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Neurochemical Mediation of Affiliation and Aggression Associated With Pair-Bonding

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ABSTRACT

BACKGROUND: The neuropeptides vasopressin and corticotropin-releasing factor facilitate, while serotonin inhibits, aggression. How the brain is wired to coordinate interactions between these functionally opposed neurotransmitters to control behavioral states is poorly understood.

METHODS: Pair-bonded male prairie voles (*Microtus ochrogaster*) were infused with a retrograde tracer, Fluoro-Gold, and tested for affiliation and aggression toward a female partner or novel female subject. Subsequent immunocytochemical experiments examined neuronal activation using Fos and neurochemical/neuroreceptor profiles on brain areas involved in these social behaviors. Finally, a series of behavioral pharmacologic and real-time in vivo brain microdialysis experiments were performed on male prairie voles displaying affiliation or aggression.

RESULTS: We localized a subpopulation of excitatory vasopressin neurons in the anterior hypothalamus that may gate corticotropin-releasing factor output from the amygdala to the anterior hypothalamus and then the lateral septum to modulate aggression associated with mate guarding. Conversely, we identified a subset of inhibitory serotonergic projection neurons in the dorsal raphe that project to the anterior hypothalamus and may mediate the spatiotemporal release of neuropeptides and their interactions in modulating aggression and affiliation.

CONCLUSIONS: Together, this study establishes the medial extended amygdala as a major neural substrate regulating the switch between positive and negative affective states, wherein several neurochemicals converge and interact to coordinate divergent social behaviors.

Keywords: Anterior hypothalamus, Corticotropin-releasing factor, Dopamine, Medial amygdala, Serotonin, Vasopressin

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A critical challenge in the psychiatry field is to determine the neurochemical circuitry underlying an individual's propensity to transition between prosocial emotional states to physical violence (1). Although preclinical neuroscience has largely focused on examining the function of individual neurochemicals, brain areas, and neuronal mechanisms therein, we know surprisingly little about the neuromodulatory microcircuits regulating emotion (2).

The posterior dorsal medial amygdala (MeAPD) projects to several subdivisions of the hypothalamus (3–5) to regulate various forms of social behavior (3–10). However, the circuitry remains largely undefined beyond these second-order projections. The integrating command centers that process sensory input and control descending motor output to program socioemotional behavior are unclear. Previous work has relied on using traditional laboratory rodents to dissect the neural circuitry involved. However, these animals do not readily display certain types of behavior and may not be appropriate for some investigations (11). For example, most laboratory animals do not exhibit strong social bonds between mates, and male animals typically do not display paternal behavior or female-directed aggression (12). Because mating naturally induces these behaviors in the socially monogamous prairie vole (*Microtus ochrogaster*), this rodent species represents a unique animal model to investigate neural circuitry programming pair-bonds (12,13).

Lesions of the vomeronasal organ (14) or MeAPD (15) impair partner preference formation and affiliation in prairie voles. In male prairie voles, parvocellular vasopressin (AVP) neurons in the nucleus circularis and medial supraoptic nucleus are both recruited during aggression (16) and release their contents in the anterior hypothalamus (AH) activating AVP 1A receptors (V1aR) to facilitate aggression selectively toward novel conspecifics but not toward a partner (17). Two weeks of sociosexual experience also induce structural plasticity of V1aR to mediate selective aggression (17). Furthermore, viral vector-mediated gene transfer of V1aR into the AH of sexually naïve male animals recapitulates pair-bonding-induced aggression (17). Finally, dopamine signaling in the rostral nucleus accumbens shell (NAcc) is also involved in selective aggression to maintain monogamous pair-bonds (18). However, despite these studies, we know little about how these brain regions, genes, and neurochemicals integrate into a network to control pair-bonding behavior (19).

Because recent work demonstrates regional overlap of molecularly specified neurons in the ventral medial hypothalamus

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that control properties characteristic of emotion states regulating social (20), sexual (21,22), and aggressive (21–25) behaviors, we investigated whether individual pair-bonding behaviors are encoded via similar or different neuronal systems. Here, we focused on examining the neurotransmitters AVP, corticotropin-releasing factor (CRF), and serotonin (5-HT) for their roles in regulating behavioral states. We proposed that AVP/CRF facilitate aggression, while 5-HT functions to inhibit the activity of the AVP/CRF systems in the AH to switch from aggression to affiliation. Our data provide necessary refinement steps toward understanding how multiple neurotransmitter systems interact within neuronal microcircuits to drive attachment.

