane potential (left) and firing activity (right) following direct stimulation of eGFP-
30 VP neurons in control ACSF (n=13), V1a antagonist (n=7), intracellular BAPTA in the
31 stimulated eGFP-VP neuron (n=5) and in the presence of L-AAA (n=5). **G1-G3**, Another

1 representative example of dual-patched and intracellularly-labeled eGFP and PVN-
2 RVLM neurons. (**G1**), Single focal plane of a confocal image showing eGFP-VP (green)
3 and PVN-RVLM (red) neurons. The recorded PVN-RVLM neuron was intracellularly
4 filled with Alexa 633 (arrow, colocalization= purple), and the recorded eGFP-VP neuron
5 was intracellularly filled with Alexa 555 (arrowhead, colocalization= yellow). In **G2**, the
6 same image as in A1, but a confocal stack of 10 images is shown to better depict the
7 dendritic processes of the recorded neurons. The blue color has been transformed to
8 white for better clarity. Asterisk points to a dendritic end of the eGFP-VP (Alexa 555
9 filled) neuron. In **G3**, the squared area in G2 is shown at a magnified scale. **P< 0.001
10 vs. AHP; ⁺P< 0.05 and [#]P< 0.01 vs. V1a antagonist and BAPTA, respectively. Scale
11 bars: A, B= 25 μ m; G1, G2= 20 μ m; G3= 10 μ m. Action potentials were cropped.

12

13 **Figure 7. An activity-dependent diffusible pool of dendritically-released VP**
14 **tonically modulates presympathetic neuronal activity.** (**A**) Sample trace showing a
15 hyperpolarization and diminished firing discharge of a presympathetic neuron following
16 bath application of the V1a receptor blocker (1 μ M). At the arrow, DC current injection
17 was applied to bring the membrane potential back to control levels, to show that the V1a
18 antagonist efficiently blocked the neuronal response to a VP puff (1 μ M, arrowhead). (**B**)
19 Summary data of the effects of the V1a receptor antagonist on firing frequency and
20 membrane potential of presympathetic neurons (n=14). (**C**) Sample trace showing a
21 blunted effect of the V1a receptor antagonist in the presence of the κ receptor agonist
22 U-50488 (1 μ M). (**D**) Summary data of the effects of the V1a antagonist on
23 presympathetic neuronal firing in control ACSF (K⁺ 2.5 mM), high K⁺ ACSF (8.5 mM) and
24 in U-50488 (K⁺ 2.5 mM) (n= 6, 7 and 8, respectively). (**E**) Sample traces and summary
25 data showing the effects of the aminopeptidase blocker amastatin (10 μ M, 10 min) in
26 control ACSF and in the presence of the V1a receptor antagonist (n=8 in each group).
27 *P< 0.05; **P< 0.01 and ***P< 0.001.

28

29 **Figure 8. Dendritic release of VP within the PVN contributes to**
30 **sympathoexcitatory homeostatic responses following a central hyperosmotic**
31 **challenge.** (**A**) Representative traces showing changes in raw (lower) and integrated

1 (upper) renal sympathetic nerve activity (RSNA) following administration of VP (16
2 pmol) onto the PVN. **(B)** Summary data showing dose-dependent increases in RSNA
3 after microinjections of VP (8, 16 and 32 pmol) onto the PVN ($P < 0.02$, one way
4 ANOVA, $n=9$). **(C)** Representative traces showing changes in RSNA following intra-
5 carotid infusions of an isosmotic (NaCl 0.3 osmol/l) or hyperosmotic (NaCl 2.1 osmol/l)
6 solution, following bilateral microinjections of ACSF or of the V1a receptor antagonist ((2
7 nmol/100 nl, 6-10 mins before the osmotic challenge) onto the PVN. Note the increase
8 in RSNA evoked by the hyperosmotic challenge in control ACSF (arrow) but not in the
9 presence of the V1a receptor antagonist. **(D)** Summary data showing dose-dependent
10 increases in RSNA after intra-carotid infusions of NaCl (0.3, 0.9 and 2.1 osmol/l) in
11 animals that received an intra-PVN microinjection of either ACSF or the V1a receptor
12 antagonist ($*P < 0.0001$ vs. respective ACSF, $n=7$). **(E)** Summary data showing
13 increased VP content in 30-min microdialysates sampled from the SON before and after
14 an intracarotid infusion of NaCl 2.1 osmol/l (arrow) ($*P < 0.05$ vs. basal levels, $n=7$).

15

1 **SUPPLEMENTARY FIGURE LEGENDS**

2 **Figure S1. V1a receptor immunoreactivity in presympathetic PVN neurons.**

3 Sample confocal images showing V1a receptor immunoreactivity (**A1, B1, C1**, green
4 Santa Cruz primary antibody) and retrogradely-labeled PVN-RVLM neurons (**A2, B2,**
5 **C2**, red, asterisks). In **A3, B3**, and **C3**, images were superimposed. Arrows point to V1a
6 immunoreactive clusters at the surface of the PVN-RVLM neurons. The *insets* in **A3**
7 and **B3** show a portion of the PVN-RVLM neurons (rectangles) at expanded scales.
8 Within the *insets*, arrows and arrowheads point to V1a cluster possibly located near the
9 neuronal surface membrane and/or at presynaptic terminals, respectively. Images in **C**
10 were obtained from tissue in which the antibody was preabsorbed with the
11 corresponding antigen (5-fold molar excess relative to IgG concentration). Scale bars:
12 A3-C3: 10 μm ; inset in B3: 2.5 μm .

13

14 **Figure S2. Expression of functional TRPM4 channels in presympathetic PVN**

15 **neurons. (A)** Sample confocal image at low magnification showing TRPM4 (green), and
16 retrogradely-labeled PVN-RVLM neurons (red). The asterisk indicates the center of the
17 lateral magnocellular subnucleus. (**B, C, D**) Three different samples at higher
18 magnification showing TRMP4 immunoreactive (left, green) PVN-RVLM neurons (right,
19 red). (**E**) Single-cell TRMP4 mRNA expression in identified PVN-RVLM neurons. A non-
20 template negative is shown in the right lane, and a small piece of a DNA ladder is
21 shown in the left lane. (**F**) Sample traces showing VP effects (puff, arrowheads) on a
22 presympathetic PVN neuronal firing activity before (left) and after (right) bath application
23 of flufenamic acid (FFA, 200 μM), a relative selective blocker of TRPM4/5 channels. The
24 right graph shows the summary data (n=9). ***P< 0.001. Scale bars in A= 50 μm ; B, C,
25 D= 10 μm . 3V= third ventricle.

26

27 **Figure S3. Identification of presympathetic and neurosecretory VP neurons using**
28 **retrograde tract tracing in eGFP-AVP transgenic rats. (A)** Sample confocal image of

29 a PVN slice showing large eGFP-VP neurons (green) in the lateral magnocellular
30 subnucleus (LM), along with smaller retrogradely-labeled PVN-RVLM neurons (red) in
31 the dorsal cap (DC) and parvocellular ventromedial (PaV) subnuclei. The inset shows

1 dense eGFP staining in the posterior pituitary (PP) of an eGFP-VP rat. **(B)** Confocal
2 micrograph showing high degree of colocalization (yellow) between eGFP (green) and
3 vasopressin (red) immunoreactivity in the PVN. **(C)** Confocal micrograph showing lack
4 of colocalization between eGFP (green) and oxytocin (red) immunoreactivity in the PVN.
5 Scale bars: (A)= 40 μm ; *inset*: 400 μm (B) and (C)= 15 μm . 3V: third ventricle. Vertical
6 and horizontal arrows point dorsally and medially, respectively.

