



A vasopressin circuit that modulates mouse social investigation and anxiety-like behavior in a sex-specific manner

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One of the largest sex differences in brain neurochemistry is the expression of the neuropeptide arginine vasopressin (AVP) within the vertebrate brain, with males having more AVP cells in the bed nucleus of the stria terminalis (BNST) than females. Despite the long-standing implication of AVP in social and anxiety-like behaviors, the circuitry underlying AVP's control of these behaviors is still not well defined. Using optogenetic approaches, we show that inhibiting AVP BNST cells reduces social investigation in males, but not in females, whereas stimulating these cells increases social investigation in both sexes, but more so in males. These cells may facilitate male social investigation through their projections to the lateral septum (LS), an area with the highest density of sexually differentiated AVP innervation in the brain, as optogenetic stimulation of BNST AVP → LS increased social investigation and anxiety-like behavior in males but not in females; the same stimulation also caused a biphasic response of LS cells ex vivo. Blocking the vasopressin 1a receptor (V1aR) in the LS eliminated all these responses. Together, these findings establish a sexually differentiated role for BNST AVP cells in the control of social investigation and anxiety-like behavior, likely mediated by their projections to the LS.

sex differences | vasopressin | social behavior | anxiety | mice

Dysfunction in social behavior and communication prominently features in psychopathologies such as autism, schizophrenia, and social anxiety (1, 2). These disorders show marked sex differences with, for example, autism and schizophrenia being more prevalent in males and social anxiety more prevalent in females (2, 3). Understanding the nature of these differences may facilitate developing broad avenues for treating these disorders. A reasonable hypothesis is that sex differences in neural circuitry that controls social behavior contribute to sex differences in dysfunctions of social behavior and communication. A problem is that there is little understanding of the role of sex differences in the neural control of social behavior, whether normal or disordered (4, 5).

Studying the role of vasopressin (AVP) innervation of the brain may provide insight in this respect. AVP has been repeatedly implicated in modulation of social behaviors in sexually differentiated ways (6–10) and is an important modulator for both animal (7, 11) and human social behavior (12). In humans, AVP has been implicated in various psychopathologies (13). For example, variations in the vasopressin V1a receptor (V1aR) gene (14, 15) as well as in neonatal AVP serum levels have been associated with autism, primarily in males (16). Pharmacological studies, primarily in laboratory rodents and several bird species, indicate that AVP acts on various brain regions to regulate social communication (17), aggression (18), maternal care (19), pair bonding (20), and social recognition (6).

Because there are distinct groups of AVP cells in the brain, each of which project to distinct but sometimes overlapping brain areas (21), identifying the extent to which these groups contribute to AVP control of social behavior has been challenging. Across tetrapods, the most prominent of these groups are found in the paraventricular nucleus of the hypothalamus, the suprachiasmatic nuclei of the hypothalamus, and the bed nucleus of the stria terminalis (BNST) and medial amygdaloid nucleus (MeA). The most sexually differentiated AVP projections come from the BNST and MeA (22). Many well-studied laboratory rodents and several species of birds, for example, have about twice as many AVP cells as females in the BNST, and their projections to areas such as the lateral septum (LS) are denser as well (21, 23, 24). Indeed, this is one of the largest and most evolutionarily conserved sexually differentiated circuits identified to date in the vertebrate brain (17, 22, 25). While sex differences have been reported in other species in other AVP circuits [e.g., in PVN projections, which are denser in female mice (21)], these differences are generally much smaller and have not been consistently reported across species.

Significance

The function of sex differences in the brain is poorly understood. Here, we test the function of the vasopressin cells of the bed nucleus of the stria terminalis, which are more numerous in males than in females, one of the most consistently found sex differences in the vertebrate brain. Using optogenetic inhibition, we demonstrate that these cells are necessary for controlling social investigation in males but not in females.

Excitation of these cells increased social investigation in both sexes, but more so in males. Excitation of their projections to the lateral septum increased both social investigation and anxiety-like behavior, but only in males. These findings demonstrate that social investigation and anxiety-like behavior is regulated in a sexually differentiated way.

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Most studies that implicated specific AVP projections in the control of social behaviors did so indirectly, e.g., by pharmacological blockade of AVP receptors in specific areas of the brains of laboratory rodent species and several bird species (7, 24). As it is nearly impossible to restrict the effects of such manipulations to AVP projections from just one source, it has remained unclear specifically which of the above cell groups contribute to AVP effects on social behavior. Recently, we have started to systematically test the role of specific AVP cell groups in social behavior in mice by blocking AVP expression in specific cell groups (26) or by lesioning AVP cells using viral vector approaches (27–29). Both of these manipulations reduced social investigation in male, but not female, mice. As both manipulations involve long-term impairment of BNST AVP cell function, which may have resulted in compensatory processes that prevented observations of deficits in social investigation specifically in females, we cannot rule out that short-term manipulations of BNST projections may reveal AVP control of social investigation in females. Moreover, we do not know whether BNST AVP cells are sufficient, by themselves, to drive social investigation nor do we know which of the numerous BNST AVP projections (21, 30) modulate social investigation in either sex, information that is needed for creating a coherent and mechanistic account of BNST AVP cell action.

In this study, we use mice to address these questions by first demonstrating that exposure to social stimuli increases Fos expression in BNST AVP cells. We then show that optogenetic inhibition of these cells reduced same-sex social investigation in males but not in females. Conversely, optogenetic stimulation of BNST AVP cells increased same- and opposite-sex social investigation in males, but had a more limited effect in females as it only increased their investigation toward males. Finally, we show that activating BNST AVP projections to the LS, one of the most conspicuous targets of BNST AVP cells (21, 30) as well as a site where AVP has consistently been shown to influence social behavior (7, 10, 31), had an overall inhibitory effect on septal neuronal activity while increasing social investigation in males, effects that could be blocked by an AVP V1a receptor antagonist. As AVP has been implicated in the control of anxiety (32), and as changes in anxiety have been linked to changes in social behavior (33, 34), we also tested whether our optogenetic manipulations of cells and fibers influenced anxiety-like behaviors. While stimulation and inhibition of BNST AVP cells did not impact anxiety-like behavior, stimulating the projections of BNST AVP cells to the LS increased both social investigation and anxiety-like behavior in males, but not in females.

Results

BNST AVP Cells Respond to Social Investigation. AVP cells in the BNST show enhanced Fos expression in response to copulatory and aggressive interactions in male mice (35), but it is unknown whether these cells are similarly responsive in females and, importantly, whether these cells show higher Fos expression following social investigation. To answer this, we measured Fos expression within BNST AVP cells of males and females exposed to a caged male or female conspecific or to empty cages in a three-chamber apparatus. Male and female mice exposed to males or females had increased BNST AVP-Fos colocalization compared to subjects not exposed to conspecifics (Fig. 1 A–C). However, females exposed to conspecifics had less AVP-Fos colocalization in the BNST compared to males in both social conditions, and females had less overall Fos expression in the BNST. To determine whether any baseline differences in Fos activation between the sexes impacted our findings, we normalized AVP-Fos

colocalization within each sex before analyzing the effects of social stimuli exposure. This did not significantly alter our finding of a sex difference in Fos activation in BNST AVP cells [$F(1, 24) = 10.69, P = 0.003$]. Overall, these results suggest that BNST AVP cells encode social cues, but perhaps differently in both sexes. Exactly what features of social interaction these cells encode await further investigation using, for example, *in vivo* calcium-imaging approaches.

Inhibiting BNST AVP Cells Reduces Male's Social Investigation of Other Males, without Affecting Social Communicative Behaviors.

Deletion of BNST AVP cells alters male, but not female, social investigation and communication in mice (27). As compensatory changes may have taken place after deletion, we cannot exclude the possibility that these cells affect social behavior in both sexes. To address this question, we tested whether acute inhibition of BNST AVP cells using an optogenetic approach (Fig. 2 A and B) influenced male and female social investigation and male-biased social communication behaviors such as urine marking (36) and ultrasonic vocalizations (37). To confirm that light application inhibits AVP BNST cells expressing the inhibitory blue-light opsin stGTACR2, we injected adeno-associated viruses (AAV) expressing Cre-dependent stGTACR2-FusionRed into the BNST of adult male and female AVP-iCre+ mice and, after 3 wk, performed patch-clamp recordings of BNST FusionRed-tagged AVP cells under blue-light stimulation. We found that continuous (10 and 90 s) blue light reliably inhibited current-injected BNST cells in both males and females (Fig. 2C).