METHODS AND MATERIALS

Subjects

Subjects were male prairie voles (90–120 days of age) that were either sexually naive or pair-bonded with a female subject for 2 weeks, which reliably induces partner preferences and selective aggression toward novel conspecifics (16–18) (Supplemental Experimental Procedures). All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Florida State University.

Behavioral Assays

Subjects' aggressive behaviors were examined using the resident-intruder test (RIT), a well-characterized and ethologically valid model of offensive aggression (26). Briefly, a conspecific intruder was introduced into the home cage of the subject (resident), and the resident was scored for 10 minutes for aggressive responses, including the frequency of lunges, bites, and chases, as well as the duration of affiliative side-by-side contact and anogenital investigation, as previously described (16,17) (Supplemental Experimental Procedures).

Monosynaptic Tracer Injection Parameters

Subjects were stereotaxically injected into the AH (coordinates from bregma: posterior 0.55 mm, lateral ± 0.75 mm, ventral 6.1 mm), rostral NAcc shell (anterior 1.60 mm, lateral ± 1.0 mm, ventral 4.5 mm), lateral septum (LS) (anterior 0.80 mm, lateral ± 0.61 mm, ventral 4.1 mm), or MeAPD (posterior 1.30 mm, lateral ± 2.70 mm, ventral 7.0 mm), respectively, with glass capillary micropipettes (A-M Systems, Inc., Carlsborg, WA) filled with 2% Fluoro-Gold (FG) (Fluorochrome, Englewood, CA) and 0.5% cresyl violet dye in 0.01 mol/L phosphate buffer solution (PBS) (pH 7.4) under sodium pentobarbital (0.1 mg/10 g body weight). Injection placement was evaluated by processing sections spanning the target area for FG immunocytochemical detection and cresyl violet dye spread. Data from the subjects with correct injection placement were included in neuroanatomical mapping (Supplemental Figure S3 and Supplemental Experimental Procedures).

Brain Microdissection and High-Performance Liquid Chromatography With Electrochemical Detection Analysis

Coronal brain sections (300 μm) were cut on a cryostat and frost mounted onto microscope slides. Bilateral tissue punches were taken using a 1-mm-diameter scalpel under 20× magnification on a Leica DMRB dissection microscope (Leica Biosystems Inc., Buffalo Grove, IL). Tissue samples were localized to the AH, medial preoptic area, and paraventricular nucleus of the hypothalamus and stored at −80°C. Subsequently, 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were measured using high-performance liquid chromatography with electrochemical detection (Supplemental Experimental Procedures).

Intra-AH Stereotaxic Cannulation and Drug Microinfusion

Subjects were anesthetized with sodium pentobarbital (0.1 mg/10 g body weight) and then stereotaxically implanted with guide cannula aimed at the AH, as described previously (17,18). All injections were made using a Hamilton syringe connected to an automatic micropump. Immediately after a 10-minute RIT test, subjects were overdosed with sodium pentobarbital and rapidly decapitated and their brains were sectioned for histologic verification of cannula placement. Subjects with correct cannula placement were included in data analysis (Supplemental Figure S2 and Supplemental Experimental Procedures).

Brain Preparation, Immunocytochemistry, and Image Analysis

Subjects were anesthetized with sodium pentobarbital and then perfused through the ascending aorta with 0.9% saline, followed by 4% paraformaldehyde in 0.1 mol/L PBS. Brains were dissected, postfixed for 2 hours in 4% paraformaldehyde, and then stored in 30% sucrose in PBS. Brains were cut into 30-μm coronal sections on a freezing microtome, and floating sections were stored in 0.1 mol/L PBS with 1% sodium azide at 4°C until immunostaining.

Different sets of floating brain sections at 150-μm intervals were processed for single- or double-immunoreactive (ir) labeling of FG, Fos, FG/Fos, FG/tyrosine hydroxylase (TH), FG/AVP, FG/5-HT, or FG/CRF. AH sections were processed for double- or triple-ir labeling for AVP, V1aR, 5-HT, 5-HT_{1A} receptors (5-HTr1a), CRF, CRF_2 receptors (CRFR2), FG, and Fos.