7
8 **Figure S4. Changes in somatodendritic $[\text{Ca}^{2+}]_i$ levels in eGFP-VP neurons in**
9 **response to laser photolysis of caged-NMDA.** **(A)** Sample showing that photolysis of
10 caged-NMDA onto the soma of a patched eGFP-VP neuron evoked a rapid and
11 transient change in dendritic $[\text{Ca}^{2+}]_i$ (red arrows). Image 1 is a projection image of the
12 recorded neuron through a Z stack (40 μm). Single confocal planes before, during, and
13 after (4 s) photolysis of caged-NMDA are shown in images 2-4, respectively. **(B)** Plots of
14 changes in dendritic $[\text{Ca}^{2+}]_i$ (F/F0) as a function of time (obtained from the ROI show in
15 **A1**), following NMDA uncaging in the soma of the eGFP-VP neuron using increasing
16 laser pixel dwelling times. Traces were purposely shifted in the X-axis for their better
17 comparison. **(C)** Summary data showing that changes in dendritic $[\text{Ca}^{2+}]_i$ following
18 somatic uncaging of NMDA was dependent on the laser pixel dwelling time (F= 4.3, P<
19 0.01, 1 way ANOVA, n=6). *P< 0.05 vs. 100 and 200 μs dwell times). Scale bar in **A** =
20 15 μm .

21
22 **Figure S5. Blockade of NMDA receptors in presympathetic PVN-RVLM neurons**
23 **does not prevent the excitatory effect mediated by photolysis of caged-NMDA**
24 **onto eGFP-VP neurons.** **(A)** Sample of a combined DIC and fluorescence image of a
25 slice containing a patched presympathetic PVN-RVLM neuron that was dialysed with
26 the NMDA receptor blocker MK801 (1 mM, asterisk), and a neighboring eGFP-VP
27 neuron (green, 1). **(B)** Lack of response to direct photolysis of caged-NMDA onto the
28 patched PVN-RVLM neuron (upper trace, yellow flash). The middle and lower traces
29 show the response of the same PVN-RVLM neuron to photolysis of caged-NMDA onto
30 the eGFP-VP neuron (1, red flashes) in control ACSF and following bath application of
31 the V1a receptor antagonist, respectively.

1
2 **Figure S6. Photolysis of caged-NMDA onto eGFP-VP neurons failed to evoke**
3 **increases in intracellular Ca^{2+} in the majority of nearby astrocytes.** (A) Sample of
4 Rhod-2 labeled astrocytes (red, 1-5) in the vicinity of an eGFP-VP neuron (green,
5 arrow) photoactivated with caged-NMDA. (B) Traces showing ΔCa^{2+} levels (F/F0) in the
6 respective astrocytes shown in A1, following NMDA uncaging onto the eGFP-VP neuron
7 (arrow and vertical line). Note the delay increase in ΔCa^{2+} (asterisks) in astrocytes 1-3.
8 (C) Summary data showing the total number of negative and positive responding
9 astrocytes following NMDA uncaging onto 33 different eGFP-VP neurons. Scale bar in
10 A1= 15 μm .

11
12 **Figure S7. Activation of κ opioid receptors robustly inhibits firing discharge in**
13 **identified eGFP-VP neurons.** (A) Sample trace showing a robust membrane
14 hyperpolarization and inhibition of firing activity of an eGFP-VP neuron evoked by bath
15 application of the κ receptor agonist U-50488 (1 μM). The summary data (n= 5) is
16 shown in (B). (C) Sample trace obtained from a presympathetic PVN-RVLM neuron in
17 the presence of the V1a receptor antagonist, showing lack of effect of U-50488 (1 μM)
18 on firing activity. The summary data (n= 4) is shown in (D). **P< 0.01.

19
20

1 ACKNOWLEDGMENTS

2 We would like to thank Professor Rainer Landgraf (Munich, Germany) for analyzing the
3 microdialysis samples, Dr. Ryoichi Teruyama (Baton Rouge, USA), for the kind donation
4 of the TRPM4 antibody; and Professor Gareth Leng (Edinburgh, UK) for critical reading
5 of the manuscript. This work was supported by NIH R01-HL090948-01 (JES) and
6 BBSRC BB/J004723/1 (ML).

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

1 REFERENCES

- 2
- 3 Antunes, V.R., Yao, S.T., Pickering, A.E., Murphy, D., and Paton, J.F. (2006). A spinal
4 vasopressinergic mechanism mediates hyperosmolality-induced sympathoexcitation. *J*
5 *Physiol* 576, 569-583.
- 6 Armstrong, W.E., Wang, L., Li, C., and Teruyama, R. (2010). Performance, properties
7 and plasticity of identified oxytocin and vasopressin neurones in vitro. *J*
8 *Neuroendocrinol* 22, 330-342.
- 9 Biancardi, V.C., Campos, R.R., and Stern, J.E. (2010). Altered balance of gamma-
10 aminobutyric acidergic and glutamatergic afferent inputs in rostral ventrolateral medulla-
11 projecting neurons in the paraventricular nucleus of the hypothalamus of renovascular
12 hypertensive rats. *J Comp Neurol* 518, 567-585.
- 13 Biancardi, V.C., Son, S.J., Sonner, P.M., Zheng, H., Patel, K.P., and Stern, J.E. (2011).
14 Contribution of central nervous system endothelial nitric oxide synthase to
15 neurohumoral activation in heart failure rats. *Hypertension* 58, 454-463.
- 16 Bourque, C.W. (2008). Central mechanisms of osmosensation and systemic
17 osmoregulation. *Nat Rev Neurosci* 9, 519-531.
- 18 Brown, C.H., Ludwig, M., and Leng, G. (1998). kappa-opioid regulation of neuronal
19 activity in the rat supraoptic nucleus in vivo. *J Neurosci* 18, 9480-9488.
- 20 Buijs, R.M., and Van Eden, C.G. (2000). The integration of stress by the hypothalamus,
21 amygdala and prefrontal cortex: balance between the autonomic nervous system and
22 the neuroendocrine system. *Prog Brain Res* 126, 117-132.
- 23 Chen, Q.H., and Toney, G.M. (2001). AT(1)-receptor blockade in the hypothalamic PVN
24 reduces central hyperosmolality-induced renal sympathoexcitation. *Am J Physiol Regul*
25 *Integr Comp Physiol* 281, R1844-1853.
- 26 Chen, X., and Pittman, Q.J. (1999). Vasopressin and amastatin induce V(1)-receptor-
27 mediated suppression of excitatory transmission in the rat parabrachial nucleus. *J*
28 *Neurophysiol* 82, 1689-1696.
- 29 Cheramy, A., Leviel, V., and Glowinski, J. (1981). Dendritic release of dopamine in the
30 substantia nigra. *Nature* 289, 537-542.
- 31 Cohn, J.N., Levine, T.B., Olivari, M.T., Garberg, V., Lura, D., Francis, G.S., Simon, A.B.,
32 and Rector, T. (1984). Plasma norepinephrine as a guide to prognosis in patients with
33 chronic congestive heart failure. *N Engl J Med* 311, 819-823.
- 34 de Kock, C.P., Burnashev, N., Lodder, J.C., Mansvelder, H.D., and Brussaard, A.B.
35 (2004). NMDA receptors induce somatodendritic secretion in hypothalamic neurones of
36 lactating female rats. *J Physiol* 561, 53-64.
- 37 de Kock, C.P., Cornelisse, L.N., Burnashev, N., Lodder, J.C., Timmerman, A.J., Couey,
38 J.J., Mansvelder, H.D., and Brussaard, A.B. (2006). NMDA receptors trigger
39 neurosecretion of 5-HT within dorsal raphe nucleus of the rat in the absence of action
40 potential firing. *J Physiol* 577, 891-905.
- 41 Ely, D.L. (1995). Organization of cardiovascular and neurohumoral responses to stress.
42 Implications for health and disease. *Ann N Y Acad Sci* 771, 594-608.
- 43 Esler, M.D., Lambert, G.W., Ferrier, C., Kaye, D.M., Wallin, B.G., Kalff, V., Kelly, M.J.,
44 and Jennings, G.L. (1995). Central nervous system noradrenergic control of
45 sympathetic outflow in normotensive and hypertensive humans. *Clin Exp Hypertens* 17,
46 409-423.

