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Vasopressin indirectly excites dorsal raphe serotonin neurons through activation of the vasopressin1A receptor

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Abstract

The neuropeptide vasopressin (AVP) is produced in a handful of brain nuclei located in the hypothalamus and extended amygdala and is released both peripherally as a hormone and within the central nervous system as a neurotransmitter. Central projections have been associated with a number of functions including regulation of physiological homeostasis, control of circadian rhythms, and modulation of social behavior. The AVP neurons located in the bed nucleus of the stria terminalis and medial amygdala (i.e., extended amygdala) in particular have been associated with affiliative social behavior in multiple species. It was recently demonstrated that in the mouse AVP projections emanating from extended amygdala neurons innervate a number of forebrain and midbrain brain regions including the dorsal raphe nucleus (DR), the site of origin of most forebrain projecting serotonin neurons. Based on the presence of AVP fibers in the DR, we hypothesized that AVP would alter the physiology of serotonin neurons via AVP 1A receptor (V_{1A}R) activation. Using whole cell electrophysiology techniques, we found that AVP increased the frequency and amplitude of excitatory post-synaptic currents (EPSCs) in serotonin neurons of male mice. The indirect stimulation of serotonin neurons was AMPA/kainate receptor dependent and blocked by the sodium channel blocker tetrodotoxin, suggesting an effect of AVP on glutamate neurons. Further, the increase in EPSC frequency induced by AVP was blocked by selective V_{1A}R antagonists. Our data suggest that AVP had an excitatory influence on serotonin neurons. This work highlights a new target (i.e., V_{1A}R) for manipulating serotonin neuron excitability. In light of our data, we propose that some of the diverse effects of AVP on physiology and behavior, including social behavior, may be due to activation of the dorsal raphe serotonin system.

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Keywords

BNST; vasopressin; serotonin; electrophysiology; V1A receptor; glutamate

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Common features of a number of mental disorders such as social anxiety, autism, and schizophrenia include disrupted social behavior, altered responses to stress, and increased predisposition to anxiety (www.nimh.nih.gov/health/topics). The vasopressin (AVP; arginine-vasopressin) and serotonin neurotransmitter systems are major foci of research to elucidate the neural underpinnings of disorders such as those listed above that affect emotion and behavior. Both AVP and serotonin have been implicated in the regulation of social behavior, stress, and anxiety (Ferris et al., 1997, Lucki, 1998, Egashira et al., 2007, Caldwell et al., 2008, Veenema, 2009). A recent study detailing AVP-immunoreactivity throughout the mouse brain indicated a moderate to dense innervation of several midbrain structures including the dorsal and median raphe (Rood and De Vries, 2011), the main source of all forebrain projecting serotonin neurons (Azmitia and Segal, 1978). Our data indicated two important features of AVP innervation to the dorsal raphe (DR): it is gonadal steroid dependent (i.e., eliminated by gonadectomy) and sexually dimorphic (Rood et al., 2012). These features strongly suggest that AVP innervation of the DR comes from the bed nucleus of the stria terminalis and/or the medial amygdala (i.e., extended amygdala, which includes both the BNST and the amygdala) as these AVP-producing nuclei are the only ones in which AVP expression is both dependent on circulating gonadal steroid hormones and sexually dimorphic (De Vries and Panzica, 2006, Rood et al., 2008, Rood et al., 2012).

AVP projections from the extended amygdala are thought to facilitate a number of affiliative and aggressive social behaviors in species ranging from rodents to primates (reviewed in Caldwell et al., 2008, Goodson, 2013). Critical brain regions and relevant receptors have been identified for a select few behaviors, including social memory in rats and mice (Dantzer et al., 1988, Landgraf et al., 1988, Bielsky et al., 2005) and pair-bond formation in prairie voles (Insel et al., 1994, Young et al., 1999, Lim and Young, 2004). However, the exact contribution of the extended amygdala versus other AVP producing regions to social behavior remains to be elucidated.

Interestingly, serotonin is known to influence many of the same behaviors and physiological functions as AVP, including social affiliation and aggression and response to stress (Insel and Winslow, 1998, Lucki, 1998, Veenema and Neumann, 2007). One reason for the functional similarities between these two neurotransmitter systems may be that they are interacting with each other. There are a few examples of interactions between the AVP and serotonin systems in the literature (Insel and Winslow, 1998, Veenema and Neumann, 2007). For example, intracerebroventricular injections of selective serotonin agonists increases peripheral AVP release (Jorgensen et al., 2003) and blocks AVP induced territorial aggression (Ferris et al., 1997, Albers et al., 2002). Conversely, bath application of AVP augments synthesis and release of serotonin from dentate gyrus slice culture (Auerbach and Lipton, 1982). In addition, serotonin and AVP systems have been discussed in parallel regarding both aggressive and affiliative behaviors (Veenema, 2009). However, there are

currently no reports of AVP action on serotonin neuron activity in the DR. Based on the dense AVP innervation of the DR, we hypothesized that AVP must have some effect on serotonin neurons.

In brain regions where a direct effect of AVP has been observed, AVP acting through the AVP 1A receptor ($V_{1A}R$) induces depolarization of the membrane by activation of non-specific cation channels (Stephens and Logan, 1986, Shewey and Dorsa, 1988, Raggenbass, 2008). However, in some systems multi-synaptic responses have been observed as well. For example, increases in postsynaptic currents (EPSCs) in spinal motor neurons and increased inhibitory postsynaptic currents (IPSCs) in lateral septal neurons have also been observed (Liu et al., 2003, Allaman-Exertier et al., 2007). Given the dense AVP innervation of the DR and current evidence regarding the action of AVP on neurons, we predicted that AVP exposure would alter the physiology of DR serotonin neurons. Our data indicated that AVP increased the frequency and amplitude of glutamatergic EPSCs in a subset of DR serotonin neurons.