We quantified the colocalization of 5-HTr1a, V1aR, CRFR2, and 5-HT on AVP-, CRF-, and/or FG-expressing neurons in the AH. Leica imaging software (Leica Biosystems Inc.) profile methods of cell counting were employed and area measurements (square millimeters) were taken on each section analyzed to determine cell densities. Photomicrographs were captured by using a Zeiss Axioskop 2 (Carl Zeiss NTS, LCC, Peabody, MA) microscope with a SPOT RT Slider (Diagnostic Instruments, Sterling Heights, MI) camera and SPOTTM (version 3.0.6; Diagnostic Instruments) software. Image files were then stored and subsequently analyzed (Supplemental Experimental Procedures).

Real-Time In Vivo Brain Microdialysis With Neurochemical Analyses

Microdialysis probe construction, cannulation, and dialysate collection were previously described (17,27,28) (Supplemental Experimental Procedures). Immediately after RIT, subjects were overdosed with sodium pentobarbital and rapidly decapitated and their brains were sectioned for histologic verification

of probe placement. Subjects with correct probe placement in the AH were included in data analysis. Microdialysis samples were processed for AVP and CRF contents using standard enzyme-linked immunosorbent assay kits and 5-HT content using high-performance liquid chromatography with electrochemical detection.

RESULTS

Neuronal Activation Associated With Opposing Behavioral States

To establish a neural framework of the circuitry associated with individual pair-bonding behaviors, we performed affiliation and aggression assays in male subjects injected with FG. Male subjects displayed offensive aggression toward novel female subjects and social affiliation with their female partner. These robust patterns of selective aggression and affiliation were observed in each of the four tracing groups (Figure 1A, B). No group differences were found in general locomotor activity, social interest, exploration, defense, or courtship behaviors (Supplemental Tables S1A–D).

The stereological parameters, anatomical coordinates, and abbreviations for each brain area quantified are summarized in (Supplemental Table S2). Stereological quantification found no significant differences in the density of FG-ir neurons among tracing groups, indicating consistent microinjection volumes (Supplemental Table S3). We have previously identified subsets of neurons selectively activated by the expression of affiliation and aggression (16,29). Therefore, we focused on this distinct subpopulation by using Fos, the protein product of an immediate early gene, *c-fos*, to assess neuronal activation in retrogradely labeled projection neurons recruited during affiliation or aggression. We added a baseline control group of handled male subjects that were not exposed to social stimuli during RIT. Male subjects displaying aggression toward a novel female subject showed a significantly higher density of Fos-ir in the AH and MeAPD than male subjects displaying affiliation, which, in turn, showed a higher density of Fos-ir than control subjects, and this pattern of Fos-ir was consistent across all tracing groups (Figure 1C–E, I–K). There was a significantly higher density of FG-ir/Fos-ir double-labeled neurons in the AH projecting to the LS (Figure 1F, I) and in the MeAPD projecting to the AH (Figure 1H, J) in male subjects displaying aggression compared with male subjects displaying affiliation and control subjects. Conversely, there was a significantly higher density of FG-ir/Fos-ir double-labeled neurons in the dorsal raphe (DR) projecting to the AH in male subjects displaying affiliation than in male subjects displaying aggression or control subjects (Figure 1H, K).

Neurochemical Microcircuit Connectivity

Multiple-label immunofluorescence experiments were performed to identify the cytochemical phenotypes of FG-ir projection neurons recruited during affiliation (DR-AH) and aggression (MeAPD-AH-LS). FG-ir neurons in the AH or MeAPD did not coexpress AVP, TH, gamma-aminobutyric acid, glutamate, or oxytocin but stained positively for CRF (Supplemental Table S4). Because the data above revealed activation of a MeAPD-AH-LS circuit during aggression, we focused on stereologically quantifying the percentage of FG-ir/CRF-ir double-labeled neurons in the AH and MeAPD from the LS and AH tracing groups. Thirty-nine percent of the total number of Fos-ir/FG-ir neurons in the AH projecting to the LS and 8% of the total number of Fos-ir/FG-ir neurons in the MeAPD projecting to the AH of aggressive male subjects stained positively for CRF (Table 1). Although we found FG-ir and CRF-ir cells in the paraventricular nucleus of the hypothalamus and bed nucleus of the stria terminalis across each injection group, we did not see any FG-ir/CRF-ir colocalized cells.