After confirming *ex vivo* efficacy of stGTACR2-mediated inhibition in BNST AVP cells, we bilaterally injected AAVs that Cre-dependently expressed stGTACR2-FusionRed or eYFP as a control in BNST AVP cells and bilaterally implanted optic fibers into the BNST of adult male and female AVP-iCre+ mice. Histological analysis indicated that FusionRed expression was limited to the BNST (Fig. 2B), and males had approximately 40 to 50% more cells labeled than females, reflecting the sex difference in the number of AVP-expressing cells in these mice that we found earlier (27). After 3 wk, subjects were introduced into a three-chamber apparatus containing either a caged male conspecific or caged female conspecific (each paired with an empty cage) and were tested twice in each condition: once with delivery of constant blue light (473 nm, 5 to 6 mW light power) and once with no light delivery (Fig. 2D). Upon light delivery, stGTACR2 males significantly decreased their time spent investigating male stimuli, compared to investigation levels during the light-off condition; investigation of female conspecifics was unaltered (Fig. 2D). In all conditions, investigation of the empty cage was unaltered by light stimulation, indicating no general changes in investigation or activity (*SI Appendix*, Fig. S1). In contrast, levels of social investigation by stGTACR2 females did not differ between light-on and light-off conditions (Fig. 2E). Social investigation by control eYFP males or females also did not differ during light-on and light-off trials. Moreover, inhibition of BNST AVP cells did not affect social communicative behaviors (i.e., urine marking and ultrasonic vocalizations) in males (*SI Appendix*, Fig. S2) or in females, which produced little to no urine marks and vocalizations in any condition.

As changes in social investigation may be explained by changes in anxiety or valence processing, both of which are affected by central AVP (39, 40), we tested whether inhibiting BNST AVP cells altered these states. First, we observed that optogenetic inhibition of BNST AVP cells did not alter time spent in the open, anxiogenic, arms of the elevated zero maze (EZM) (*SI Appendix*, Fig. S3), indicating no obvious change in anxiety-like states.

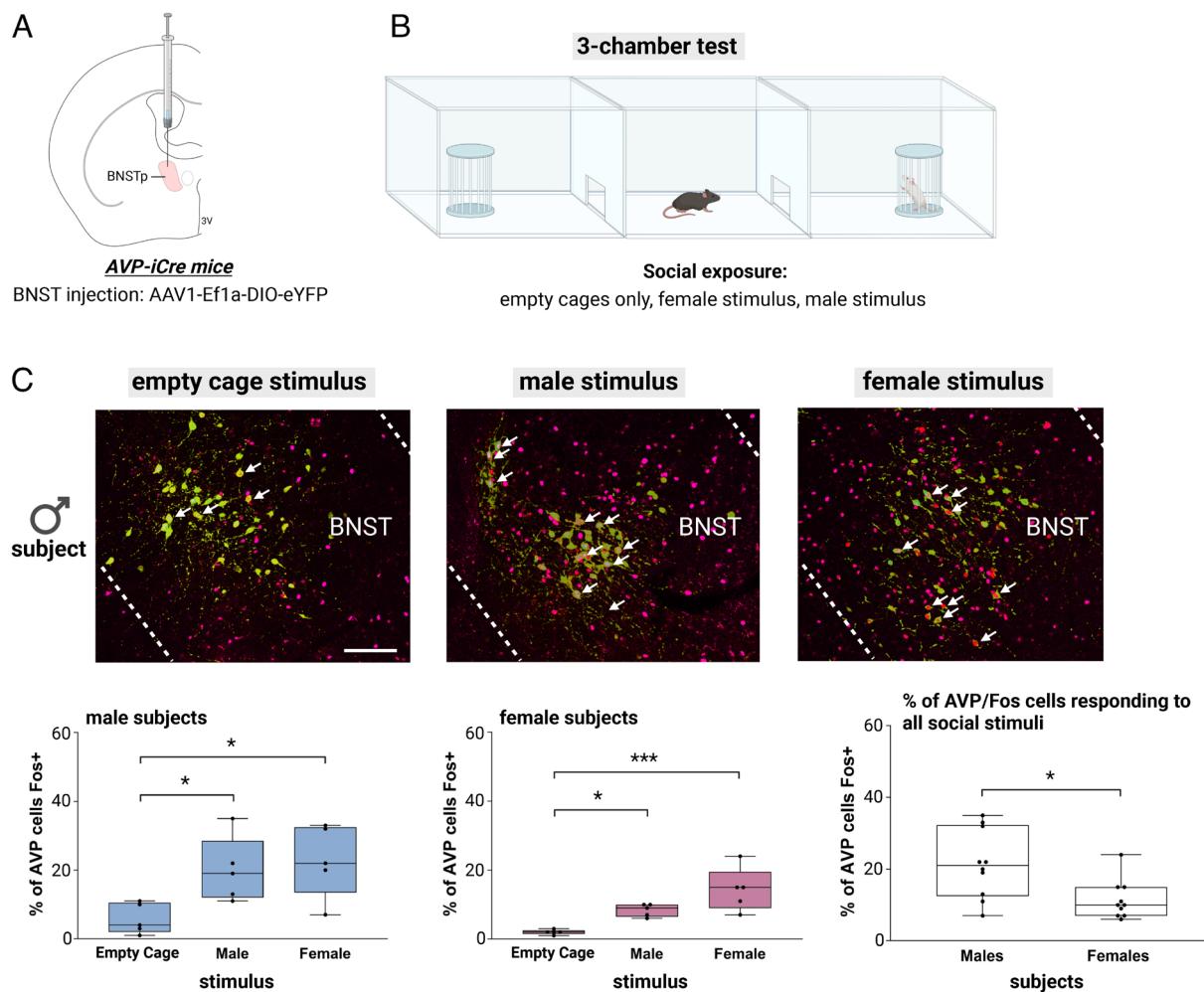


Fig. 1. BNST AVP cell and Fos colocalization during social exposure in the three-chamber test (A) BNST injection site and virus. (B) Three-chamber social testing. (C) (Top) Example images of merged BNST AVP cells (green) and Fos+ cells (magenta). (Bottom) Boxplots of the percentage of AVP cells colocalized with Fos in each three-chamber stimulus condition (empty cage, male, female) for male and female subjects. A two-way ANOVA revealed a significant effect when comparing activated BNST AVP cells with the type of stimulus received [$F(1,24) = 12.89, P = 0.00016, \eta^2 = 0.8$], a significant effect when comparing the sex of the subjects [$F(1,24) = 10.69, P = 0.003, \eta^2 = 0.62$], yet there was no interaction between sex and stimulus type. A post hoc ANOVA followed by Bonferroni-corrected pairwise tests showed that male mice exposed to male or female conspecifics had increased BNST AVP-Fos colocalization compared to males kept in an empty three-chamber apparatus containing empty stimulus cages [$F(1,12) = 5.8, P = 0.017, \eta^2 = 0.4$; male vs. empty cage: $P = 0.05$, female vs. empty cage: $P = 0.025$]. Similarly, female mice also showed Fos activation to social cues in BNST AVP cells [$F(1,12) = 13.23, P = 0.0009, \eta^2 = 0.69$; male vs. empty cage: $P = 0.05$, female vs. empty cage: $P = 0.0007$]. Mean \pm SEM data represented. Dots indicate individual data points. White arrows indicate Fos and AVP cell colocalization, and the dotted lines indicate boundaries of the BNST. (Scale bar: 25 μ m.) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Second, we examined whether inhibiting BNST AVP cells is intrinsically rewarding or aversive using a real-time place preference test (RTTP). During this test stGTACR2 females, but not males, spent more time in the light-paired chamber compared to eYFP control females, suggesting that inhibition of BNST AVP cells was moderately rewarding to females (*SI Appendix*, Fig. S3).

Exciting BNST AVP Cells Increases Social Investigation in Both Sexes As Well As Social Communication in Males. The increased Fos expression in BNST AVP cells in response to social cues found in male and female mice in the present study and in male mice previously (35) suggests that these cells are activated during social encounters. To test whether increasing BNST AVP cell activity boosts social investigation and communication, we first confirmed ex vivo that blue light stimulates AVP BNST cells in male and female AVP-iCre+ mice that Cre-dependently expressed the excitatory opsin ChR2-eYFP (Fig. 3D). Increasing frequency of light stimulation indeed increased spiking activity in BNST AVP cells in both male and female tissue (Fig. 3D). However, stimulation beyond 10 Hz reduced spike fidelity (Fig. 3D). Consequently, we

used 10 Hz stimulation, a frequency known to cause neuropeptide release from terminals (41), for all subsequent *in vivo* procedures.

We then bilaterally injected AAVs into the BNST of male and female AVP-iCre+ mice that Cre-dependently expressed ChR2-eYFP or, as a control, eYFP in BNST AVP cells and bilaterally implanted optic fibers above the injection sites (Fig. 3B). Using RNAscope *in situ* hybridization, we confirmed that ChR2-eYFP was only expressed in BNST AVP cells (*SI Appendix*, Fig. S4). Histological analysis indicated that males had about 50% more eYFP cells labeled than females, again reflecting the sex differences found in BNST AVP expression (22, 27). Subjects were then allowed to investigate caged male or caged female conspecifics (each paired with an empty cage) twice in a three-chamber apparatus: once during delivery of pulsed blue light (20 ms pulse width, 473 nm, 5 to 6 mW light power) and once with no light delivery. Blue-light activation of BNST AVP cells strongly increased the time that ChR2 males spent investigating male and female stimuli compared to their investigation during the light-off condition (Fig. 3E). Surprisingly, activating BNST AVP cells in ChR2 females also increased their investigation time of male, but not female, stimuli (Fig. 3E). We did not notice