1 Filosa, J.A., Bonev, A.D., Straub, S.V., Meredith, A.L., Wilkerson, M.K., Aldrich, R.W.,
2 and Nelson, M.T. (2006). Local potassium signaling couples neuronal activity to
3 vasodilation in the brain. *Nat Neurosci* 9, 1397-1403.

4 Fuxe, K., Dahlstrom, A., Hoistad, M., Marcellino, D., Jansson, A., Rivera, A., Diaz-
5 Cabiale, Z., Jacobsen, K., Tinner-Staines, B., Hagman, B., *et al.* (2007). From the Golgi-
6 Cajal mapping to the transmitter-based characterization of the neuronal networks
7 leading to two modes of brain communication: wiring and volume transmission. *Brain*
8 *Res Rev* 55, 17-54.

9 Galarreta, M., and Hestrin, S. (2001). Spike transmission and synchrony detection in
10 networks of GABAergic interneurons. *Science* 292, 2295-2299.

11 Ghamari-Langroudi, M., and Bourque, C.W. (2002). Flufenamic acid blocks depolarizing
12 afterpotentials and phasic firing in rat supraoptic neurones. *J Physiol* 545, 537-542.

13 Gouzenes, L., Desarmenien, M.G., Hussy, N., Richard, P., and Moos, F.C. (1998).
14 Vasopressin regularizes the phasic firing pattern of rat hypothalamic magnocellular
15 vasopressin neurons. *J Neurosci* 18, 1879-1885.

16 Guan, J.L., Kageyama, H., Wang, Q.P., Takenoya, F., Kita, T., Matsumoto, H., Ohtaki,
17 T., and Shioda, S. (2005). Electron microscopy examination of galanin-like peptide
18 (GALP)-containing neurons in the rat hypothalamus. *Regul Pept* 126, 73-78.

19 Hatton, G.I., Cobbett, P., and Salm, A.K. (1985). Extranuclear axon collaterals of
20 paraventricular neurons in the rat hypothalamus: intracellular staining,
21 immunocytochemistry and electrophysiology. *Brain Res Bull* 14, 123-132.

22 Kombian, S.B., Mougnot, D., and Pittman, Q.J. (1997). Dendritically released peptides
23 act as retrograde modulators of afferent excitation in the supraoptic nucleus in vitro.
24 *Neuron* 19, 903-912.

25 Landgraf, R., Neumann, I., Holsboer, F., and Pittman, Q.J. (1995). Interleukin-1 beta
26 stimulates both central and peripheral release of vasopressin and oxytocin in the rat.
27 *Eur J Neurosci* 7, 592-598.

28 Landgraf, R., and Neumann, I.D. (2004). Vasopressin and oxytocin release within the
29 brain: a dynamic concept of multiple and variable modes of neuropeptide
30 communication. *Front Neuroendocrinol* 25, 150-176.

31 Leng, G., Brown, C.H., Bull, P.M., Brown, D., Scullion, S., Currie, J., Blackburn-Munro,
32 R.E., Feng, J., Onaka, T., Verbalis, J.G., *et al.* (2001). Responses of magnocellular
33 neurons to osmotic stimulation involves coactivation of excitatory and inhibitory input: an
34 experimental and theoretical analysis. *J Neurosci* 21, 6967-6977.

35 Leng, G., and Ludwig, M. (2008). Neurotransmitters and peptides: whispered secrets
36 and public announcements. *J Physiol* 586, 5625-5632.

37 Ludwig, M. (1998). Dendritic release of vasopressin and oxytocin. *J Neuroendocrinol*
38 10, 881-895.

39 Ludwig, M., and Leng, G. (1997). Autoinhibition of supraoptic nucleus vasopressin
40 neurons in vivo: a combined retrodialysis/electrophysiological study in rats. *Eur J*
41 *Neurosci* 9, 2532-2540.

42 Ludwig, M., and Leng, G. (2006). Dendritic peptide release and peptide-dependent
43 behaviours. *Nat Rev Neurosci* 7, 126-136.

44 Ludwig, M., Sabatier, N., Bull, P.M., Landgraf, R., Dayanithi, G., and Leng, G. (2002).
45 Intracellular calcium stores regulate activity-dependent neuropeptide release from
46 dendrites. *Nature* 418, 85-89.

1 McBean, G.J. (1994). Inhibition of the glutamate transporter and glial enzymes in rat
2 striatum by the gliotoxin, alpha amino adipate. *Br J Pharmacol* 113, 536-540.

3 Mens, W.B., Witter, A., and van Wimersma Greidanus, T.B. (1983). Penetration of
4 neurohypophyseal hormones from plasma into cerebrospinal fluid (CSF): half-times of
5 disappearance of these neuropeptides from CSF. *Brain Res* 262, 143-149.

6 Min, M.Y., Rusakov, D.A., and Kullmann, D.M. (1998). Activation of AMPA, kainate, and
7 metabotropic receptors at hippocampal mossy fiber synapses: role of glutamate
8 diffusion. *Neuron* 21, 561-570.

9 Oliet, S.H., and Bourque, C.W. (1993). Mechanosensitive channels transduce
10 osmosensitivity in supraoptic neurons. *Nature* 364, 341-343.

11 Petersen, O.H. (2002). Cation channels: homing in on the elusive CAN channels. *Curr*
12 *Biol* 12, R520-522.

13 Piet, R., Vargova, L., Sykova, E., Poulain, D.A., and Oliet, S.H. (2004). Physiological
14 contribution of the astrocytic environment of neurons to intersynaptic crosstalk. *Proc*
15 *Natl Acad Sci U S A* 101, 2151-2155.

16 Pittman, Q.J., Veale, W.L., and Lederis, K. (1982). Central neurohypophyseal peptide
17 pathways: interactions with endocrine and other autonomic functions. *Peptides* 3, 515-
18 520.

19 Pliquett, R.U., Fasshauer, M., Blüher, M., and Paschke, R. (2004). Neurohumoral
20 stimulation in type-2-diabetes as an emerging disease concept. *Cardiovasc Diabetol* 3,
21 4.