Furthermore, previous mapping studies indicated the source of 5-HT innervation to the medial supraoptic nucleus and paraventricular nucleus of the hypothalamus-AVP systems (30). Here, we found that 16% of the total number of Fos-ir/FG-ir neurons in the DR projected to the AH and coexpressed 5-HT in bonded male subjects displaying affiliation (Table 1). Finally, because the catecholaminergic circuit from the ventral tegmental area (VTA) to NAcc has been well established in a variety of species including voles (31,32), we processed sections spanning the VTA taken from the NAcc FG injection group for TH and FG double-labeling, as an internal control for validating our retrograde tract-tracing methods. Twenty-seven percent of the FG-ir neurons in the VTA projecting to the NAcc coexpressed TH (Table 1), implicating a population of mesolimbic dopaminergic neurons extending from the VTA to the NAcc (33,34). Together, our data unraveled a novel circuit associated with affective behavior outside the hypothalamic-pituitary-adrenal axis (35).

5-HT and AVP Modulation of Selective Aggression via 5-HTr1a Activation

AVP in the AH mediates offensive aggression in rodent species (36), including prairie voles (17), and this AVP effect is attenuated by activation of the 5-HT system (37,38). Results from our tract-tracing data suggested that a DR-AH 5-HT circuit was activated during affiliation but not aggression. Therefore, we tested the hypothesis that 5-HT in the AH mediates AVP-induced or pair-bonding-induced aggression.

To increase accumulation of extracellular 5-HT, we used fluoxetine, a commonly used selective serotonin reuptake inhibitor, which blocks mating-induced aggression in male prairie voles (39) and agonistic behavior in other species (40–47). Results from meta-analytic studies demonstrated that increased 5-HT had the strongest inhibitory effect on aggression in rodent species when aggression was offensive, fluoxetine was used, injection was intraperitoneal, and treatment was acute (48). Therefore, we followed a similar selective serotonin reuptake inhibitor injection and dosing regimen.

To induce aggression in sexually naïve male subjects, we used intra-AH administration of AVP (500 ng/side), which induces offensive aggression in male prairie voles (17). Sexually naïve male subjects that received bilateral intra-AH injections of AVP (in 200 nL cerebrospinal fluid [CSF]) were divided into three groups that received intraperitoneal injections of saline or saline containing a low (1 mg/kg) or high (6 mg/kg) dose of fluoxetine followed by a 10-minute RIT toward a novel female subject. Both doses of fluoxetine blocked AH-AVP-induced aggression (Supplemental Figure S1A), while the high dose of fluoxetine also increased affiliation