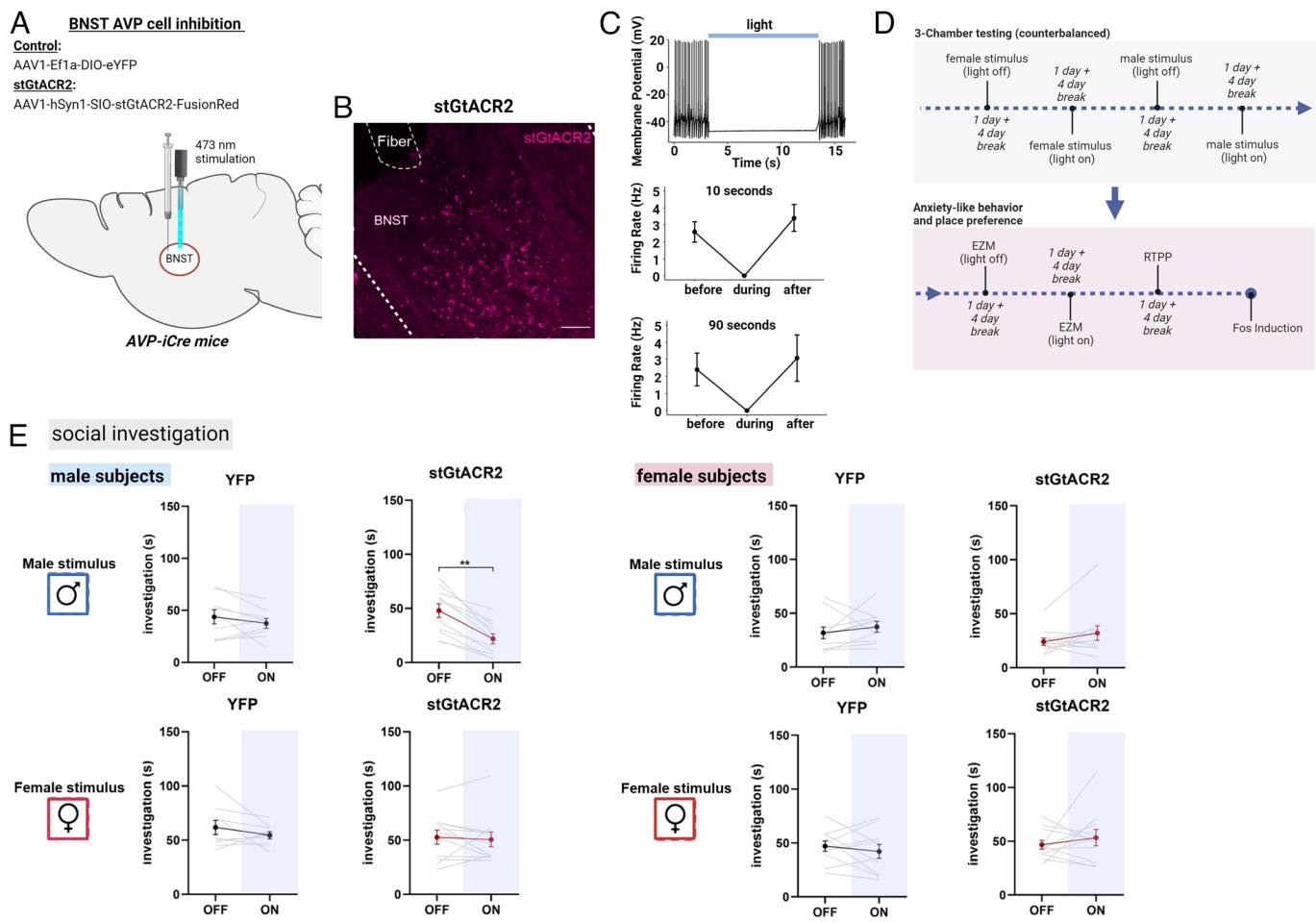


Fig. 2. Optogenetic inhibition of BNST AVP cells decreases male-male social investigation. (A) Bilateral BNST injection and fiber implantation site; coordinates: DV: -4.4, AP: +0.15, ML: ±0.8; modified from ref. 38; dotted lines indicate boundaries of the BNST. (B) Example image of BNST AVP cells infected with inhibitory stGtACR adeno-associated virus (FusionRed). (C) Representative trace from whole-cell current-clamp recording of stGtACR2-expressing BNST cell silenced by light application at 10 Hz for 10 s and firing rate of stGtACR2-expressing BNST cell suppressed by the blue light for 10 s ($n = 8$ cells) and 90 s ($n = 9$ cells). (D) Experimental timeline. All subjects were tested within the three-chamber apparatus on 4 separate days (4-d break in between) with all stimuli and light conditions counterbalanced. The same subjects were further tested within the elevated-zero maze (EZM) (counterbalanced) followed by a real-time place preference test (RTPP) and Fos induction. (E) Social investigation (in seconds) by male and female subjects during the three-chamber test [male subjects: YFP, $n = 9$, and stGtACR2, $n = 11$ female subjects (YFP, $n = 10$, and stGtACR2, $n = 11$)] during light-OFF and light-ON conditions, counterbalanced. Blue light inhibition (ON) of BNST AVP cells in stGtACR2 males significantly decreased time spent investigating male stimuli, but not female stimuli, compared to investigation during light-OFF condition [mixed model ANOVA, treatment*light*stimulus interaction ($F(1,37) = 4.11, P = 0.05, \eta^2 = 0.6$; post hoc: $P = 0.001$)]. Blue light inhibition (ON) of BNST AVP cells in stGtACR2 females did not affect time spent investigating male or female stimuli. Light stimulation did not affect investigation times of YFP male and female subjects to either stimulus type (female or male). Bonferroni post hoc tests were used. Each point and horizontal line represent individual within-subject data. Overlapping data are represented as one point/line. (Scale bar: 50 μm.) ** $P < 0.01$.

changes in locomotion or time spent investigating the clean stimulus cage (*SI Appendix*, Fig. S5). eYFP-expressing control males or females did not change their investigatory behavior across light-on and light-off trials (Fig. 3E). Light stimulation of ChR2-expressing BNST AVP cells in males also increased male urine marking (total area) in the presence of female, but not male, stimuli (Fig. 3F). This increase in marking was unrelated, at an individual level, to increases in social investigation ($r^2 = 0.27, P = 0.15$), suggesting that marking was not directly linked to increased social interaction. In contrast to males, urine marking by females was low and was unaffected by optogenetic activation of BNST AVP cells. Control eYFP males and females did not change their marking behavior across light-on and light-off trials (Fig. 3F). Activation of BNST AVP cells did not influence USV production (*SI Appendix*, Fig. S2). We also did not observe changes in anxiety-like behavior or real-time place preference across light-on/light-off conditions in either ChR2- or eYFP-expressing males and females, suggesting that the stimulation-induced changes in social investigation are not readily explained by changes in these behavioral states (*SI Appendix*, Fig. S6).

Following behavioral testing, a subset ($n = 34$) of subjects were stimulated with blue-light, killed, and processed for Fos/eYFP immunohistochemistry. We confirmed that blue-light stimulation of BNST AVP cells significantly increased Fos expression in these cells compared to eYFP controls (Fig. 3B and C). Moreover, the number of excited BNST AVP cells (Fos/eYFP colocalized) in ChR2 males positively correlated with their time spent investigating female, but not male, stimuli (*SI Appendix*, Fig. S7). One explanation is that increased (artificial) recruitment of BNST AVP cells is needed for driving investigation of females, but that increased male–male investigation is, instead, triggered by a relatively low threshold of active cells.

Activating AVP-BNST Outputs to Lateral Septum Increases Social Investigation and Anxiety-Like Behavior in Males, but Not Females. While more indirect approaches have supported the BNST as the major source of AVP innervation of the LS in rodents and birds (22), we recently confirmed that mouse BNST AVP cells send some of its strongest projections to the LS (30).