22 Pow, D.V., and Morris, J.F. (1989). Dendrites of hypothalamic magnocellular neurons
23 release neurohypophysial peptides by exocytosis. *Neuroscience* 32, 435-439.

24 Salio, C., Lossi, L., Ferrini, F., and Merighi, A. (2006). Neuropeptides as synaptic
25 transmitters. *Cell Tissue Res* 326, 583-598.

26 Shi, P., Stocker, S.D., and Toney, G.M. (2007). Organum vasculosum laminae
27 terminalis contributes to increased sympathetic nerve activity induced by central
28 hyperosmolality. *Am J Physiol Regul Integr Comp Physiol* 293, R2279-2289.

29 Sonner, P.M., Lee, S., Ryu, P.D., Lee, S.Y., and Stern, J.E. (2011). Imbalanced K⁺ and
30 Ca²⁺ subthreshold interactions contribute to increased hypothalamic presympathetic
31 neuronal excitability in hypertensive rats. *J Physiol* 589, 667-683.

32 Swanson, L.W., and Kuypers, H.G. (1980). The paraventricular nucleus of the
33 hypothalamus: cytoarchitectonic subdivisions and organization of projections to the
34 pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde
35 fluorescence double-labeling methods. *J Comp Neurol* 194, 555-570.

36 Swanson, L.W., and Sawchenko, P.E. (1980). Paraventricular nucleus: a site for the
37 integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* 31,
38 410-417.

39 Swanson, L.W., Sawchenko, P.E., Wiegand, S.J., and Price, J.L. (1980). Separate
40 neurons in the paraventricular nucleus project to the median eminence and to the
41 medulla or spinal cord. *Brain Res* 198, 190-195.

42 Teruyama, R., and Armstrong, W.E. (2007). Calcium-dependent fast depolarizing
43 afterpotentials in vasopressin neurons in the rat supraoptic nucleus. *J Neurophysiol* 98,
44 2612-2621.

1 Teruyama, R., Sakuraba, M., Kurotaki, H., and Armstrong, W.E. (2011). Transient
2 receptor potential channel m4 and m5 in magnocellular cells in rat supraoptic and
3 paraventricular nuclei. *J Neuroendocrinol* 23, 1204-1213.

4 Tobin, V.A., Hashimoto, H., Wacker, D.W., Takayanagi, Y., Langnaese, K., Caquineau,
5 C., Noack, J., Landgraf, R., Onaka, T., Leng, G., *et al.* (2010). An intrinsic vasopressin
6 system in the olfactory bulb is involved in social recognition. *Nature* 464, 413-417.

7 Toney, G.M., and Stocker, S.D. (2010). Hyperosmotic activation of CNS sympathetic
8 drive: implications for cardiovascular disease. *J Physiol* 588, 3375-3384.

9 Ueta, Y., Fujihara, H., Serino, R., Dayanithi, G., Ozawa, H., Matsuda, K.-i., Kawata, M.,
10 Yamada, J., Ueno, S., Fukuda, A., and Murphy, D. (2005). Transgenic expression of
11 enhanced green fluorescent protein enables direct visualization for physiological studies
12 of vasopressin neurons and isolated nerve terminals of the rat. *Endocrinology* 146, 406-
13 413.

14 Ullrich, N.D., Voets, T., Prenen, J., Vennekens, R., Talavera, K., Droogmans, G., and
15 Nilius, B. (2005). Comparison of functional properties of the Ca²⁺-activated cation
16 channels TRPM4 and TRPM5 from mice. *Cell Calcium* 37, 267-278.

17 Xu, H.L., Mao, L., Ye, S., Paisansathan, C., Vetri, F., and Pelligrino, D.A. (2008).
18 Astrocytes are a key conduit for upstream signaling of vasodilation during cerebral
19 cortical neuronal activation in vivo. *Am J Physiol Heart Circ Physiol* 294, H622-632.