2. Experimental Procedures

2.1 Animals

Adult male Pet-1::YFP transgenic mice, 63 total, were used for all experiments. Heterozygous and homozygous Pet-1::YFP mice express yellow fluorescent protein (YFP) driven by a Pet-1 promoter, a transcription factor unique to serotonin neurons, enabling visualization of serotonin neurons prior to recording (Scott et al., 2005, Crawford et al., 2010, 2011). This transgenic line has been backcrossed to the C57BL6 strain for more than 10 generations. Animals were derived from breeding pairs housed in our AAALAC accredited facility at the Children's Hospital of Philadelphia. All animals were used in accordance with the National Institutes of Health guide for the care and use of laboratory animals, and all experiments were approved by the institutional IACUC committee.

2.2 Immunohistochemistry

2.2.1 Tissue preparation—Animals (n=10) were perfused with 0.9% NaCl followed by 30ml of 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB) under isoflurane anesthesia. Following perfusion, brains were removed and placed in 4% paraformaldehyde overnight and then placed in 30% sucrose in 0.1M PB. Brains were sectioned at 40 μ m on a cryostat; sections were stored in cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1M PB) at -20°C until processing.

2.2.2 Immunohistochemistry—Brain sections were washed in PBS (0.9% NaCl in PB) 3 $\times$ 10 minutes, incubated in blocking solution (PBST-BSA, PBS with 0.5% Triton X-100 and 0.04% bovine serum albumin) for 30 min, and then incubated overnight at room temperature in PBST-BSA containing mouse anti-tryptophan hydroxylase (TPH; 1:500, Sigma-Aldrich, St. Louis, MO) and guinea pig anti-AVP (1:8000; Bachem, Bubendorf, Switzerland). After overnight incubation, sections were washed with PBST-BSA 3 $\times$ 10 min and then incubated for 2 hours in donkey anti-mouse Alexa Fluor 488 (1:250, Invitrogen, Carlsbad, CA) and biotinylated goat anti-guinea pig (1:250; Vector Labs, Burlingame, CA)

in PBST-BSA. Sections were again washed 3×10 min with PBST-BSA, incubated for 2 hours in streptavidin Alexa Fluor 647 (1:500, Invitrogen) in PBST-BSA, and then washed a final 3×10 min in PBS. Immuno-labeled sections were mounted to Colorfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and coverslipped using Fluoromount-G mounting medium (Electron Microscopy Sciences, Hatfield, PA). Slides were sealed with generic clear nail polish.

2.3 Electrophysiology

2.3.1 Number of animals—A total of 53 mice were used for electrophysiological experiments. From these 53 mice, 35 AVP responsive cells from 31 different mice were identified and included in various experiments. Thus the number (n) of cells reported in the results is roughly the same as the number of animals for each experimental condition. In no case were all data for a given condition derived from a single animal, and in no case were more than two cells from single animal included in a given experiment.

2.3.2 Brain slice preparation—Mice were rapidly decapitated, and the head was immediately placed in carbogen (95% O₂, 5% CO₂) bubbled ice cold sucrose artificial cerebrospinal fluid (aCSF, see below, 248 mM sucrose substituted for NaCl) for approximately 30 min. Anesthesia is not used as it causes increases in corticosterone release that can affect serotonin neuron physiology (Akana et al., 1988, Lorens et al., 1990). Following the cooling period, the brain was removed and 200–220 μ m coronal sections were made through the midbrain on a VT1000 vibratome (Leica Microsystems, Wetzlar, Germany) as previously described (Crawford et al., 2010, 2011). Brain slices were maintained for one hour at 37°C in aCSF (in mM, NaCl 124, KCl 2.5, NaH₂PO₄ 1.25, MgSO₄ 2.0, CaCl₂ 2.5, dextrose 10 and NaHCO₃ 26) bubbled with carbogen, and then at room temperature until recording.

2.3.3 Recording—As previously described (Crawford et al., 2010, 2011), slices were placed into a recording chamber (Warner Instruments, Hamden, CT) continuously perfused with carbogen bubbled aCSF warmed to 32°C with an inline heater (Warner Instruments). Fluorescing serotonin neurons were visualized using a Nikon E600 upright microscope (Nikon, Tokyo, Japan), and then targeted under DIC. Recordings were made using glass pipettes filled with electrolyte and placed into a pipette holder equipped with an Ag-Cl electrode. Standard electrolyte (mM; K-gluconate, 130; NaCl, 5; Na phosphocreatine, 10; MgCl₂, 1; EGTA, 0.02; HEPES, 10; MgATP, 2; Na₂GTP, 0.5; and biocytin, 0.1%; pH 7.3) was used for most recordings, but for some experiments modified high [Cl⁻] electrolyte (mM; K-gluconate, 75; K-Cl 75; NaCl, 5; Na phosphocreatine, 10; MgCl₂, 1; EGTA, 0.02; HEPES, 10; MgATP, 2; and Na₂GTP, 0.5; with biocytin, 0.1%; pH 7.3) was used to enhance the magnitude of IPSCs. Chemicals for buffers and electrolytes were purchased from Sigma-Aldrich.

For each cell, membrane characteristics (i.e., resting membrane potential, membrane resistance, and membrane tau) and action potential characteristics (threshold, amplitude, duration), and after-hyperpolarization (amplitude and decay time) were first recorded in current clamp mode. Then in voltage clamp mode, with cells held at -60mV, the change in

current in response to bath applied ligands was measured. Using multiple pharmacological approaches, we identified the receptor mediating the observed response to AVP. Drugs are listed here, and experimental details are included with the results.

2.3.4 Drugs—[Arg⁸]-vasopressin (AVP, 200nM; Sigma-Aldrich). Bicuculline, GABA_A antagonist (20μM, Sigma-Aldrich). DNQX, AMPA/kainate glutamate receptor antagonist (20μM, Sigma-Aldrich). Tetrodotoxin (TTX), voltage-gated sodium channel antagonist (1μM, Abcam, Cambridge, MA). d(CH₂)₅[Tyr(Me)²]AVP (Manning Compound), selective V_{1A}R antagonist with some oxytocin receptor antagonism (300 nM; Bachem). d(CH₂)₅[Tyr(Me)², Dab⁵]AVP, selective V_{1A}R antagonist with no affinity for the oxytocin receptor (500 nM; generously donated by Dr. Maurice Manning, University of Toledo, Toledo, OH). SSR149415, V_{1B}R antagonist (50nM - 1mM; Axon Medchem, Gronigen, Netherlands). (Deamino-Cys¹, Val⁴, D-Arg⁸)-AVP (dVD-AVP), selective V₂R agonist (200 nM; Bachem).