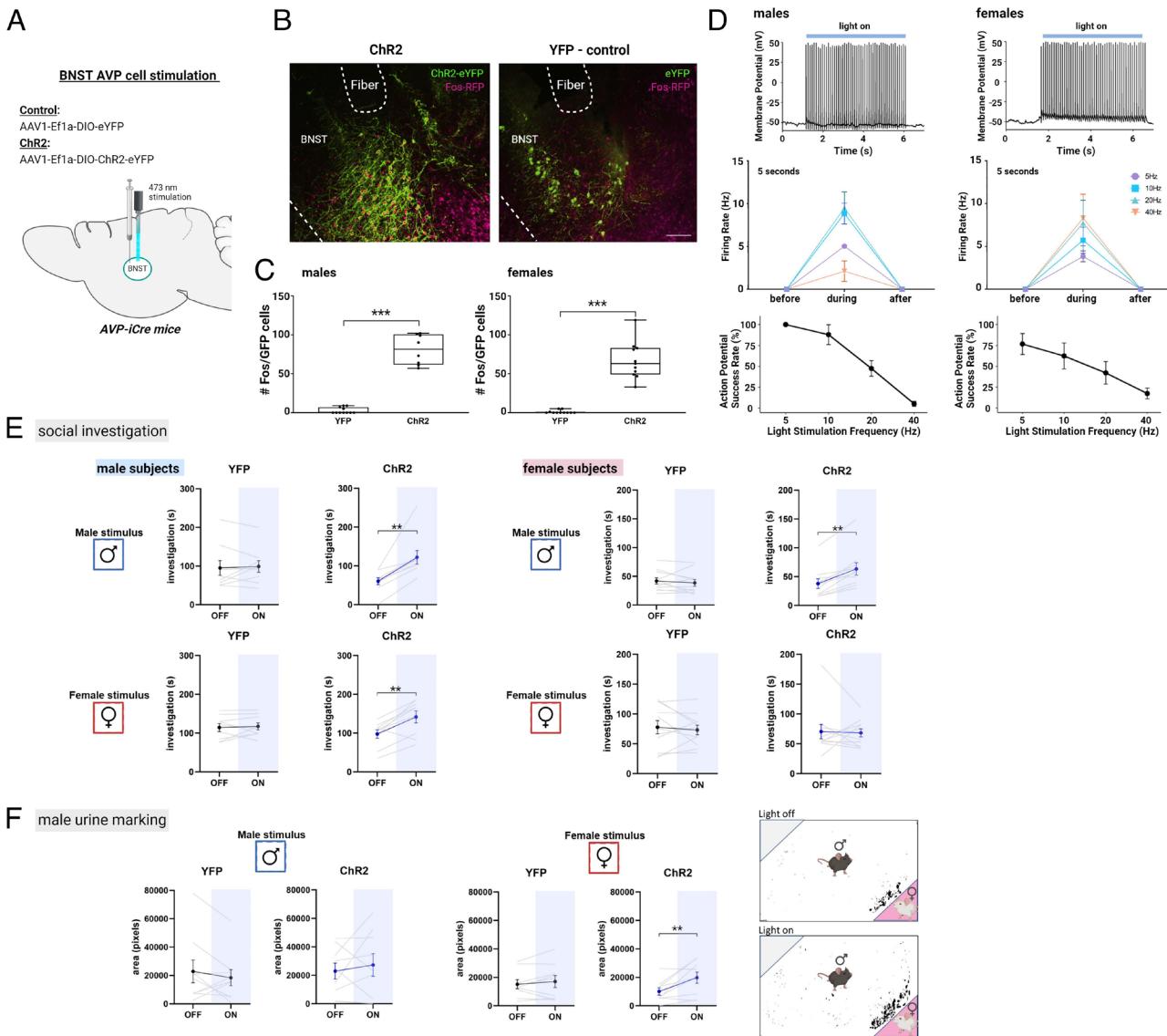


Fig. 3. Optogenetic activation of BNST AVP cells increases social investigation in both sexes and male urine marking toward female stimuli. (A and B) Bilateral BNST injection and fiber implantation site; coordinates: DV: -4.4, AP: +0.15, ML: ±0.8; modified from Paxinos and Franklin (2012). (B) Example images of merged BNST AVP cells infected with either the excitatory ChR2 adeno-associated virus (ChR2) or YFP control virus, both colocalized with Fos+ cells (magenta); dotted lines indicate boundaries of the BNST. (C) Boxplots of the number of BNST AVP ChR2/YFP cells colocalized with Fos. Blue light stimulation robustly increased the number of BNST AVP labeled cells colocalized with Fos [F (1,35) = 234.17, $P < 0.000001$; $\eta^2 = 0.87$]. (D) Representative trace from whole-cell current-clamp recording of ChR2-mCherry-expressing cells activated by light application at 10 Hz for 5 s and the response to 10 ms pulse delivered at 5, 10, 20, 40 Hz for 5 s, with the probability of action potential (AP) in response to an individual pulse [action potential success rate in male and female subjects (E) Social investigation (in seconds) by male and female subjects during the three-chamber test] [male subjects: YFP, n = 9, and ChR2, n = 9; female subjects (YFP, n = 10, and ChR2, n = 11)] during light-OFF and light-ON conditions, counterbalanced. Blue light stimulation (ON) of BNST AVP cells in ChR2 males significantly increased time spent investigating male and female stimuli compared to investigation during light-OFF condition [mixed model ANOVA, treatment*light*sex interaction, F (1,36) = 7.02, $P = 0.012$; $\eta^2 = 0.5$; post hoc: $P = 0.002$ (male stimuli), $P = 0.004$ (female stimuli)]. Blue light stimulation (ON) of BNST AVP cells in ChR2 females significantly increased time spent investigating male stimuli compared to investigation during light-OFF condition [post hoc: $P = 0.002$ (male stimuli)]. Light stimulation did not affect investigation times of YFP male and female subjects to either stimulus type (female or male). (F) Total area of urine marking by male subjects during the three-chamber test. Blue light stimulation (ON) of BNST AVP cells in ChR2 males significantly increased urine marking in the presence of a female stimulus [mixed model ANOVA, treatment*light*sex interaction, (F 1,36) = 4.5, $P = 0.04$; $\eta^2 = 0.4$; post hoc: $P = 0.009$]. Bonferroni post hoc tests were used. Each point and horizontal line represent individual within-subject data. Overlapping data are represented as one point/line. ** $P < 0.01$.

Here, we tested whether these projections are sufficient to influence social behavior. First, to establish that exciting these projections indeed affects LS neuronal activity, we injected AAVs expressing Cre-dependent ChR2-mCherry into the BNST of adult male AVP-iCre+ mice and conducted patch-clamp recordings of LS cells adjacent to mCherry fibers under blue-light stimulation. We found that light stimulation of BNST AVP fibers within the LS led to a biphasic effect on all recorded AVP-fiber adjacent LS cells, involving an initial (early) excitation, followed by a more pronounced (late) inhibition (Fig. 4 A–C). These responses were observed in all sampled neurons. The initial excitation peaked

at 1.35 ± 0.31 min (mean/SEM) from the start of stimulation, whereas the inhibitory response started later at 5.29 ± 0.59 min (mean/SEM) and lasted >5 min. This biphasic response is broadly similar to that seen after exogenous application of AVP to LS ex vivo (42). As optogenetic stimulation of BNST AVP → LS terminals likely releases neurotransmitters other than AVP, which may drive the complex pattern of response, we tested BNST AVP → LS stimulation in the presence or absence of a highly selective V1aR antagonist. Intriguingly, we found that both excitatory and inhibitory responses in LS neurons induced by BNST AVP terminal stimulation were eliminated by the V1aR antagonist (Fig. 4C).