20 Zingg, H.H. (1996). Vasopressin and oxytocin receptors. *Baillieres Clin Endocrinol*
21 *Metab* 10, 75-96.

22
23

Figure 1

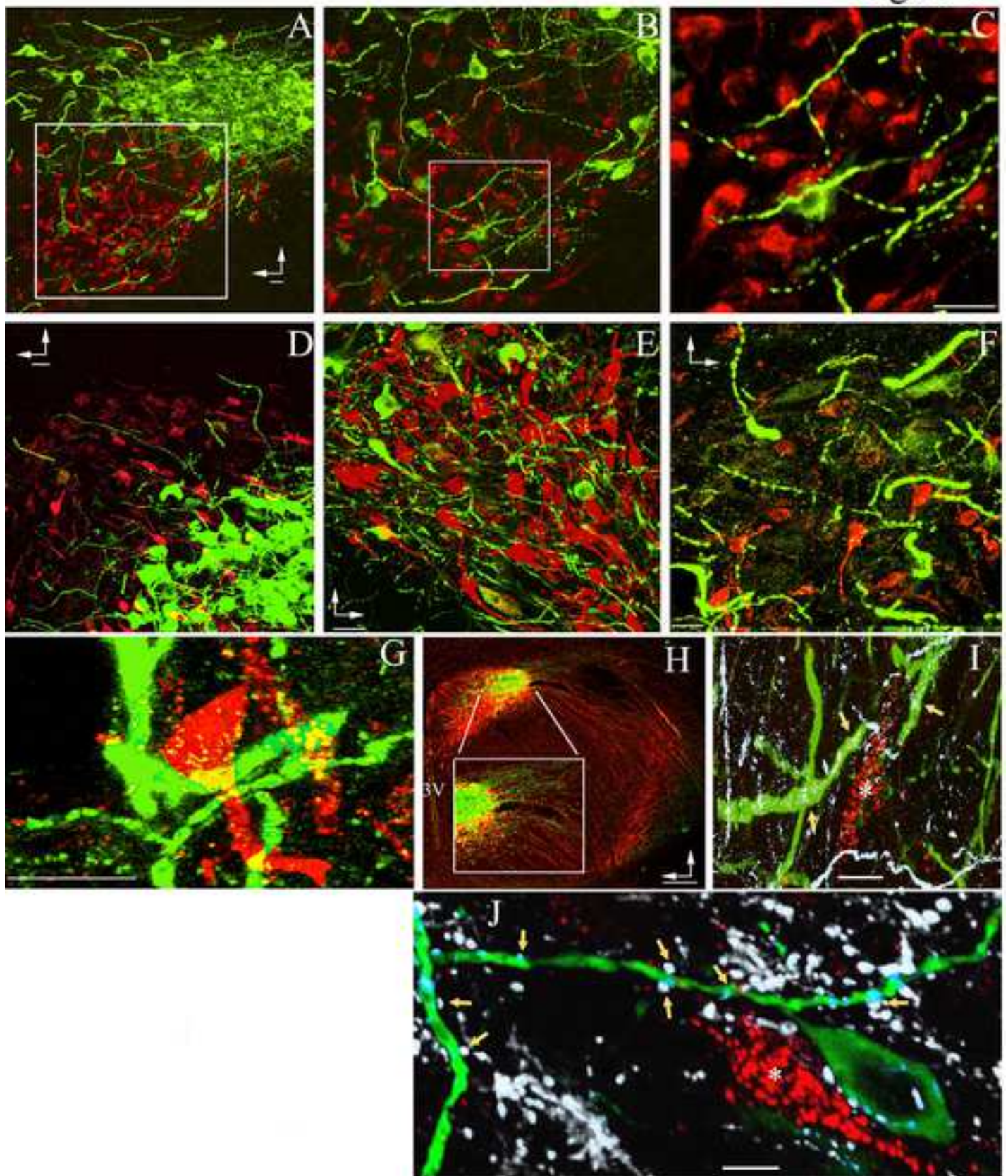


Figure 2

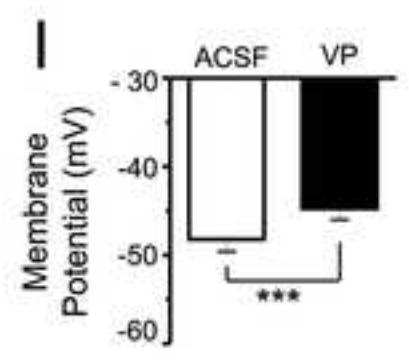
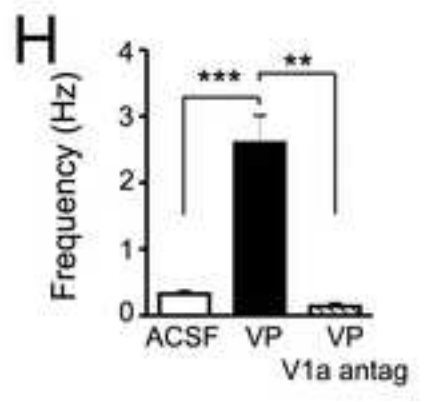
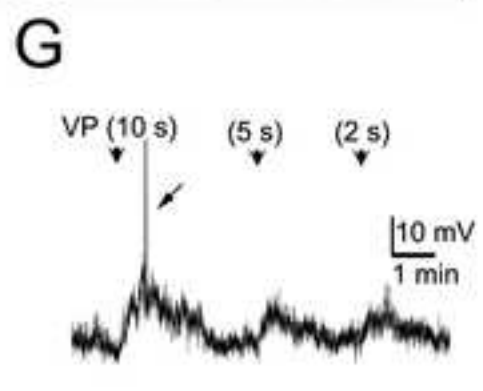
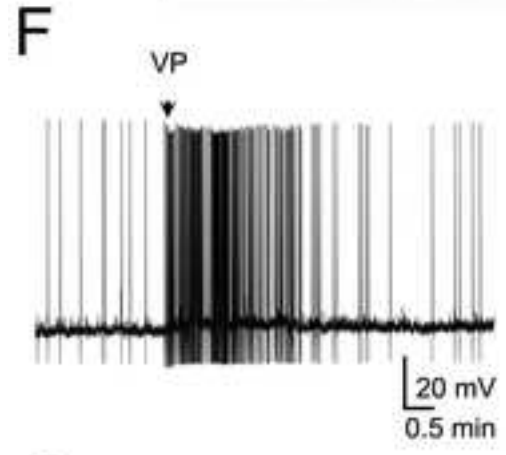
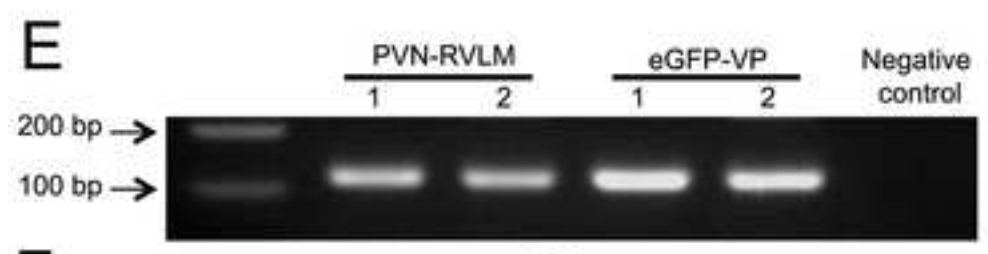
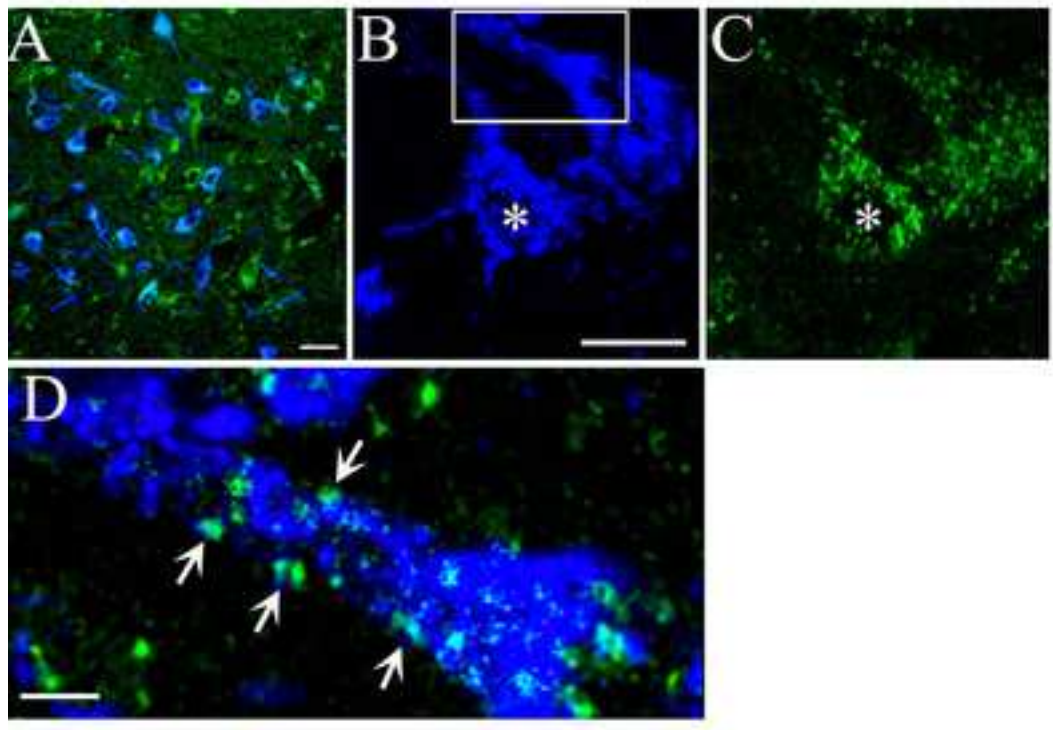


Figure 3
[Click here to download high resolution image](#)