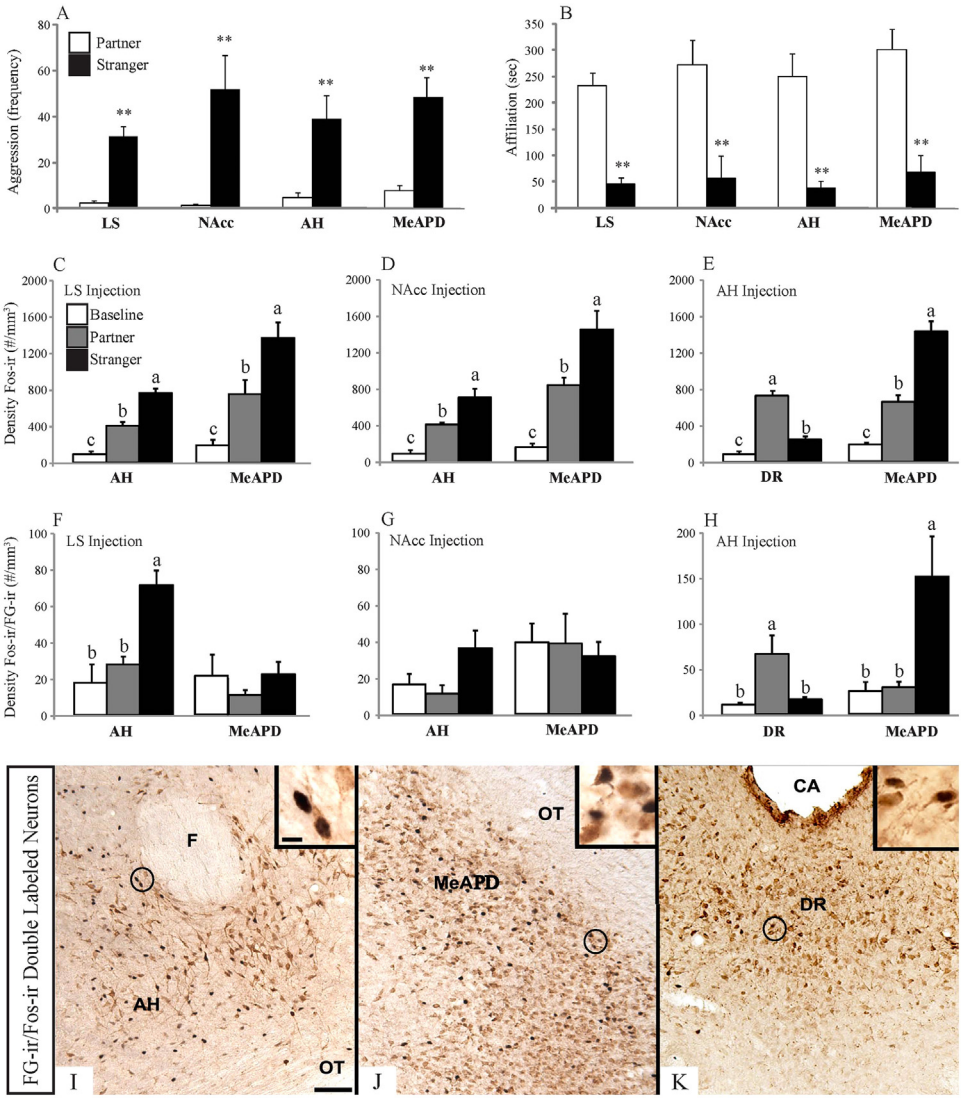

Figure 1. Differential limbic circuit activation associated with affiliation and aggression. **(A)** Male prairie voles that pair-bonded with a female subject for 2 weeks displayed robust aggression against a novel female subject but **(B)** high levels of affiliation toward their familiar female partner. This behavioral pattern was consistent across animals that received Fluoro-Gold (FG) injections into the lateral septum (LS), nucleus accumbens (NAcc), anterior hypothalamus (AH), or posterior dorsal medial amygdala (MeAPD) with significant main ($F_{2,4,20} = 32.58$, $p < .001$ for aggression and $F_{2,4,20} = 37.29$, $p < .001$ for affiliation) but not interaction ($F_{2,4,20} = 1.37$, nonsignificant [ns] for aggression and $F_{2,4,20} = 1.79$, ns for affiliation) effects. In these four FG injection groups, aggression against a novel female subject induced a significant increase in the density of Fos-immunoreactive (ir) neurons in the AH **(C, D)** and MeAPD (**C–E**), while affiliation induced a significant increase in the density of Fos-ir neurons in the dorsal raphe (DR) compared with control subjects with significant main ($F_{2,4,20} = 6.85$, $p < .05$ for aggression and $F_{2,4,20} = 7.64$, $p < .05$ for affiliation) but not interaction ($F_{2,4,20} = 1.13$, ns for aggression and $F_{2,4,20} = 1.32$, ns for affiliation) effects. In addition, aggression induced a significant increase in the density of neurons double-labeled for Fos-ir/FG-ir in the AH **(F)** and MeAPD **(H)** compared with affiliation or control subjects with significant main ($F_{2,4,20} = 5.93$, $p < .05$) but not interaction ($F_{2,4,20} = 1.68$, ns) effects. Conversely, male subjects displaying affiliation toward their female partner had an increased density of Fos-ir/FG-ir double-labeling in the DR compared with male subjects displaying aggression against a novel female subject and control subjects with significant main ($F_{2,4,20} = 5.43$, $p < .05$) but not interaction ($F_{2,4,20} = 1.09$, ns) effects **(H)**. Light-field photomicrographs (30 μm stack) of neurons labeled for FG-ir (brown cytoplasmic staining), Fos-ir (black nuclear staining), or both in the AH (FG injected into LS) **(I)**, MeAPD (FG injected into AH) **(J)**, and DR (FG injected into AH) **(K)** of male subjects exposed to a stranger female subject. The open black circles shown in panels **(I)**, **(J)**, and **(K)** depict the area at higher magnification (5 μm stack) in the inset. Bars indicate means ± standard error of the mean. $^{**}p < .01$ **(A, B)** Bars labeled with different letters **(C–H)** differ significantly by post hoc Student Newman-Keuls tests of significance examining both main effects and interactions with analysis of variance p value set to $< .05$. Scale bar = 100 μm. The insert within each panel shows neurons double-labeled for Fos-ir/FG-ir, while scale bar = 10 μm. See also Supplemental Table S1. CA, cerebral aqueduct; F, fornix; OT, optic tract.