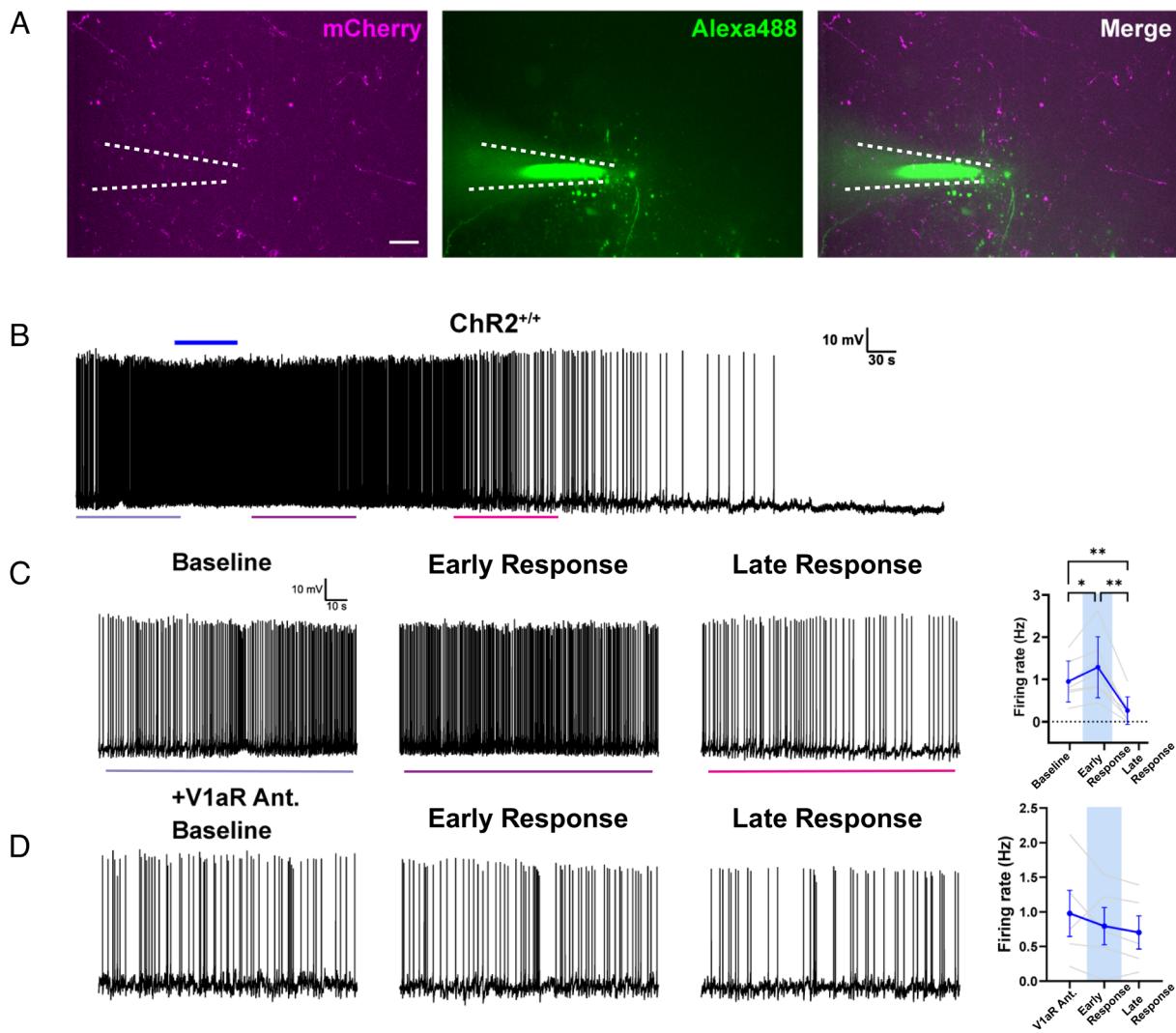


Fig. 4. Optogenetic activation of BNST AVP cell projections to the lateral septum (LS) biphasically modulates neurons in LS. (A) Visualization of mCherry reporter tagged to ChR2-AVP indicating BNST AVP → LS fibers in the LS (*Left*) and a patched neuron dialyzed with Alexa 488 dye (*Middle*), with a merged image on the right. (B) Representative example of electrophysiological activity of a patched LS neuron showing continuous ongoing firing activity before and after laser stimulation (blue line). (C) Firing segments for baseline, early response, and late response time points [indicated by the color lines under the trace in (B)], are shown at an expanded time scale to better display the changes in firing evoked following ChR2 stimulation. Note that BNST AVP → LS terminal activation biphasically modulates LS neuron firing activity. (Baseline vs. Early Response $P < 0.05$, Baseline vs. Late Response $P < 0.01$, Early Response vs. Late Response $P < 0.01$, one-way repeated measures ANOVA). (D) Bath application of V1aR antagonist (d(CH2)₅[Tyr(Me)₂, Dab5] AVP) blocks biphasic ChR2 modulation of LS neurons. ($P > 0.05$, one-way repeated measures ANOVA). (Scale bar: 25 μ m.)

Overall, these results suggest that optogenetic stimulation of BNST AVP → LS terminals increases overall inhibition within the LS, an effect mediated by AVP acting on V1aRs.

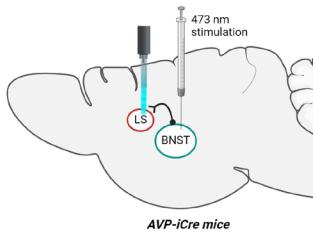
Next, we examined the behavioral effects of stimulating BNST AVP afferents to the LS in male and female mice. We bilaterally injected AAVs expressing Cre-dependent ChR2-eYFP or eYFP as a control into the BNST of adult male and female AVP-iCre+ mice and implanted fibers in the LS (Fig. 5*A*). Blue light stimulation of BNST AVP → LS terminals in ChR2 males significantly increased the time they spent investigating both male and female stimuli, compared to the time during the light-off conditions; light stimulation did not affect the time males spent investigating clean cages (Fig. 5*B* and *SI Appendix*, Fig. S8). Blue light stimulation of BNST AVP → LS terminals did not affect social investigation in ChR2 females or eYFP subjects (Fig. 5*B*). Male investigation of male and female stimuli showed a significant increase only after 5 to 6 min following onset of BNST AVP → LS terminal stimulation (*SI Appendix*, Fig. S9). This corresponds to the time it took before photostimulation significantly inhibited recorded LS cells ex vivo. The total number of visits to the stimuli

did not differ between groups, indicating that the photostimulated increases in social investigation were not driven by an increase in general activity (*SI Appendix*, Fig. S9). Similar to the effects of stimulating BNST AVP cells, blue light stimulation BNST AVP → LS terminals increased male urine marking. Unlike the prior experiment, BNST AVP → LS stimulation increased the number of marks, not the deposition area, in the presence of a female (*SI Appendix*, Fig. S10). However, as with BNST AVP stimulation, changes in scent marking levels were also not correlated with levels of social investigation ($r^2 = 0.01$, $P = 0.89$), again suggesting that urine marking is not tightly linked to increased social investigation. As seen in prior experiments (26, 27, 43), urine marking and USV production by female mice was low and unaffected by optogenetic stimulation. In contrast to the lack of effect of BNST AVP cell stimulation (or inhibition) on anxiety-like behavior, stimulation of BNST AVP terminals in LS in ChR2 males significantly decreased time spent in the open arm of the EZM, indicating a possible anxiogenic effect (Fig. 5*C*). However, increased anxiety-like behavior in subjects did not correlate with their increased social investigation (*SI Appendix*, Fig. S7). In females or

A**BNST AVP cell → LS stimulation**

Control:
AAV1-Ef1a-DIO-eYFP

ChR2:
AAV1-Ef1a-DIO-ChR2-eYFP



Lateral Septum ChR2 fibers

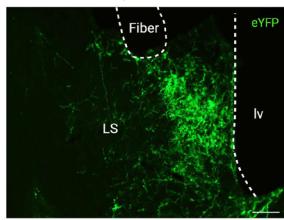
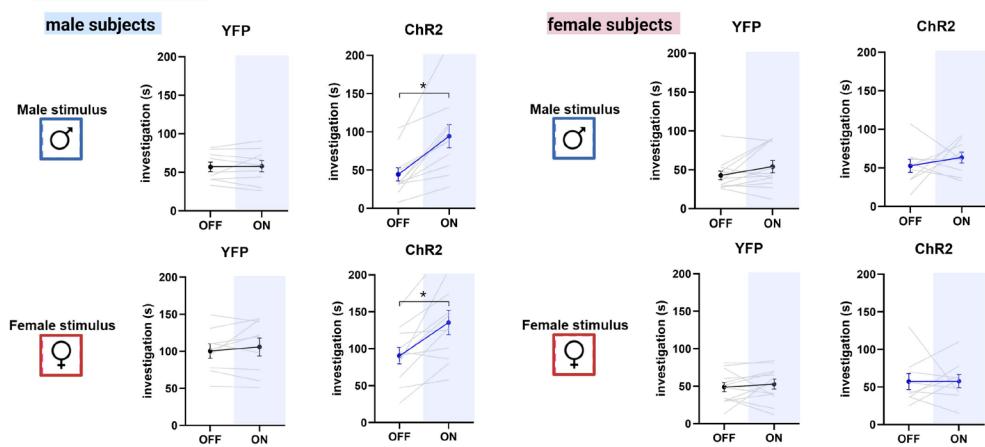
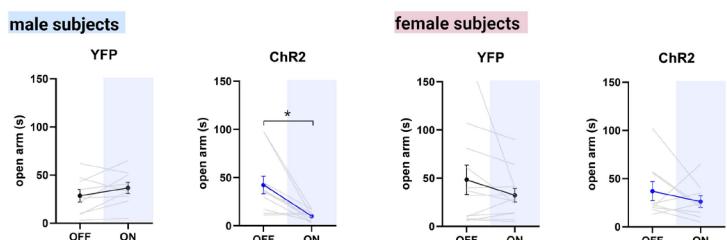
**B social investigation****C elevated-zero maze**

Fig. 5. Optogenetic activation of BNST AVP cell projections to the lateral septum (LS) increases social investigation and anxiety-like behavior in males, but not females. (A) Bilateral BNST injection of ChR2 adeno-associated virus (ChR2) and fiber implantation within the lateral septum (intermediate zone). (B) Social investigation (in seconds) by male and female subjects during the three-chamber test (male subjects: YFP, $n = 9$, and ChR2, $n = 11$; female subjects: YFP, $n = 12$, and ChR2, $n = 9$) during light-OFF and light-ON conditions, counterbalanced. Blue light stimulation (ON) of BNST AVP → LS terminals in ChR2 males significantly increased time spent investigating male and female stimuli compared to investigation during light-OFF condition [mixed model ANOVA, treatment*light interaction, $(F(1,17) = 6.9, P = 0.01; \eta^2 = 0.6$; post hoc: $P = 0.001$ (male stimuli), $P = 0.02$ (female stimuli)]. Blue light stimulation (ON) of BNST AVP → LS terminals in ChR2 females did not affect investigation times compared to investigation during light-OFF condition. Light stimulation did not affect investigation times of YFP male and female subjects to either stimulus type (female or male). (C) Time spent in the open arm of the elevated-zero maze (EZM). Blue light stimulation (ON) of BNST AVP → LS terminals in ChR2 males significantly decreased time spent in the open arm of the EZM. In females, blue light stimulation had no effect on time spent in the open arm of the EZM [mixed model ANOVA, treatment*light*sex interaction, $(F(1,17) = 6.2, P = 0.024; \eta^2 = 0.5$; post hoc: $P = 0.008$]. Bonferroni post hoc tests were used. Each point and horizontal line represent individual within-subject data. Overlapping data are represented as one point/line. * $P < 0.05$.

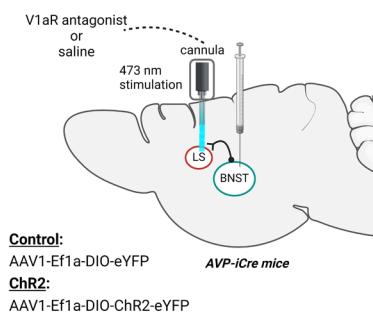
eYFP controls, blue light stimulation had no effect on time spent in the open arm of the EZM (Fig. 5C). Light stimulation was neither aversive nor rewarding in any group in the RTTP (*SI Appendix*, Fig. S6).