Figure 3

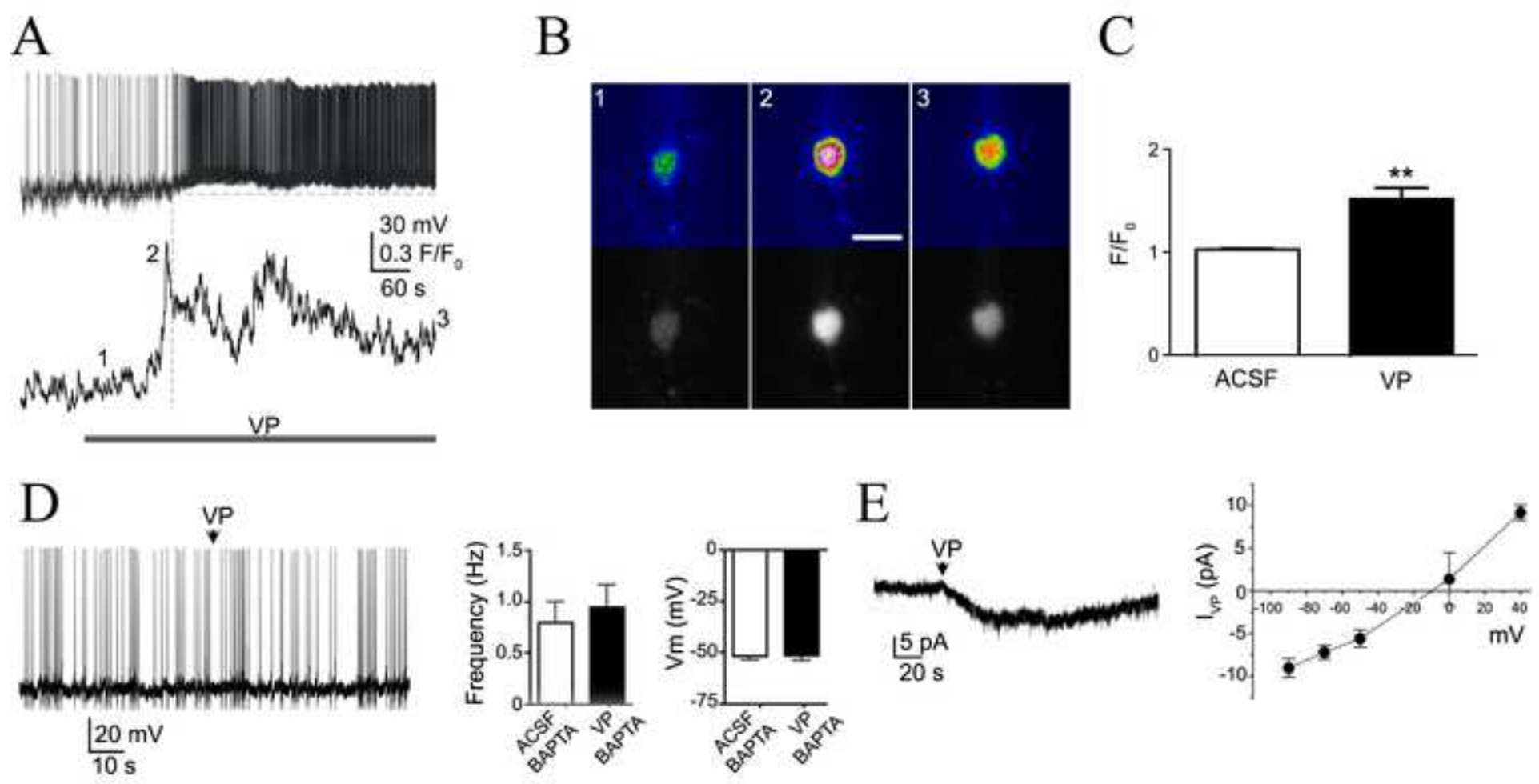


Figure 4

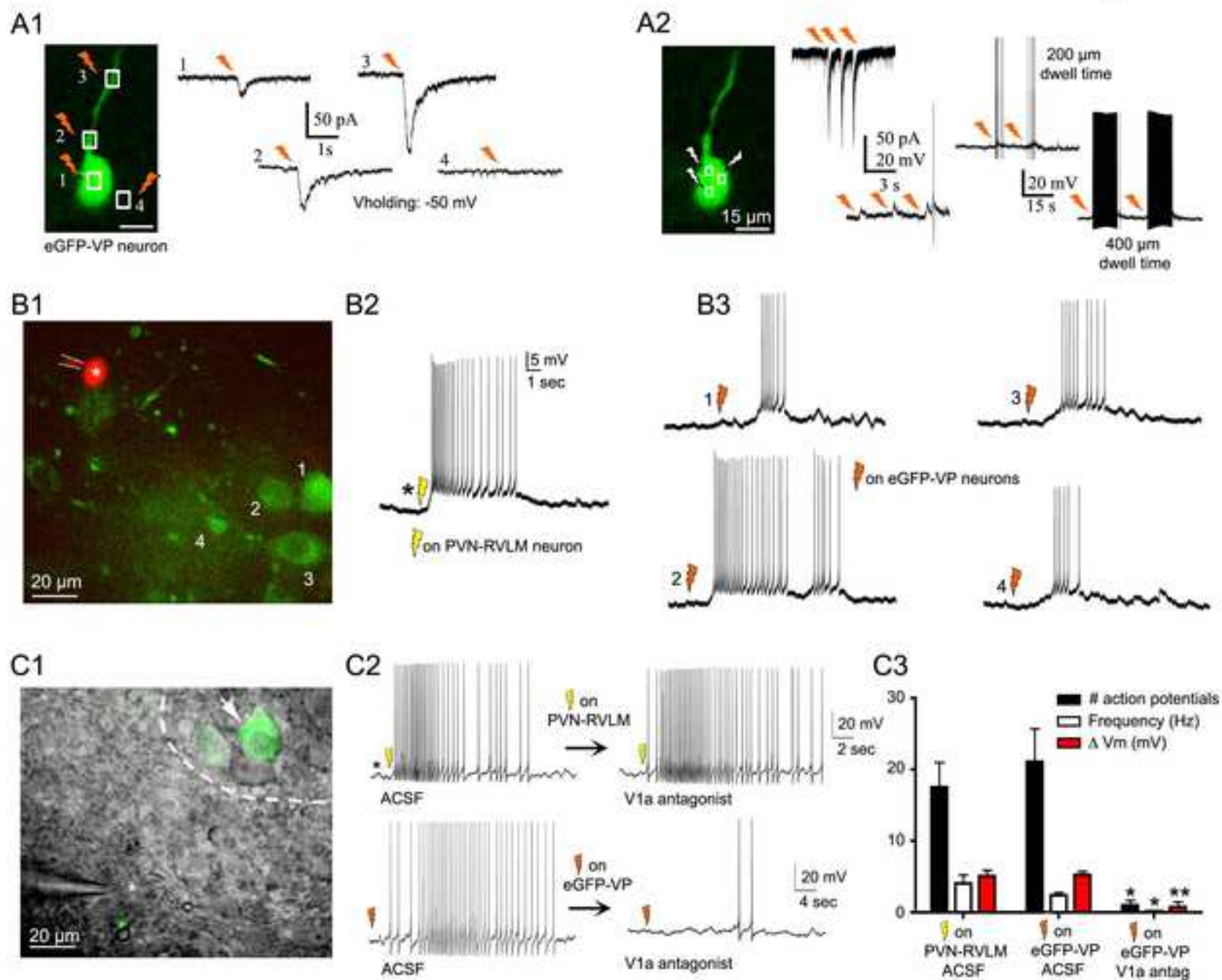


Figure 5

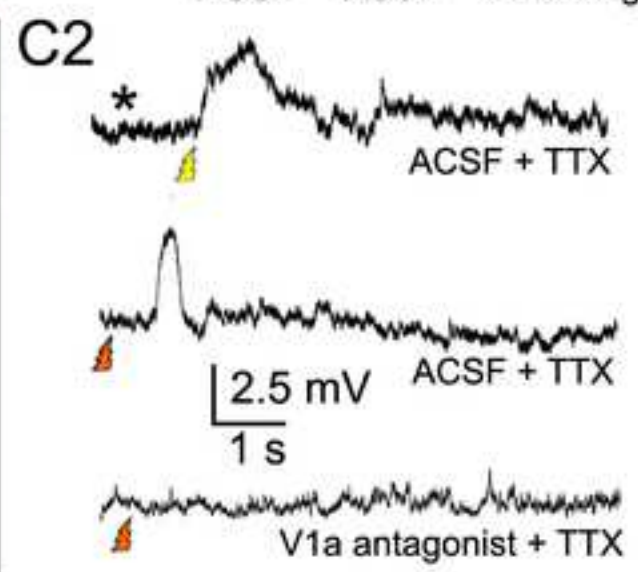