2.3.5 Histology—Recorded slices were stored in 4% paraformaldehyde until labeling to confirm the location of recorded cells. Immunohistochemistry was carried out as described above with the following exceptions: 1) slices were incubated overnight in just mouse anti-TPH (1:500; Sigma Aldrich) in PBST-BSA, and 2) secondary labeling included a single 2-hour incubation with donkey anti-mouse Alexa Fluor 488 (1:250, Invitrogen) and streptavidin Alexa Fluor 647 (1:500; Invitrogen) prior to a final 3 × 10 min wash in PBS. Sections were mounted on slides and coverslipped as described above.

2.4 Image analysis

Analyses of the AVP innervation of the DR were carried out using images of AVP and TPH immunofluorescence taken on a Leica DM5000B (Leica Microsystems, Wetzlar, Germany) fluorescent microscope using a 10x objective. In ImageJ (imagej.nih.gov/ij; NIH, Bethesda Maryland), photomicrographs were sharpened and adjusted for brightness and contrast to minimize background. Then the areas occupied by AVP-immunoreactivity (-ir) and TPH-ir were calculated by thresholding and measuring the covered area in pixels. Finally, the percent of overlapping pixels was determined using ImageJ.

Further qualitative analyses were carried out using confocal image stacks (1μm interval) obtained using an Olympus FluoView FV1000 confocal microscope with FluoView FV10-ASW software (ver. 1.7; Olympus, Center Valley, PA) and with 20x and 40x objectives. Photomicrographs of DR in Fig 1A-D are derived from confocal stacks. In each case, stacks of each channel were made into a z-projection using the standard deviation method in ImageJ. The separate AVP and TPH channels were then combined using blending options and adjusted for contrast to reduce background in Photoshop (ver. 12.1; Adobe Systems, San Jose, CA).

2.5 Data analysis and statistics

For each recorded cell, cellular characteristics were analyzed from current clamp data as previously described using Clampfit software (Ver.9.2, Molecular Devices, Sunnyvale, CA; (Beck et al., 2004)). Characteristics included membrane tau, resting membrane resistance

and membrane potential, action potential threshold, amplitude, and duration, and after-hyperpolarization amplitude and half-decay time. Differences in membrane characteristics were determined between AVP responsive and non-responsive cells using t-tests.

Voltage clamp data characterizing the response to AVP were analyzed using Minianalysis (Synaptosoft, Decatur, GA; (Lemos et al., 2006)). Event detection was carried out using a threshold level (8-12 pA) defined as 2x the noise amplitude. In Minianalysis, user-defined parameters such as peak amplitude threshold were used with event detection algorithms to isolate baseline, rise, peak, and decay components of potential events, post-synaptic currents in this case. Following automatic detection to find potential events, traces were scanned by eye for accuracy to make sure no peaks were missed. Then peaks were examined individually to eliminate non-events picked up in the automated search. A one-factor repeated measure ANOVA was used to analyze post-synaptic current characteristics before and after AVP bath application. When examining more specific attributes of the AVP response within subsets of cells, PSCs from different conditions were analyzed using the Kolmogorov-Smirnov (K-S) Two Sample Test. For K-S tests, data from all cells from the condition being analyzed were added into appropriate data arrays and analyzed within Minianalysis. $p = 0.05$ was considered significant throughout.

3. Results

3.1 Immunohistochemistry

In the rostral DR, AVP-ir (immunoreactive/immunoreactivity) fibers and puncta form a bilateral plexus on either side of TPH-ir neurons in the dorsomedial subdivision (Fig 1A). This fiber plexus becomes less dense briefly (Fig 1B) before converging in a large dense fiber plexus at the midline in more caudal sections where the dorsomedial and ventromedial subdivisions are bifurcated (Fig 1C). A low magnification analysis of the overlap of AVP-ir fibers and TPH-ir ($n=15$, 15 , and 10 sections analyzed from 10 mice) showed that only $15\% \pm 2$, $46 \pm 3\%$, and $48 \pm 3\%$ of the area occupied by AVP-ir in the rostral, middle, and caudal sections of the DR overlapped with regions occupied by TPH-ir. In some cases, multiple sections from the same animal met the anatomical selection criteria and were thus included in the analysis. No more than 2 sections from a given animal were used for a given region (i.e., rostral, middle, or caudal).

The lower than expected overlap of AVP-ir and TPH-ir prompted us to further examine immunostained tissue using confocal imaging. Qualitative examination of z-stacks suggested that even in regions of overlap, very few AVP-ir puncta were close enough to TPH-ir dendrites or cell bodies to form contacts (Fig 1D). Because of the very low number of co-localized puncta, we did not undertake a quantitative analysis.

3.2 AVP altered post-synaptic current (PSC) activity in serotonin neurons

The response to bath application (i.e., added to the ACSF reservoir being perfused into the recording chamber) of AVP in serotonin neurons was recorded from 122 serotonin neurons spread across the dorsomedial (dm) and ventromedial (vm) subfields of the DR that derived from a total of 53 male mice (Fig 1E). In initial voltage clamp recordings from serotonin neurons in the DR, we observed a striking increase in PSC frequency (187% of baseline; Fig

2A and B; $n=6$, Kolmogorov-Smirnov Two Sample Test (K-S) $Z = 7.36$, $p < 0.0001$) and amplitude (148% of baseline; Fig 2A and C; $n=6$, K-S $Z = 5.85$, $p < 0.0001$) following bath application of 200 nM AVP. Recordings typically lasted 4-5 minutes beyond the addition of AVP. As depicted in Fig2A, there is a delay of about 90 s to 2 min between AVP administration and the observed response. This delay is due to the time needed for the peptide to travel from the reservoir to the chamber. These initial experiments were conducted using high $[Cl^-]$ electrolyte to allow both glutamate-mediated excitatory (E) and GABA-mediated inhibitory (I)PSCs to be recorded as inward currents; this increases the detectability of IPSCs. Subsequent experiments characterized this response. Response data and statistics for all experiments are summarized in Table 1.