It is possible that BNST AVP → LS photostimulation altered behavior by acting directly on BNST AVP cell bodies, either by photic stimulation penetrating to BNST AVP cell bodies or via antidromic stimulation. However, we believe this is unlikely for two reasons. First, there are differences in behavioral effects between terminal and cell body stimulation: Female investigation was partially increased by cell body stimulation, but not by terminal stimulation whereas male anxiety-like behavior was increased during terminal stimulation, but not during cell body photostimulation. Second, we analyzed BNST AVP/Fos colocalization following BNST AVP → LS stimulation but observed no differences between stimulated and nonstimulated groups (*SI Appendix*, Fig. S11), indicating that, in contrast to photic stimulation of BNST cells, photostimulation of LS terminals was insufficient to trigger Fos in BNST cell bodies. Consequently, our stimulation effects were likely specific to activating BNST AVP → LS terminals.

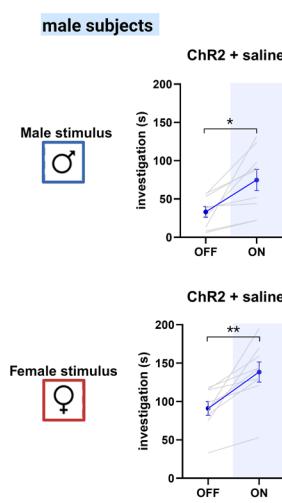
Increases in Social Investigation and Anxiety-Like Behavior Caused by BNST AVP LS Stimulation in Males Depend on the Vasopressin 1a Receptor. Although blocking V1aR action in LS reduces the impact of BNST AVP stimulation *ex vivo*, the behavioral effects of BNST AVP → LS stimulation may be due to

the release of other neuroactive substances coexpressed in BNST AVP cells. Consequently, we tested whether V1aR action was required for BNST AVP → LS optogenetic-mediated increases in male social investigation and anxiety-like behavior. Following Cre-dependent viral delivery of ChR2-eYFP to BNST AVP cells of male mice, cannulas with interchangeable fibers and fluid injectors were implanted in the LS to allow for dual delivery of V1aR antagonist (or saline control) and light stimulation in LS (Fig. 6A). Two groups of ChR2 males were assigned to interact with either caged novel male or female conspecifics in the three-chamber apparatus. We tested subjects with their respective stimuli in four separate counterbalanced tests: light-off + saline infusion, light-on + saline infusion, light-off + V1aR antagonist infusion, light-on + V1aR antagonist infusion. Blue light stimulation of AVP BNST → LS terminals in both groups following saline injection significantly increased their time spent investigating male and female stimuli compared to their investigation during the light-off condition, replicating the behavioral effect of BNST AVP → LS stimulation (Fig. 6B). In a test of nonsocial novel object investigation, a separate set of ChR2 male subjects ($n = 9$) did not alter their investigation of nonsocial stimuli (toys) during BNST AVP → LS stimulation (*SI Appendix*, Fig. S12), indicating that optogenetic-mediated increases in male social investigation were limited to social targets rather than reflecting nonspecific increases in investigatory behavior. Importantly, during pharmacological blockade of LS V1aR, BNST AVP → LS stimulation-mediated increases in social investigation of both male and female stimuli

A

LS terminal stimulation + V1aR blockade

B social investigation



Male stimulus

Female stimulus

C elevated-zero maze

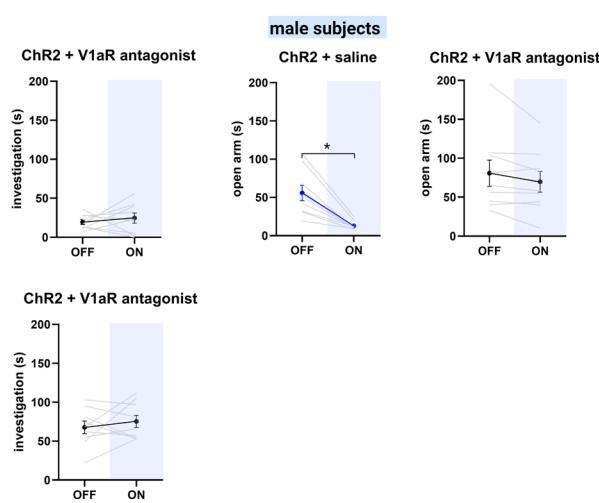


Fig. 6. Antagonism of V1aR in the LS blocked optogenetic-mediated increases in male social investigation and anxiety-like behavior. (A) Bilateral BNST injection of ChR2 adeno-associated virus (ChR2) and fiber implantation within the lateral septum (intermediate zone). (B) Social investigation (in seconds) by male subjects during the three-chamber test. Two groups of ChR2+ injected males were tested with either a male or female stimulus (LIGHT OFF/ON) and received the same type of stimulus (i.e., novel male or female) with LIGHT OFF/ON + a highly selective V1aR antagonist (subjects tested with a male stimulus: n = 9; subjects tested with a female stimulus: n = 9). All conditions were counterbalanced. Blue light stimulation (ON) of BNST AVP → LS terminals in both ChR2 + saline male groups significantly increased time spent investigating male and female stimuli compared to investigation during light-OFF condition [mixed model ANOVA, drug*light interaction, ($F(1,16) = 11.76, P = 0.003; \eta^2 = 0.42$; post hoc: $P = 0.01$ (male stimuli), $P = 0.006$ (female stimuli)]. In the same male subjects, antagonism of V1aR in the LS blocked optogenetic-mediated increases in male social investigation. (C) Time spent in the open arm of the elevated-zero maze (EZM). Blue light stimulation (ON) of BNST AVP → LS terminals in ChR2 males significantly decreased time spent in the open arm of the EZM, and in the same males, antagonism of V1aR in the LS blocked optogenetic-mediated increases in male anxiety-like behavior [mixed model ANOVA, treatment*light interaction, ($F(1,8) = 10.58, P = 0.012; \eta^2 = 0.5$)]. Bonferroni post hoc tests were used. * $P < 0.05$, ** $P < 0.01$.

were eliminated (Fig. 6B). We did note that V1aR antagonism by itself, during light-off conditions, reduced males' investigation of both male and female stimuli compared to the saline control condition (SI Appendix, Fig. S13), further supporting the role of V1aR LS cells in social investigation. We were, however, unable to replicate the effect of BNST AVP → LS stimulation on male urine marking nor did we see any effect of our manipulation on USV production (SI Appendix, Fig. S8). LS V1aR antagonism by itself did not alter male communicative behavior.

As was previously observed, BNST AVP → LS stimulation significantly decreased time spent in the open arm of the EZM, indicating this stimulation increased anxiety-like behavior (Fig. 6C) but did not correlate with increased social investigation (SI Appendix, Fig. S6), indicating differences in mechanism. Surprisingly, given the role of the AVP-V1aR system in rodent models of anxiety (7), blocking V1aR in the LS during the light-off condition did not alter anxiety-like behavior (Fig. 6C). However, blocking LS V1aR during BNST AVP → LS stimulation prevented stimulus-induced increases in anxiety-like behavior, suggesting that this effect is mediated by V1aR.

Discussion

Previous experiments had indicated that lesioning AVP cells of the BNST or inhibiting their AVP synthesis affected social investigation in male, but not female, mice. Unresolved was whether real-time excitation or inhibition of these cells affects behavior in both sexes. Here, we show that engaging in social investigation drives Fos expression in BNST AVP cells in males as well as females. Acute optogenetic excitation and inhibition of BNST AVP cells indicate that these cells play a role in social behavior in both sexes, but much more prominently so in males. In males, optogenetic inhibition of these cells reduced social investigation of other males but not of females, while stimulation increased social investigation of males and females as well as male communication toward females. In

females, optogenetic inhibition did not affect social investigation, and stimulation only increased female investigation of males. In a follow-up experiment, we found that optogenetic stimulation of BNST AVP cell axon terminals in the lateral septum (LS) in males increased their social investigation and anxiety-like behavior in the elevated-zero maze, but had no effect in females. These effects are likely mediated by V1aR since V1aR antagonism in the LS blocked the increases in male social investigation and anxiety-like behavior as well as optogenetically induced changes in LS cell activity. Our findings indicate that the sexually differentiated AVP cells in the BNST contribute to sex-specific aspects of social approach and anxiety-like behavior through their connections with the LS, via V1aR receptor-mediated mechanisms.

One possible limitation of this study is that potentially lower levels of Cre expression per cell in females in the present study might have reduced the success rate of opsin expression per cell, which might have lowered the percentage of AVP cells in males and females that can be manipulated optogenetically. This does not appear to be the case as we find that the ratio of cells expressing opsins in males and females is similar to the normal sex-biased ratio of AVP-expressing cells in BNST of these mice in an earlier study (~50% more AVP cells in males than in females) (27). In addition, the level of opsin expression per cell likely does not appear to differ significantly in males and females; there are no differences in ex vivo (physiological responses to optogenetic stimulation/inhibition) and in vivo (Fos expression after ChR2-based excitation) cellular responses in males and females. In addition, in previous studies, we were equally able to delete AVP cells in male and female mice with Cre-dependent activated caspase (27), indicating that Cre-dependent viral vectors in our mice are equally effective in inducing gene expression in males and females. This suggests that our ability to excite or inhibit individual BNST AVP cells in males and in females does not differ and that, therefore, differences in the effect of such manipulation on behavior probably reflect differences in the number and behavioral impact of these cells.