(Supplemental Figure S1B) relative to saline control subjects. Fluoxetine treatment did not affect general locomotor activity, social interest, exploration, defense, or courtship behaviors (Supplemental Table S5).

To assess the effect of fluoxetine treatment on 5-HT activity in the brain, three groups of sexually naïve male prairie voles were injected (intraperitoneally) with saline, a low (1 mg/kg) dose of fluoxetine, or a high (6 mg/kg) dose of fluoxetine, and

Table 1. Neurochemical Microcircuit Phenotyping

FG Injection	Area	Markers	Cells	%
LS	AH	CRF/Fos/FG	152	39[a]
AH	MeAPD	CRF/Fos/FG	66	8[a]
AH	DR	5-HT/Fos/FG	58	16[a]
NAcc	VTA	TH/FG	774	27[b]

AH, anterior hypothalamus; CRF, corticotropin-releasing factor; DR, dorsal raphe; FG, Fluoro-Gold; 5-HT, serotonin; ir, immunoreactive; LS, lateral septum; MeAPD, posterior dorsal medial amygdala; NAcc, nucleus accumbens; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

[a]Percent of the total Fos-ir/FG-ir double-labeled cells.

[b]Percent of the total FG-ir cells.

their brain tissue was microdissected (Supplemental Figure S1F) for 5-HT and its metabolite 5-HIAA measurement using high-performance liquid chromatography with electrochemical detection. Treatment of fluoxetine at the high dose increased levels of 5-HT (Supplemental Figure S1C) and 5-HIAA (Supplemental Figure S1D) in the AH. Both fluoxetine doses decreased 5-HT turnover, indicated by a low 5-HIAA/5-HT ratio in the AH (Supplemental Figure S1E). This effect of fluoxetine on 5-HT turnover has been corroborated in previous work (39,49–51).

We then focused on the AH to determine the receptor-specific role of 5-HT attenuating AH-AVP-induced aggression. We focused on 5-HTr1a because of their antiaggressive properties (52) and site-specific effects in the AH on modulating offensive aggression (53). 5-HTr1a were expressed on AVP-ir neurons (Figure 2A, C, D) surrounded by a dense network of 5-HT-ir boutons (Figure 2B). Sexually naïve male subjects received bilateral intra-AH injections of AVP (500 ng/side) in CSF or CSF containing a low (0.5 μg/side) or high (5 μg/side) dose of a 5-HTr1a agonist [R(+)-8-OH-DPAT] followed by a 10-minute RIT toward a novel female subject. Doses were determined based on previous studies (52). Intra-AH microinjections of the 5-HTr1a agonist attenuated AVP-induced aggression (Figure 2E) and enhanced social affiliation compared with CSF control subjects (Figure 2F). Treatment with R(+)-8-OH-DPAT did not affect other behaviors (Supplemental Table S6). These data indicate that 5-HTr1a activation by R(+)-8-OH-DPAT abolishes selective aggression that was pharmacologically induced by intra-AH AVP administration. We then tested whether manipulation of 5-HTr1a in the AH influenced aggression naturally induced by pair-bonding (16–18). Pair-bonded male subjects received intra-AH injections of CSF (control) or CSF containing a 5-HTr1a agonist [R(+)-8-OH-DPAT; 5 μg/side] or antagonist [4-(2′-methoxy-phenyl)-1-[2′-(n-2′′-pyridinyl)-p-iodobenzamido]-ethyl-piperazine; 5 μg/side] followed by a 10-minute RIT toward a novel female subject. Compared with CSF control subjects, intra-AH infusions of the 5-HTr1a agonist abolished aggression (Figure 2G) and facilitated affiliation (Figure 2H). Conversely, blocking 5-HTr1a in the AH enhanced aggression above vehicle controls. Other behaviors were not affected (Supplemental Table S7).