In all, 35 of 122 serotonin neurons responded to AVP administration with an increase in frequency of PSCs greater than 1 Hz, which we set as the benchmark for a response based on the observation that large (> 10 pA) increases in amplitude were only observed when frequency increased by 1Hz or more. One factor (Response: responders vs. non-responders) repeated measures (Treatment: baseline vs. AVP) ANOVA demonstrated the change in frequency between the two groups (Table 2; Response X Treatment, $F_{1, 120} = 111.7$, $p < 0.0001$). AVP also increased average current amplitude (Table 2; Response X Treatment, $F_{1, 120} = 37.6$, $p < 0.0001$). Not surprisingly given changes in frequency and amplitude, AVP also increased average current charge and phasic current (average charge X frequency; Table 2; Response X Treatment, $F_{1, 120} = 13.2$, $p < 0.0001$ and $F_{1, 120} = 62.2$, $p < 0.0001$). PSC kinetics, i.e., rise and decay time did not change in response to AVP and did not differ between groups (Table 2). Cell characteristics of AVP-responsive and non-responsive neurons were compared using t-tests. Responders had higher tau values ($t = -2.9$, $p = 0.004$), lower resting membrane potentials ($t = -4.0$, $p = 0.0001$), and shorter action potential (AP) duration ($t = 3.2$, $p = 0.002$). Resting membrane resistance, AP threshold, AP amplitude, after-hyperpolarization (AHP) amplitude, and AHP decay time did not differ. AVP responsive neurons were found in both ventromedial and dorsomedial subfields of the DR. The locations of recorded neurons within the DR are illustrated in Fig 1E.

Finally, it was important to demonstrate that AVP could reliably elicit repeated responses in the same cell without tachyphylaxis. A one-factor (Trial: 1st vs. 2nd administration) repeated-measures (Baseline vs. AVP) ANOVA showed a significant response of AVP administration (Fig 2D and E; $n = 8$; $F_{1, 14} = 21.07$, $p < 0.001$), but no main effect of Trial and no interaction. The observed repeatability of the AVP response allowed us to check cells for AVP responsiveness prior to examining characteristics of the response in different experiments as described below.

3.3 Increased PSCs were mediated by glutamate

To determine whether the AVP-induced increase in PSCs was mediated by glutamate (EPSCs) or GABA (IPSCs), serotonin neurons were recorded in the presence of antagonists to AMPA/kainate glutamate receptors (20 μ M DNQX) or GABA_A receptors (20 μ M bicuculline); combined the two antagonists eliminate PSCs in DR serotonin neurons. In AVP-responsive neurons, DNQX dramatically blocked the AVP-induced increase in PSC frequency (Fig 3A and 3B). DNQX reduced event frequency by 56% (Fig 3C; 44% of AVP

response with DNQX added; $n = 4$; K-S $Z = 7.6$, $p < 0.0001$). EPSC frequency was reduced compared to baseline as well (61% of baseline with DNQX and AVP added; $n = 4$; K-S $Z = 4.9$, $p < 0.0001$), most likely because DNQX blocks glutamate mediated events including both spontaneous and basal (non-TTX sensitive) PSCs. In contrast, increases in PSC frequency and amplitude were observed following addition of AVP in the presence of 20 μM bicuculline (see Fig 2E; Frequency: 209% of baseline/bicuculline with AVP added; $n = 6$; K-S $Z = 7.9$, $p < 0.0001$ and Amplitude: 171% of baseline/bicuculline with AVP added; $n = 6$; K-S $Z = 7.6$, $p < 0.0001$). These data strongly indicated that AVP increased the frequency of excitatory EPSCs, but not IPSCs. Because AVP specifically increased EPSCs, all subsequent recordings were performed with unmodified aCSF in the presence of 20 μM bicuculline. Amplitudes were not compared in the DNQX experiment as baseline and AVP measures included both EPSCs and IPSCs, but only IPSCs are present with DNQX. Thus the comparison would be examining very different event populations making it invalid.

3.4 Increase in EPSCs was action potential dependent

To determine whether AVP increased EPSCs by acting at the synapse (i.e., action potential independent) or at the cell body or dendrites (i.e., action potential dependent) to induce glutamate release, we examined the effect of tetrodotoxin (TTX, 1 μM) on the AVP response. TTX blocks voltage-gated sodium channels thereby eliminating action potentials. TTX eliminated the AVP induced increase in PSCs (Fig 4A and B; 19% of AVP response with TTX added; $n = 3$, K-S $Z = 11.9$, $p < 0.0001$), suggesting that the process was action potential dependent. Therefore the effect of AVP was not mediated directly at the post synaptic membrane of serotonin neurons or presynaptically on glutamate terminals. TTX also reduced EPSC frequency below baseline, hence the significant K-S value shown. This is likely due to the presence of spontaneous glutamate cell activity in untreated slices. The amplitude of EPSCs was reduced in the presence of TTX as compared to both baseline and following AVP administration (Fig 4A; 83% of baseline and 53% of AVP response respectively; $n = 3$; VS. baseline: K-S $Z = 3.2$, $p < 0.0001$ and VS. AVP: K-S $Z = 8.9$, $p < 0.0001$). The reduced amplitude is likely due to the elimination of AVP induced and spontaneous EPSCs leaving only TTX-insensitive EPSCs, typically referred to as mini-EPSCs, which tend to have smaller amplitudes.