Another limitation of our study is that optogenetic inhibition or excitation of BNST AVP cell bodies likely affects the release of transmitters other than AVP, such as galanin (44), which has also been implicated in control of murine social behavior (45). Previously, however, we have shown that blocking AVP synthesis in BNST cells reduces the same behaviors that are blocked by optogenetic inhibition in this study (26), supporting the idea that changes in AVP release play an important role in the changes observed in this study. An even stronger argument that changes in AVP release underlie these effects is that the effects of stimulating BNST → LS terminals on anxiety-like behavior could be blocked by AVP antagonists in this study.

The increase in Fos expression in BNST AVP cells suggests that even brief social contact with either sex can increase activity of these cells in males as well as in females. Our findings are broadly consistent with earlier studies demonstrating increased Fos expression over baseline in BNST AVP cells of male mice and finches following agonistic or sexual interactions (24, 35, 46, 47). Overall, we observed ~20% AVP/Fos colocalization in the BNST in males following social investigation of caged conspecifics, a percentage that is not out of line with that observed in BNST oxytocin cells following aggressive behavior in deer mice [~30%; (48)] but lower than that observed following copulatory behavior in male mice [~60%; (35)]. In the latter study, AVP/Fos colocalization might have been higher than it was in our study because, in addition to social investigation, subjects were in direct contact with conspecifics and engaging in reproductive behavior. It is unclear whether the greater stimulus-related increase in Fos expression in males than in females observed in the present study is simply related to differences in intrinsic properties of these cells, e.g., the types of receptors and intracellular transduction pathways employed, the types of immediate-early genes activated, or in the nature of their inputs. The latter is a distinct possibility, because previously we showed several sex differences in input. For example, male BNST AVP cells receive more input from regions such as the medial amygdala, and females receive more input from other brain areas, such as the medial preoptic area (30). Our Fos data suggest social stimuli activate BNST AVP cells more so in males than in females. But even if male and female cells were activated to the same extent, the impact of this activation on the control of social behavior is still likely to differ, as the outputs of these cells differ as well (30). This is indeed what the results of our optogenetic experiments suggest.

Our finding that optogenetic inhibition of BNST AVP cell activity decreased male, but not female, social investigation and specifically toward males aligns very well with results of earlier studies where lesions of these cells or reduction of their AVP synthesis produced almost identical effects (26, 27). Similar reductions of male-typical social proximity have been observed after AVP knockdown in the BNST of several finch species (49–51). Combined, these effects indicate the necessity of these cells for normative social investigation in males. In our study, excitation of these cells, however, affected social investigation in both sexes, although not necessarily in the same way. In males, it increased investigation of males as well as females, indicating that artificial stimulation of these cells can also drive male social investigation in females as well eliciting increased communicative behavior (urine marking) toward them. As the endogenous activity patterns of BNST AVP cells during social behavior are unknown, it is possible that our stimulation parameters drove these cells abnormally, overriding normal patterns of activity and inhibitory control, and thereby drove increases in investigation of females. In females, stimulation effects were limited to an increase in social investigation of males; there was no increase in social investigation

of other females. As females expressed less Fos in BNST AVP cells during social investigation, their cells may normally be less active during social behavior (30). However, it is not clear why BNST AVP cell stimulation should increase female investigation of males, but not females, given that both stimuli are investigated at similar levels at baseline. It may be that in females, unlike in males, AVP acts on downstream structures, such as the LS, to suppress female–female interactions (52).

Overall, these manipulations suggest a much more central role for BNST AVP cells in social investigative behavior and certain communicative behaviors in males than in females. In addition to the differences in inputs mentioned above, the output of these cells in mice show important sex differences as well (30) and track the differences in density of vasopressin fibers in brain areas, such as the LS, that receive steroid-sensitive AVP innervation in rats and mice (higher in males than in females) (21, 53). We opted for testing the effects of local excitation of terminals in this set of projections, because previous research in rodent and bird species indicates an important role of AVP within the LS (7, 10, 31). The increase in social investigation that we observed after optogenetic activation of BNST AVP → LS terminals indicates the potential and sufficiency of this pathway to drive social investigation in males. This finding is consistent with AVP's facilitatory action within the LS on social recognition in rats (6), pair-bonding and parental care in prairie voles (54, 55), aggression in mice and rats (52, 56, 57), social interactions in rats (58), and hamster social communication (59). Indeed, we observed a strong overlap in the effects of excitation of BNST AVP cell bodies and BNST AVP projection to the LS on male social investigation and urine marking, indicating that the LS is the major output structure regulating BNST AVP action on these behaviors.

The stimulatory effects of optogenetic excitation of BNST AVP → LS terminals on investigation are probably mediated by AVP acting on V1aR-expressing cells in the LS, as these effects could be blocked by V1aR antagonists. Stimulatory effects on urine marking, however, could not be blocked by V1aR antagonists, suggesting that AVP may exert its effects on urine marking via oxytocin receptors or V1b receptors, both of which are present in the LS of mice (56, 60) and both of which can mediate central actions of AVP (56, 61). Not all effects of BNST AVP projections on social behavior appear to be mediated by the LS. For example, in females, excitation of cell bodies stimulated social investigation, but excitation of BNST AVP → LS did not. It may be that BNST AVP projections to other areas other than the LS may play a more important role in the control of social behavior in female mice.

The fact that inhibition of AVP BNST cells affected social investigation in males only, whereas excitation affected it in both sexes, suggests that AVP BNST cells may chronically maintain a behavioral state more conducive for social investigation in male, but not in female, mice, perhaps by controlling anxiety or arousal states. In fact, central AVP has been consistently implicated in the generation of anxiety-like states in laboratory rodents (31, 32), and AVP in the LS controls anxious states by acting on V1a receptors (62–65) and more prominently in male than in female lab rodents (39, 66). We were, therefore, surprised that we did not observe changes in anxiety-like behavior during BNST AVP cell inhibition or stimulation. However, when we stimulated BNST → LS AVP terminals, we did find an increase in anxiety-like behavior within the EZM that could be blocked with a V1aR antagonist, but only in males, consistent with prior research. If there is a link between the effects of manipulating BNST → LS AVP projections on anxiety and social behavior, this link is tenuous as we did not find a correlation between anxiety-like behavior and social investigation in individual mice.

While it is clear that the BNST AVP system regulates male-typical social investigation in mice, especially toward other males, the exact motivational state induced by this system's activity is not clear. The discrepancy of effects of optogenetic manipulation of BNST AVP cells on social investigation (which was increased or reduced after optogenetic stimulation or inhibition, respectively) and anxiety-like behaviors, which were not affected, suggest that changes in investigation were not based on general changes in anxiety. However, it is possible that this system normally dampens social anxiety-like states during stressful social encounters, as may occur when two males interact, which then may influence the intensity and shape of social behaviors. This idea is supported by observations that inhibiting the BNST AVP system preferentially reduces male investigation of other males in mice, while leaving investigation of females, presumably a nonstressful event intact (26, 27). Indeed, central AVP has been shown to promote active coping in stressful situations (67). Another possibility is that the BNST AVP system functions in males to support their evaluation of potential competitors during acute interactions (68) or to facilitate formation of social memories, which appears to require this circuit (69, 70).

Our ex vivo physiological recordings support the notion that BNST → LS AVP projections act on LS target cells via V1aR. Ex vivo stimulation of these projections initially increased the firing frequency of cells adjacent to BNST AVP fibers and then depressed them, changes that were not seen when the V1aR antagonist was present. These observations align with the reported bimodal effects of exogenous AVP on LS neurons in rats, which caused an initial excitation in a subset of neurons, followed by a more generalized depression of most LS neurons (71, 72). Although we did not measure activity of all LS cells, one mechanistic interpretation for our observation of the V1aR-mediated biphasic response is that the initial excitatory effect works as a “switch” to engage a downstream inhibitory network that feedbacks onto the same set of activated neurons, resulting in a prolonged inhibition. Notably, we observed that the increase in male social investigation during BNST AVP → LS photostimulation occurred with a delay, coinciding with the timeframe of ex vivo LS cell inhibition during photostimulation. This suggests that inhibition of at least a subset of LS cells may be the mechanism whereby AVP facilitates high levels of social investigation in males. It is unknown whether inhibiting these AVP-responsive cells alters social investigation through action on other local LS circuits, such as oxytocin receptor-expressing cells (52), or via direct disinhibitory action on LS output structures such as the hypothalamus (73). Identifying the inputs and targets of these AVP-responsive neurons may provide insight into how BNST AVP → LS projections control social behavior and anxiety-like states.