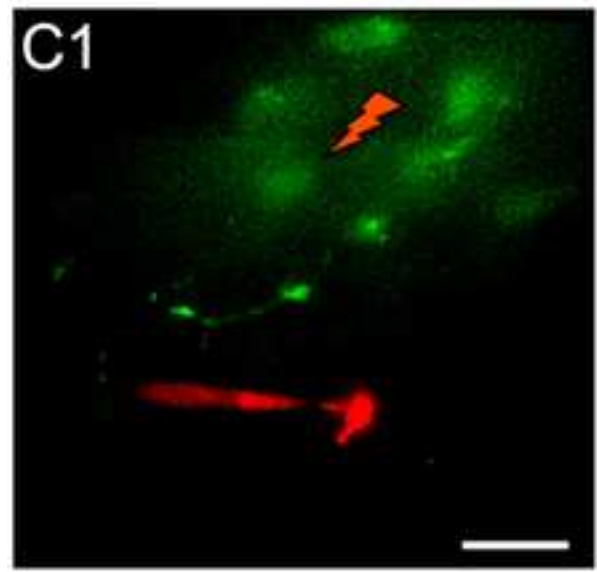
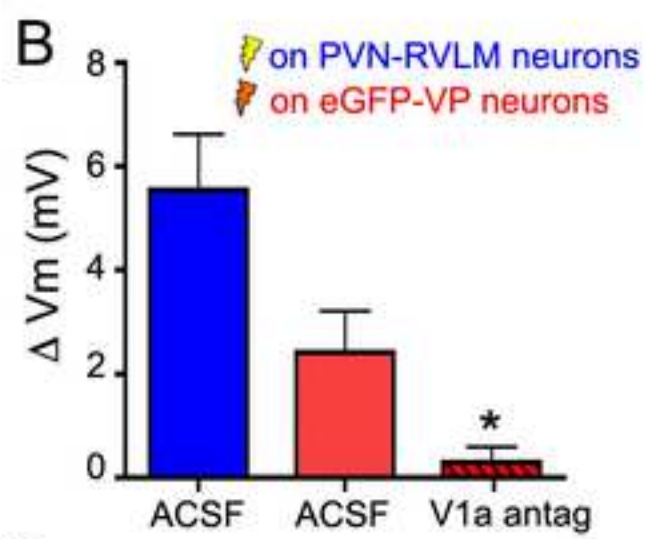
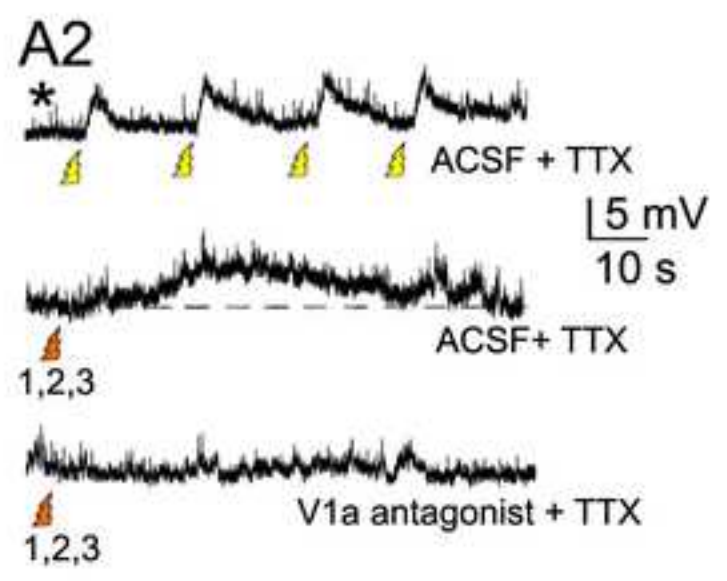
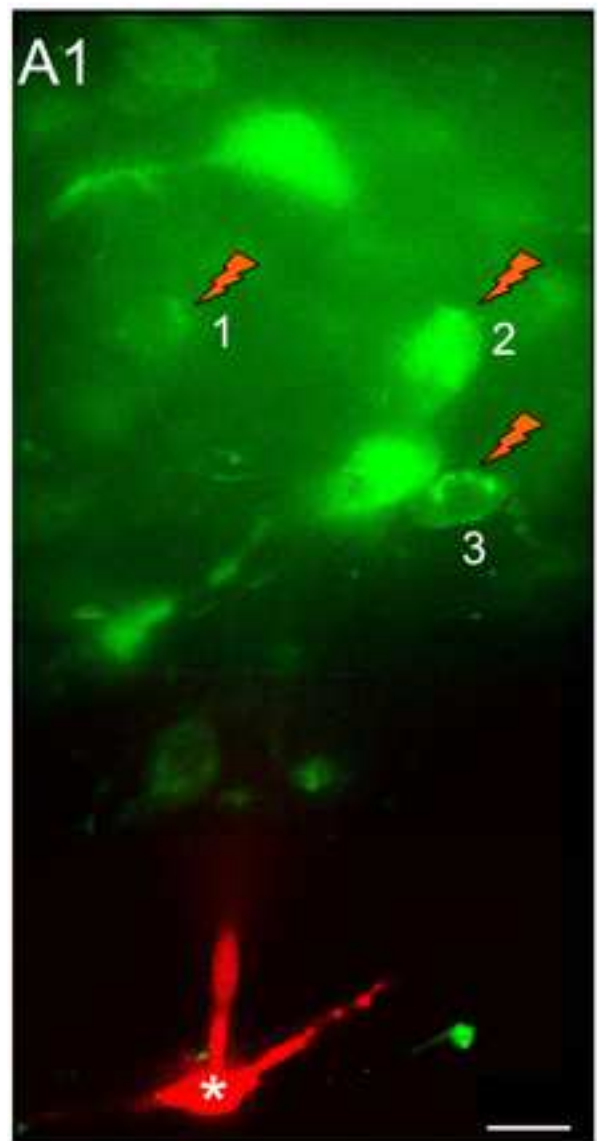