3.5 Increase in EPSCs was mediated by $V_{1A}R$

Currently, there are three known AVP receptor subtypes: $V_{1A}R$, $V_{1B}R$, and V_2R . The $V_{1A}R$ antagonist $d(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2]\text{AVP}$ (300 nM, Manning compound) blocked the AVP-mediated increase in EPSCs (Fig 5A and 5B; 233% of baseline with AVP only and 86% of baseline with AVP plus antagonist; $n = 4$, K-S $Z = 2.0$, $p = 0.008$, less than baseline) as well as the increase in EPSC amplitude (Fig 5C; 170% of baseline with AVP only and 96% of baseline with AVP plus antagonist; K-S $Z = 1.2$, $p = 0.09$). In contrast, $V_{1B}R$ antagonist SSR149415 (50 nM - 1 μM) failed to block the AVP induced increase in EPSC frequency (Fig 5D; 177% of baseline with AVP only and 129% of baseline with AVP plus antagonist; $n = 6$, K-S $Z = 3.4$, $p < 0.0001$) and amplitude (188% of baseline with AVP only and 127% of baseline with AVP plus antagonist; $n = 6$, K-S $Z = 3.4$, $p < 0.0001$). The potent V_2 receptor agonist dVD-AVP (200 nM) failed to increase either EPSC frequency (Fig 5E; 137% of baseline with AVP only and 94% with dVD-AVP; $n = 3$, K-S $Z = 1.5$, $p = 0.02$, less than

baseline) or amplitude (149% of baseline with AVP only and 82% with dVD-AVP only; $n=3$, $K-S\ Z = 1.9$, $p = 0.002$, smaller than baseline). AVP is also known to bind to and activate oxytocin receptors, and the Manning compound exhibits some oxytocin receptor antagonism (Manning et al., 2008). Therefore to confirm the role of the $V_{1A}R$, we also performed experiments with a different $V_{1A}R$ antagonist $d(CH_2)_5[Tyr(Me)^2, Dab^5]AVP$ (500nM) that does not exhibit detectable oxytocin receptor antagonism (Manning et al., 2008). AVP failed to increase EPSC frequency or amplitude in the presence of this more selective antagonist as well (Fig 5F; frequency: 193% of baseline with AVP only and 86% of baseline with AVP plus antagonist; $n=4$, $K-S\ Z = 0.75$, $p = 0.63$ and amplitude: 111% of baseline with AVP only and 102% of baseline with AVP plus antagonist; $n=4$, $K-S\ Z = 0.64$, $p = 0.81$) suggesting that the response is mediated by $V_{1A}R$ and not the oxytocin receptor.

4. Discussion

AVP-ir fibers originating in the extended amygdala densely innervate the DR (Rood and De Vries, 2011, Rood et al., 2012). In the current study, double-label immunohistochemistry for AVP and TPH revealed that most AVP-ir puncta in the DR were not close enough to serotonin neurons or dendrites to make direct synaptic connections. Although direct connections between AVP neurons and serotonin cells were not apparent, electrophysiological data indicated that AVP did in fact affect serotonin neurons causing a robust increase in the frequency and amplitude of glutamatergic excitatory post-synaptic currents. Our data indicated that this effect was action potential dependent and mediated by $V_{1A}R$. All of these data combined lead to the conclusion that AVP exerts a multi-synaptic stimulatory effect on serotonin neurons in the DR.

One of the challenges in carrying out the work described here was the low percentage of responding cells (35 of 122 or 29%). Under the current conditions, it is unclear whether this is a biological phenomenon or a technical artifact. It is possible that only a subset of serotonin neurons in the raphe is innervated by AVP-responsive glutamate neurons. However, it is also possible that in the process of tissue sectioning connections were severed thereby eliminating responsiveness to AVP. One piece of evidence that supports the idea of a sub-population of AVP-responsive neurons is the fact that there were significant differences in neural characteristics (i.e., membrane time constant, resting membrane potential, and action potential duration) between AVP responsive and non-responsive serotonin neurons. Multiple reports have indicated the existence of differences between sub-populations of serotonin neurons in rats and mice in both neuronal characteristics and topographic projections (Beck et al., 2004, Crawford et al., 2010, Calizo et al., 2011, Bang et al., 2012). The implication of there being a subset of AVP responsive serotonin neurons is that serotonin release could be directed to discrete regions of the brain as opposed to a global release. This is especially relevant given the fact that there is evidence for functionally opposing roles (i.e., anxiogenic versus anxiolytic) of different serotonin neuron populations in the dorsal raphe (Hale et al., 2012). Further research is needed to determine whether or not a functionally distinct sub-group of AVP-responsive serotonin neurons actually exists.

Bath applied AVP induced an increase in the frequency and amplitude of PSCs recorded from serotonin neurons. This response was not observed in the presence of DNQX, an AMPA/Kainate glutamate receptor antagonist, but was in the presence of bicuculline, a GABA_A receptor antagonist. Thus while a change in GABA-mediated IPSCs cannot be ruled out for all serotonin neurons, our data indicate that glutamate neurons or glutamate nerve terminals within the brain slice mediated the observed effect of AVP. If vasopressin acted on nerve terminals only, i.e., presynaptically, then the process would be independent of action potentials. However, the AVP response was completely blocked by TTX, which blocks voltage gated sodium channels thereby blocking action potentials. Multiple conclusions can be drawn from this fact. First, AVP must be acting somewhere upstream of the axon of glutamatergic cells that synapse on serotonin neurons. This means that the cell bodies and or dendrites of the AVP-responsive cells must be located within the same 200 μ m thick slice as the affected serotonin neurons. In addition, because the increase in amplitude was also blocked by TTX, it is unlikely that changes to post-synaptic AMPA receptors or pre-synaptic changes on glutamate terminals are responsible for the increased EPSC amplitude. Rather AVP must elicit an action potential in a glutamate neuron that through an action potential dependent process releases more frequent and larger pulses of glutamate.