Overall, our results suggest a sexually differentiated role for BNST AVP neuronal circuitry, with these neurons primarily driving male-specific social investigation and anxiety-like behaviors through one of its major outputs, the LS. Importantly, the role of this system also appears to be sex-specific with respect to the targets of social investigation, in that it primarily regulates male-male social investigation, adding to our increasing awareness of how the lateral septum regulates social and emotional behavior (74, 75). Several other sexually differentiated neurochemical systems have been shown to play different roles in social behavior. For example, female-biased tyrosine hydroxylase neurons in the anteroventral periventricular nucleus (AVPV) drive maternal behavior in female mice but inhibit aggressive behavior in males (76); male-biased BNST aromatase cells control social behavior in male, but not female, mice (77); and female-biased cholecystokinin receptor-expressing cells in the VMHvl are critical for

female, but not male mouse sexual behavior (78). In many of these cases, sexually differentiated systems drive behaviors in one, but not the other sex, even if these behaviors do not necessarily differ, such as anxiety-like behaviors in the current and other studies (79). In other words, the neurochemistry underlying these behaviors differs by sex. Understanding the function of the large and phylogenetically conserved sex differences in BNST AVP cell number and in the density of their projections to key social decision-making network structures (80) facilitates a broader understanding of how and why sex differences in neuronal structure exist and influence social and emotional behavior.

Materials and Methods

Refer to *SI Appendix* for surgical procedures, behavioral procedures, immunohistochemistry, RNAscope assay, in vitro electrophysiology, and statistical analysis.

Animals and Husbandry. All mice were maintained at 22 °C on a 12/12 h reversed light/dark cycle with food and water available ad libitum, housed in individually ventilated cages (Animal Care Systems), and provided with corn cob bedding, a nestlet square, and a housing tube. All animal procedures were performed in accordance with Georgia State University Institutional Animal Care and Use Committee regulations and the NIH Guide for the Care and Use of Laboratory Animals.

Subject Animals. Founding AVP-iCre mice were obtained from Dr. Michihiro Mieda (Kanazawa University, Japan). These mice were generated using a bacterial artificial chromosome that expressed codon-improved Cre recombinase under the transcriptional control of the AVP promoter (AVP-iCre mice). In these animals, iCre expression is found in the bed nucleus of the stria terminalis (BNST) and the medial amygdala (MeA), as well as in hypothalamic areas (81). Subjects were derived by crossing heterozygous iCre mutants to wild-type C57Bl/6 J mice and genotyped (ear punch) by PCR at 21 to 24 d of age (Transnetyx). A total of 176 adult iCre+ mice (2 to 4 mo old) were used for in vivo behavioral experiments (91 males, 79 females): Fos mapping: n = 15 males, n = 15 females; RNAscope to test virus specificity: n = 3 males, n = 3 females, BNST AVP cell stimulation: n = 18 males, n = 22 females; BNST AVP cell inhibition: n = 20 males, n = 21 females; BNST AVP → LS stimulation: n = 20 males, n = 21 females; BNST AVP → LS stimulation with V1aR antagonist: n = 18 males. AVP-iCre+ mice [BNST AVP cell stimulation: n = 8 cells (males), n = 8 cells (females); BNST AVP cell inhibition: n = 16 cells (males and females); BNST AVP → LS stimulation: n = 7 cells (males); BNST AVP → LS stimulation with V1aR antagonist: n = 5 cells (males)] were used for ex vivo electrophysiological recordings. All subject mice were socially experienced (see below) and singly housed for a minimum of 1 wk prior to experimental use.

Stimulus Animals. CD1 (ICR; Charles River Laboratories) mice were used as stimuli for behavioral testing and to provide male and female subjects with social experience because strain differences between subjects and stimulus mice increase social investigation. Mice were used at 9 to 16 wk of age and were novel to the subject to which they were exposed. For detailed methods, refer to *SI Appendix*.

Viral Vectors.

Fos and AVP/ChR2 mapping. AVP driven-, Cre-expressing BNST neurons were induced to express eYFP (AAV-EF1a-DIO-eYFP; serotype 5; Karl Deisseroth; Addgene_27056) for visualization of BNST AVP neurons.

Cell inhibition. AVP driven-, Cre-expressing BNST neurons were induced to express the blue-light activated inhibitory opsin, *guillardia theta* anion-conducting channelrhodopsin (stGtACR; hSyn1-S10-stGtACR2-FusionRed; serotype 1; RRID: Addgene_105677) or eYFP alone as a control (AAV-EF1a-DIO-eYFP; serotype 5; Karl Deisseroth; RRID: Addgene_27056).

Cell and terminal excitation. AVP driven-, Cre-expressing BNST neurons were induced to express the blue-light activated excitatory opsin, channelrhodopsin-2 (ChR2) fused to eYFP (in vivo) or mCherry (ex vivo): AAV-EF1a-double floxed-hChR2; serotype 5; eYFP: RRID: Addgene_20298, mCherry: RRID: Addgene_20297 or eYFP alone as a control (AAV-EF1a-DIO-eYFP; serotype 5; Karl Deisseroth; RRID: Addgene_27056).

Social Experience. As opposite-sex sexual experience and attaining competitive status (“social dominance”) promote male and female social and communicative behaviors (82), subject mice received adult social experience. This experience consisted of two of the following sequences: an opposite-sex encounter (sexual experience) followed by a same-sex encounter (aggressive experience) the following day with at least 1 d between social experiences. For detailed methods, refer to *SI Appendix*.

Experimental Procedures (In Vivo). All testing occurred during the dark phase under red light illumination, except for the elevated zero maze (EZM) which was conducted during the light phase. Two to six weeks after viral injection and implantation surgery (or viral injection alone), subjects were habituated to the testing room and apparatus by handling and placing mice (for 3 to 5 min) in the three-chamber apparatus (see below) each day for 3 d. On experimental days, subjects were adapted to the experimental room for 15 min before testing. Social investigation, social communication (i.e., urine marking and ultrasonic vocalizations), and locomotion was measured during three-chamber testing, followed by an elevated-zero maze test to evaluate anxiety-like behavior and a real-time place preference test. For detailed methods, refer to *SI Appendix*. All behavioral tests were scored by an experimenter blind to the viral and drug manipulation of the subject. To assess cellular activity in response to social stimulation, a group of AVP-iCre+ animals were injected within the BNST with Cre-dependent eYFP virus (see above) and exposed for 10 min to either a caged male, caged female, or an empty cage (with another adjacent empty cage) within a three-chamber apparatus. We used a Cre-dependent viral labeling approach rather than AVP immunocytochemistry as levels of AVP in BNST cell bodies are low (83), presumably because there is rapid translocation of AVP to terminals (84). Mice were removed and killed 70 min later; brain tissue was extracted, sectioned, and processed for Fos/eYFP immunohistochemistry, with Fos as a marker for neural activity. For detailed methods, refer to *SI Appendix*.

Light Stimulation. A dual fiber-optic patch cord (Doric Lenses, DFP_200/230/900-0.48_1m_DF1.6-2FC) was coupled, using a zirconia sleeve (Doric Lenses, SLEEVE_ZR_2.5), to dual optic fiber cannulas chronically implanted in subjects. During testing, the dual-fiber optic patch cord was connected via a FC/PC connector to a rotary joint (Doric Lenses, FRJ_1x2i_FC-2FC_0.22) to minimize torque on the animal’s head.

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The rotary joint was mounted above the center of testing arenas using a gimbal holder (Doric Lenses, GH_FRJ) to further minimize torsional stress on subjects during behavior testing. A monofiber optic patch cord (Doric Lenses, MFP_200/240/LWMJ-0.22_1m_FC-FC) connected the rotary joint to a 473 nm blue diode pumped solid-state laser (Shanghai Laser & Optics Century Co., BL473T8-150FC) via FC/PC connectors. Blue-light laser pulses were generated via a controller (PlexBright 4-Channel Optogenetics Controller) and Plexon Radiant Software (2.2.0.19). During ChR2 experiments, 10 Hz pulses of blue light (20 ms pulse width, 473 nm, 5 to 6 mW light power) were delivered at 5 s on/off intervals over a 10-min (three-chamber tests) or 5-min period (EZM tests). Mice in the stGtACR2 cell inhibition experiment received constant light (473 nm, 5 to 6 mW light power) for the duration of each 5-min test. Light intensity optic fiber was verified prior to each test using a light sensor (Thor Labs, S140C); light power ranged from 5 to 6 mW at the tip of the dual optic fiber implant.

V1aR Antagonist Injections into LS. The highly specific V1aR antagonist (d(CH2)5[Tyr(Me)2, Dab5] AVP; Bachem) was diluted in sterile saline and 0.1% acetic acid to a final injected dose of 2.5 μM and stored at –20 °C until use. This antagonist is exceptionally selective for V1aR, eliciting no detectable anti-OT activity in vitro or in vivo (85). Thirty to forty-five minutes before behavioral testing, subjects were briefly anesthetized (1.5 to 3% isoflurane gas) and a 33-gauge needle was inserted through implanted guide cannula, extending a total length of 3.7 mm. Subjects were then injected with 300 nL of V1aR antagonist or sterile saline (vehicle) at 100 nL/min (10 μL Hamilton syringe; Harvard Apparatus PHD 22/2000 syringe pump) via the guide cannula. The injection needle was left in place for 1 min to allow the drug to diffuse away from the tip of the injection needle, followed by replacement with optical fibers extending 3.2 mm.

Data, Materials, and Software Availability. Behavior and histology data have been deposited in Figshare: <https://figshare.com/s/b851f54722a244b4e8d4> (86).

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