Figure 6

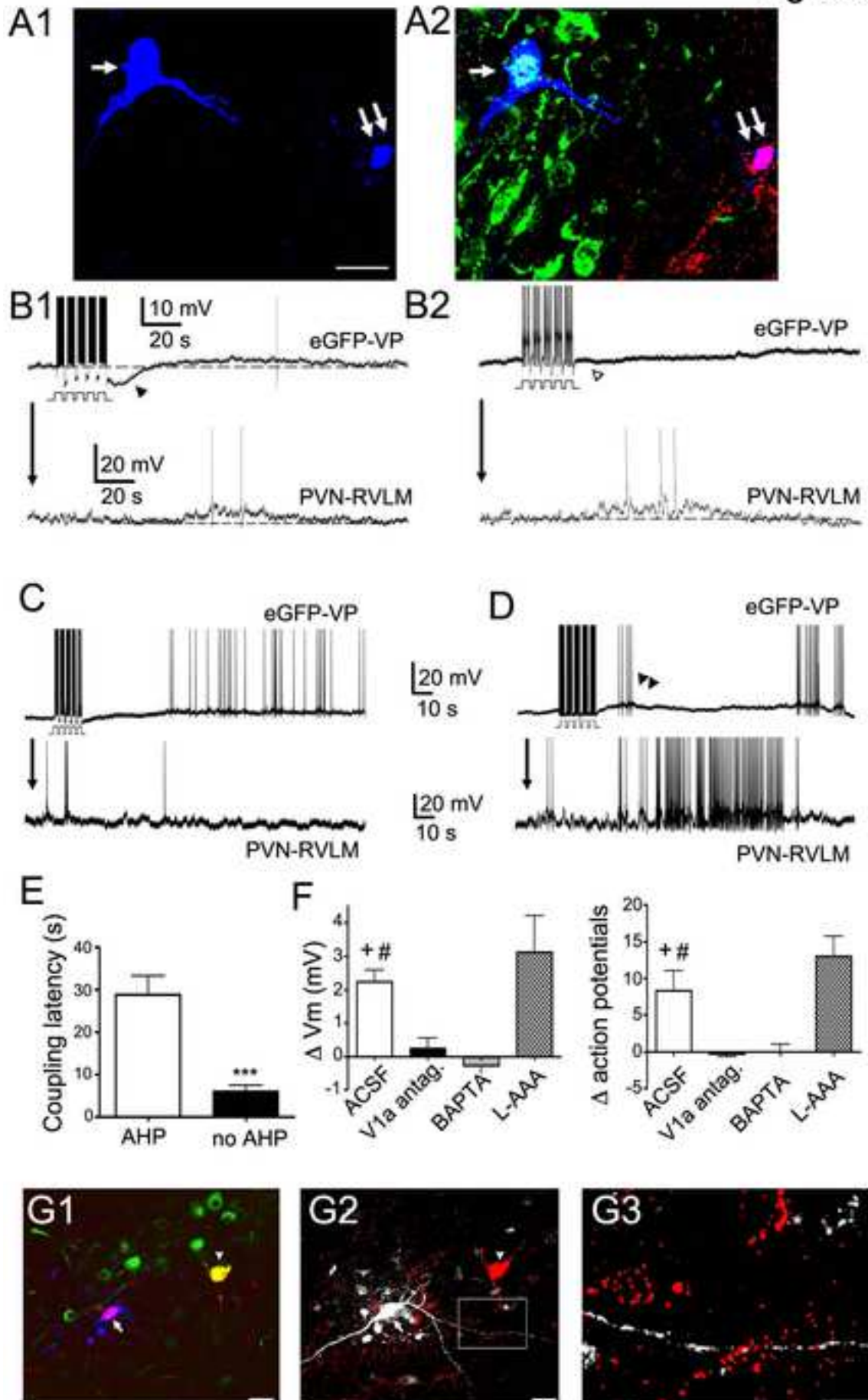


Figure 7

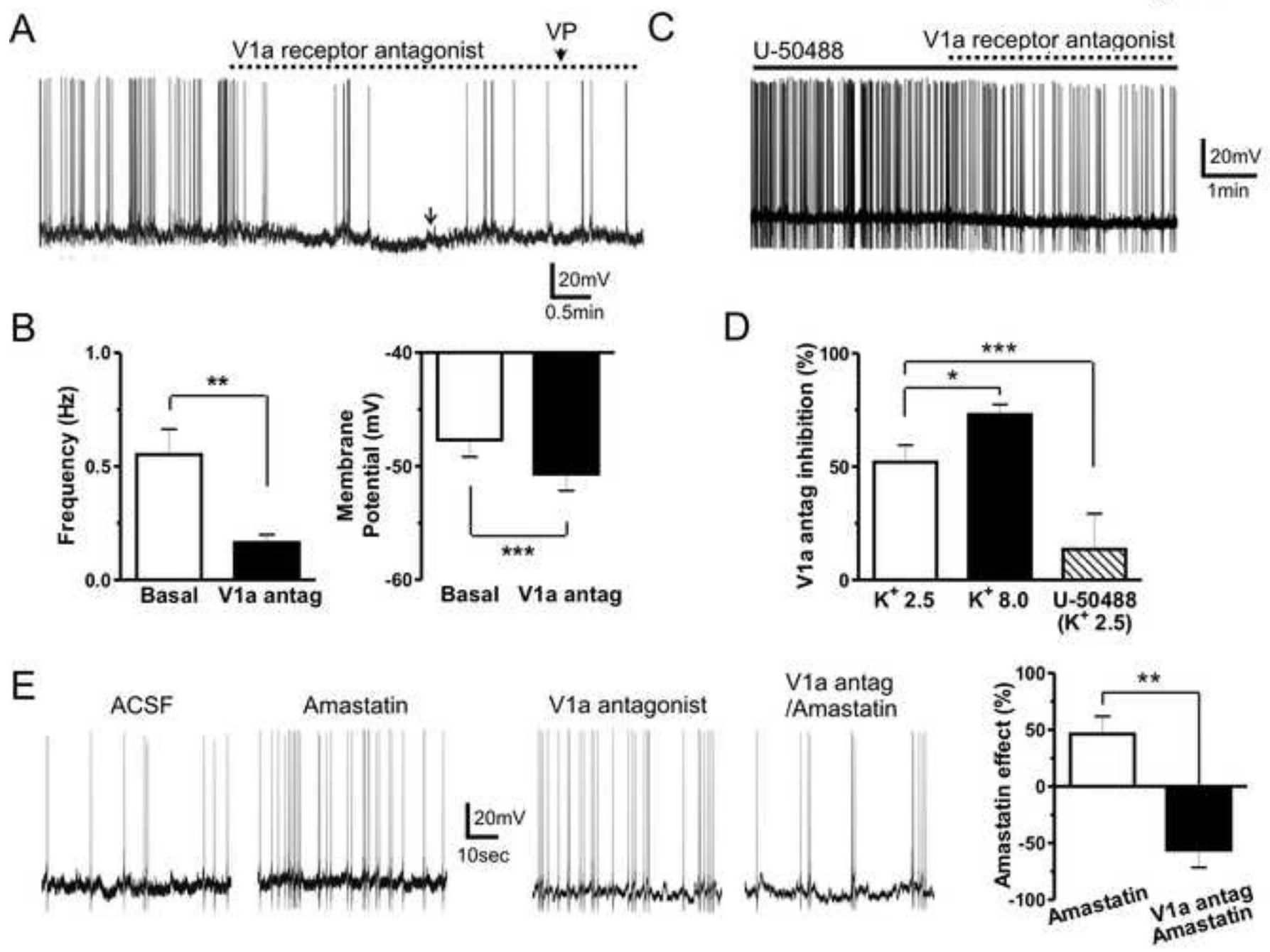


Figure 8