The next challenge in understanding this phenomenon will be to identify the population of glutamate neurons that responds directly to AVP. Our data indicated that there must be at least one upstream neuron (i.e., the glutamate neuron), but it is possible that AVP acts even farther upstream. It is also possible that the effect on serotonin neurons is mediated by some other neuron outside of the raphe itself. Other brain regions within the 200 μ m thick section such as the inferior colliculus, periaqueductal gray, or ventral tegmentum could potentially house the AVP-responsive glutamate neurons that are upstream of the serotonin neurons. One notable example is the caudal ventrolateral periaqueductal gray area, which is dorsolateral to the DR and is innervated by hypothalamic AVP neurons originating in the paraventricular nucleus (Rood et al., 2012). In fact, some behavioral effects of AVP are likely due to release from the paraventricular nucleus, especially the parvocellular subdivision, which is thought to modulate both the pituitary and central nervous system responses to stress (Wersinger et al., 2002, Aguilera et al., 2008, Rood et al., 2012). However, based on the location of AVP innervation, the most parsimonious explanation is that the population of glutamate neurons resides adjacent to serotonin neurons within the DR itself. The region of the dorsomedial and upper ventromedial rostral DR where AVP-responsive serotonin neurons were most abundant is the region most clearly encapsulated by AVP-ir fibers from the extended amygdala (see Figure 1). The DR contains numerous non-serotonergic neurons that express vesicular glutamate transporter 3 that are located intermixed with the serotonin neurons of the dorsal raphe (Freneau et al., 2002, Crawford et al., 2011). The most plausible supposition is that these are the glutamate neurons that were being excited by AVP receptor activation. Although a discrete neural circuit (i.e., AVP neuron innervates glutamate neuron that innervates serotonin neurons) is likely to exist, we cannot rule out the possibility that AVP release occurring some distance away from the site of action is responsible. Volumetric release of AVP has been observed in other brain regions, primarily the paraventricular nucleus (Pow and Morris, 1989, Ludwig and Leng, 2006). Interestingly, AVP receptor binding density using autoradiography is often greater in

areas with few AVP-ir fibers suggesting the necessity of volumetric release (Young et al., 1999, Rood and De Vries, 2011). Our goal here was to test the action of AVP specifically on serotonin neurons. However, based on our results, future efforts will need to focus on identifying neurons within or around the dorsal raphe that respond directly to AVP, i.e., contain the AVP receptor.

Our data indicated that V_{1A}R and not V_{1B}R or V₂R was responsible for increasing EPSC activity in serotonin neurons. This result was expected as *in situ* hybridization studies indicate that V_{1A}R is the most abundant AVP receptor in the brain (Ostrowski et al., 1992). V₂R is found predominantly in the collecting ducts of the kidneys (Ostrowski et al., 1992). V_{1B}R expression is most dense in the anterior pituitary, although there are reports of V_{1B}R expression in some brain regions such as the hypothalamus, amygdala, and circumventricular zones (Saito et al., 1995, Hernando et al., 2001). V_{1A}R mediated responses have been recorded in a number of brain regions including the paraventricular nucleus, amygdala, lateral septum, facial nucleus, hypoglossal nucleus, nucleus of the solitary tract, and thoracolumbar spinal cord (Raggenbass, 2008). In each of these brain regions, V_{1A}R increases neuronal excitability as evidenced by inward (i.e., depolarizing) currents or increases in firing rate (Raggenbass, 2008). Indirect responses or changes in synaptic activity have also been observed. In the lateral septum most cells responded to AVP with an increase in IPSC frequency; only a small subset of a cells show a direct depolarization (Allaman-Exertier et al., 2007). A similar multi-synaptic effect has been observed in brainstem and spinal cord motor neurons where increases in EPSCs and IPSCs have been observed in different neuron populations (Liu et al., 2003, Reymond-Marron et al., 2005). It is also important to note here that the observed response was not due to oxytocin receptors; the response could be fully blocked by d(CH₂)₅[Tyr(Me)², Dab⁵]AVP, a V_{1A}R antagonist with no affinity for the oxytocin receptor (Manning et al., 2008). This is important because AVP is able to bind and activate oxytocin receptors (Elands et al., 1988).

AVP has been shown to act via the V_{1A}R in a diverse set of brain regions that are likely to have an equally diverse set of functions. Although V_{1A}R activation is the predominant receptor (Ostrowski et al., 1992, Ostrowski et al., 1994), the source of AVP innervation differs greatly across brain regions (De Vries and Buijs, 1983, Rood et al., 2012). Innervation of the brainstem and spinal cord as well as the central amygdala comes primarily from the paraventricular nucleus (Sawchenko and Swanson, 1982, Rood et al., 2012), whereas innervation to the lateral septum and DR comes primarily from the extended amygdala (De Vries and Buijs, 1983, Rood et al., 2012). The differences in AVP innervation based on the origin of fibers highlights the importance of considering the AVP system in its entirety when thinking about function. For example, the BNST and medial amygdala share reciprocal connections and process and integrate sensory information, especially from the olfactory bulb (Kang et al., 2011). Thus extended amygdala AVP neurons would be uniquely poised to bridge the gap between sensory stimuli and behavioral state by modulating serotonin neuron excitability. In contrast, other regions affected by AVP such as those in the hindbrain and spinal cord are innervated by the hypothalamic paraventricular nucleus (Raggenbass, 2008, Rood et al., 2012), which receives sensory signals relating to

homeostasis and stress (Dallman et al., 1987, Fujio et al., 2006, Aguilera et al., 2008). Thus these caudal AVP inputs may be involved in the regulation of the stress response.

Our data indicated that AVP increased glutamatergic EPSCs in serotonin neurons. The increase in EPSCs *in vivo* would likely result in increased serotonin neuron excitability and increased serotonin release. The fact that AVP and serotonin influence multiple behavioral and physiological functions including social behavior is clear throughout the literature (Azmitia and Segal, 1978, Lucki, 1998, Albers et al., 2002). However, the mechanisms of action at the cellular level and the organization of relevant neural circuits through which these neurotransmitters act to control or regulate social behavior are still being discovered. To the best of our knowledge, the only data regarding the effect of AVP on neurons innervated by the extended amygdala are from the lateral septum (Allaman-Exertier et al., 2007) and the dorsal raphe as laid out here.

With increasing attention to the cellular and molecular mechanisms underlying AVP action in the brain, the role and mechanism of AVP action in a wide variety of physiological and behavioral processes is starting to be clarified. Our data promoted this goal by uncovering a novel site of AVP action in the DR, i.e., demonstrating that AVP increased EPSC activity in serotonin neurons through V_{1A}R most likely located on glutamate neurons. As such AVP may mediate some of its effects through activation of the serotonin system. Future studies will be required to demonstrate AVP-mediated activation of the serotonin system *in vivo*. In addition, future studies should examine AVP function in non-serotonergic glutamate neurons in or around the DR to determine which population of neurons is responsible for the increase in EPSC activity. Data presented here in conjunction with future studies will help to unravel the complex neural control of social behavior and anxiety, increase our understanding of local circuitry in the DR, and identify new targets for studying the function of the serotonin system.

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Highlights

- We tested effects of the neuropeptide vasopressin on dorsal raphe serotonin neurons.
- Vasopressin increased frequency and amplitude of post-synaptic currents.
- Vasopressin effect was glutamate receptor and action potential dependent.
- Vasopressin induced EPSCs were blocked by vasopressin1A receptor antagonists.
- Vasopressin stimulates serotonin neurons through a nearby glutamate intermediary.

Figure 1. AVP fibers and puncta in the DR do not interact directly with serotonin neurons

A) AVP-immunoreactive (-ir) fibers form bilateral plexuses in the rostral dorsal raphe (bregma -4.36; Franklin and Paxinos, 2008). B) At bregma -4.48, these plexuses become less dense for a short distance. C) Then, around bregma -4.72 where the dorsomedial and ventromedial subfields separate, AVP-ir fibers form a dense plexus at the midline. D) A magnification of the region outlined in (A) suggests that AVP fibers avoid areas of TPH-ir cells and, for the most part, dendrites. This location was chosen for magnification as it was where the highest concentration of AVP-responsive serotonin neurons was found. E) Recordings were made from 122 serotonin neurons spread across the rostral to caudal extent of the ventromedial (vm) and dorsomedial (dm) subfields of the DR. Most AVP responsive neurons were located rostrally in the dmDR and upper part of the vmDR. The relative locations of recorded cells are shown with yellow dots indicating neurons that had an increase in PSC frequency > 1 Hz following AVP administration and red dots indicating a response less than 1 Hz or a decrease in PSC frequency. Representative sections through the

DR were imaged using differential interference contrast optics, and the fluorescent signals from the same sections were overlaid (green pseudo-coloring) to indicate relative distribution of serotonin neurons for cell map background. Scale bars in the bottom right corner of all images are equal to 100 μm . Aq = cerebral aqueduct; lwDR = later wings of the DR; mlf = medial longitudinal fasciculus; PAG = periaqueductal gray.

Figure 2. AVP increased the frequency and amplitude of PSCs in serotonin neurons

A) Current trace using voltage clamp techniques (-60 mV) shows the administration of 200 nM AVP (indicated by line over trace) followed by a remarkable increase in PSC activity. The delay was due to the flow rate of solution from the reservoir to the slice chamber (1-2 minutes). B) AVP bath administration induced an increase in the occurrence of shorter inter-event intervals compared to baseline as indicated by a shift to the left in the cumulative histogram (*K-S Z = 7.4, $p < 0.0001$, $n = 6$). Shorter inter-event interval is equivalent to higher frequency of events. C) The amplitude of PSCs also increased following AVP administration as indicated by the shift to the right in the cumulative histogram (*K-S Z = 5.8, $p < 0.0001$, $n = 6$). D) The AVP response could be washed out and induced multiple times. In a one-factor (Trial: 1st vs. 2nd administration) repeated-measures (Baseline vs. AVP) ANOVA there was a main effect of AVP administration $*F_{1, 14} = 21.07$, $p < 0.001$, $n = 8$, but no main effect of Trial and no interaction. Washout in between trials lasted for approximately 8-10 min. E) Current trace showing multiple responses to AVP separated by

a washout of about 8 min (i.e., no AVP present). Solid lines represent the presence of 20 μ M bicuculline (BIC; lower) and 200 nM AVP (upper). The dotted line represents washout of both drugs. BIC was added about 3 min prior to the second AVP administration.

Fig 3. AVP-induced increase in frequency was blocked by an AMPA/kainate receptor antagonist
 A) Current traces recorded in voltage clamp (-60mV) show PSC activity in response to 200 nM AVP in the absence (upper trace) and presence (lower trace) of the AMPA/kainate glutamate receptor antagonist DNQX (20μM). B) 2 sec segments from the above traces (A) are shown to better illustrate the individual postsynaptic events. These recordings were obtained with an electrode containing high [Cl⁻] electrolyte causing GABA IPSCs to occur as downward spikes best seen in the absence of glutamate activity (lower trace). Some GABA events are likely present in the baseline and AVP traces; potential IPSCs can be identified by their longer decay time. C) AVP induced an increase in the occurrence of shorter inter-event intervals compared to baseline as indicated by a shift to the left in the cumulative histogram (*K-S Z = 3.2, $p < 0.0001$, $n = 4$). However, EPSC frequency was significantly reduced (#) following application of DNQX as compared to both baseline (K-S Z = 4.9, $p < 0.0001$, $n = 4$) and AVP alone (K-S Z = 7.6, $p < 0.0001$, $n = 4$).

Figure 4. TTX eliminated AVP mediated increase in EPSC frequency and amplitude
 A) Voltage clamp current trace demonstrated the increase in EPSCs following AVP administration (200 nM), and the subsequent elimination of the response by the voltage-gated sodium channel antagonist tetrodotoxin (TTX, 1 μ M) that blocks action potentials. B) AVP induced an increase in the occurrence of shorter inter-event intervals compared to baseline as indicated by a shift to the left in the cumulative histogram (*K-S Z = 10.2, $p < 0.0001$, $n = 3$). EPSC frequency was greatly reduced (#) in the presence of 1 μ M TTX plus AVP as compared to both baseline (K-S Z = 3.15, $p < 0.0001$, $n = 3$) and AVP alone (K-S Z = 11.91, $p < 0.0001$, $n = 3$).

Figure 5. AVP induced increase in EPSCs in DR serotonin neurons was mediated by V_{1A} receptors ($V_{1A}R$)

All experimental data were collected in the presence of 20 μ M bicuculline (BIC). A) Voltage clamp current traces show the increase in frequency and amplitude of EPSCs following AVP administration (upper trace) and the lack of response in the presence of a $V_{1A}R$ antagonist in the same cell (lower trace; Manning Compound). B) The left cumulative histogram shows the increase in shorter inter-event intervals (left shift equals increased frequency) compared to baseline (*K-S $Z = 6.3$, $p < 0.0001$, $n = 4$). The right histogram shows the lack of AVP influence in the presence of the Manning Compound, although there was a small but significant shift toward longer inter-event intervals (#K-S $Z = 2.0$, $P = 0.0008$, $n = 4$). C) The left cumulative histogram shows the increase in EPSC amplitude (right shift) following AVP administration (*K-S $Z = 7.6$, $p < 0.0001$, $n = 4$), and the right histogram shows the absence of amplitude change in the presence of the Manning Compound (K-S $Z = 1.2$, $p = 0.09$, $n = 4$). D) The $V_{1B}R$ antagonist SSR149415 (50 nM, 300 nM, and 1 μ M data combined) failed to block the AVP-mediated increase in EPSC frequency (*K-S $Z = 3.4$, $p < 0.0001$, $n = 6$); inset shows data for initial AVP response (*K-S $Z = 5.4$, $p < 0.0001$, $n = 6$). E) The potent V_{2R} agonist dVD-AVP failed to increase frequency; rather there was a small but significant decrease in frequency (#K-S $Z = 1.5$, $p = 0.02$, $n = 3$); inset shows data for initial AVP response (*K-S $Z = 2.7$, $p < 0.0001$, $n = 3$). F) The $V_{1A}R$ antagonist d(CH₂)₅[Tyr(Me)², Dab⁵]AVP, which has no affinity for oxytocin receptors, also fully blocked the AVP-mediated increase in short inter-event intervals (K-S $Z = 0.7$, $p = 0.63$, $n = 4$); inset shows data for initial AVP response (*K-S $Z = 4.4$, $p < 0.0001$, $n = 4$).

Table 1

Percent response to 200 nM AVP alone or in the presence of various antagonists

200 nM AVP % of Baseline	K-S*	P				
Freq. 187	7.36	<0.0001				
Amp. 148	5.85	<0.0001				
200 nM AVP						
% of Baseline	K-S ^b	P	200 nM AVP in the presence of...	(A) % of Baseline (B) % AVP alone	K-S ^b	P
Freq. 140	3.21	<0.0001	20 μ M	(A) Freq. 61	4.86	<0.0001 ^a
Amp. 152	4.35	<0.0001	DNQX	(B) Freq. 44	7.55	<0.0001
Freq. 348	10.21	<0.0001	1 μ M	(A) Freq. 66	3.15	<0.0001
Amp. 198	10.11	<0.0001	TTX	(A) Amp. 83	3.17	<0.0001
				(B) Freq. 19	11.91	<0.0001
				(B) Amp. 53	8.88	<0.0001
200 nM AVP			200 nM AVP in the presence of...	% of Baseline ^e	K-S ^b	P
% of Baseline ^c	K-S ^b	P				
			20 μ M bicuculline			
Freq. 233	6.30	<0.0001	200 nM	Freq. 209	7.93	<0.0001
Amp. 170	7.58	<0.0001	Manning Compound	Amp. 171	7.82	<0.0001
Freq. 193	4.41	<0.0001	500 nM d(CH ₂) ₅	Freq. 86	1.98	0.0008
Amp. 111	2.61	<0.0001	[Tyr(Me) ² , Dab ⁵]AVP	Amp. 96	1.24	0.09
Freq. 177	5.38	<0.0001	50 nM to 1 μ M	Freq. 86	0.75	0.63
Amp. 188	5.74	<0.0001	SSR149415	Amp. 102	.64	0.81
Freq. 137	2.70	<0.0001	200 nM	Freq. 129	3.36	<0.0001
Amp. 149	1.64	0.009	dVDAVP ^d	Amp. 127	2.86	<0.0001
				Freq. 94	1.52	0.02
				Amp. 82	1.86	0.002

^a Shaded cells indicate conditions in which the effect of AVP was blocked or in which a receptor agonist failed to elicit a response (i.e., response was at or below baseline).

^b Kolmogorov-Smirnov Two Sample Z-score

^c Baseline was measured in the presence of 20 μ M bicuculline.

*d*_{VDA VP} is a potent V₂R agonist and was applied in the absence of AVP

^g Baseline used to calculate the % change in response to AVP was measured in the presence of receptor antagonist and 20 μ M bicuculline or bicuculline alone.

Table 2

Post-synaptic current characteristics in responsive and non-responsive serotonin neurons at baseline and in the presence of 200 nM AVP

	Responsive (N = 35)		Non-responsive (N = 87)	
	Baseline	AVP	Baseline	AVP
Frequency (Hz)	11.75 ± 1.44	21.90 ± 1.93 ^a	9.95 ± 0.65	8.20 ± 0.55
Amplitude (pA)	27.47 ± 1.6	38.79 ± 3.69 ^b	28.24 ± 0.66	28.88 ± 0.72
Rise (ms)	1.86 ± 0.04	1.86 ± 0.05	1.73 ± 0.44	1.84 ± 0.03
Decay (ms)	1.81 ± 0.12	2.01 ± 0.26	1.84 ± 0.47	1.47 ± 0.06
Charge (pA/ms)	75.66 ± 3.43	114.06 ± 12.16 ^c	65.18 ± 2.52	75.45 ± 3.08
Phasic Current (pA)	0.89 ± 0.12	0.26 ± 0.37 ^d	0.63 ± 0.48	0.60 ± 0.48

Values are Mean ± SEM

^aResponse X Treatment, $F_{1, 120} = 111.7$, $p < 0.0001$.

^bResponse X Treatment, $F_{1, 120} = 37.56$, $p < 0.0001$.

^cResponse X Treatment, $F_{1, 120} = 13.22$, $p < 0.0001$.

^dResponse X Treatment, $F_{1, 120} = 62.19$, $p < 0.0001$.