Lastly, we tested the behavioral consequences of AVP administration in the AH of male subjects treated with a combination of intra-AH 5-HTr1a agonist/antagonist infusions. Pair-bonded male subjects were divided into one of four groups that received intra-AH infusions of AVP (500 ng in 200 nL CSF/side) in CSF (control, $n = 8$) or CSF containing a 5-HTr1a agonist [R(+)-8-OH-DPAT; 5 μg/side, $n = 9$], antagonist (4-(2′-methoxy-phenyl)-1-[2′-(n-2′′-pyridinyl)-p-iodobenzamido]-ethyl-piperazine; 5 μg/side, $n = 7$), or both [R(+)-8-OH-DPAT + 4-(2′′-methoxy-phenyl)-1-[2′-(n-2′′-pyridinyl)-p-iodobenzamido]-ethyl-piperazine; 5 μg/side, $n = 8$] followed by a 10-minute RIT toward a novel female subject. Overall, male subjects displayed significantly higher levels of offensive aggression and low levels of affiliation toward a novel female subject (Figure 2I, J). Male subjects treated with the 5-HTr1a 5-μg antagonist exhibited significantly higher levels of offensive aggression (Figure 2I). No group differences were found in affiliation (Figure 2J) or other behaviors measured (Supplemental Table S8), extending previous findings (37–39).

Cytochemical Profiling of AH Neurons

As AVP, 5-HT, and CRF coordinate patterns of affiliation and aggression, we histochemically profiled the AH for these neurochemical markers and their receptors (Table 2). Twenty percent of AVP-ir neurons in the AH coexpressed 5-HTr1a-ir (Figure 3A–C; Table 2), which confirms our triple-labeling experiments (Figure 2A–D). Sixteen percent of CRF-ir neurons in the AH coexpressed 5-HTr1a-ir (Figure 3D–F; Table 2) and 45% of AVP-ir neurons coexpressed CRFR2 (Figure 3G–I; Table 2). Finally, 14% of CRF-ir neurons coexpressed V1aR-ir (Figure 3J, K, M; Table 2). In addition, 45% of neurons double-labeled for CRF-ir/V1aR-ir coexpressed FG-ir (Figure 3J–M) in male subjects that received FG injections into the LS.

AH 5-HT Mediates Neuropeptide Release to Modulate Behavioral Switches

Pair-bonded male subjects were implanted with a microdialysis probe aimed at the AH. After 1-week recovery, subjects were randomly divided into four pharmacologic treatment groups and received reverse microdialysis infusion of CSF or CSF containing a receptor agonist or antagonist for V1aR, CRFR2, or 5-HTr1a, while their behavior toward a familiar partner or a novel female subject was examined using RIT (Figure 4A). Microdialysis samples were collected and subsequently processed for AVP, CRF, and 5-HT contents. In control male subjects (CSF), aggression levels were low when they were reunited with their female partner but high when they were exposed to a novel female subject (Figure 4B), and a reverse pattern was found in affiliative behavior (Figure 4C). Pharmacologic inactivation of V1aR or CRFR2, as well as activation of 5-HTr1a, in the AH diminished aggression and facilitated affiliation toward novel female subjects. Conversely, activation of V1aR or CRFR2 induced aggression and decreased affiliation toward their female partner (Figure 4B, C). Furthermore, blockade of 5-HTr1a in the AH impaired affiliation toward female partners (Figure 4C). None of the drug compounds affected other behaviors measured (Supplemental Tables S9–S11).

Changes in behavioral responses toward a partner or novel female subject were associated with dynamic neurochemical release patterns in the AH. Enhanced 5-HT release coupled with decreased AVP and CRF release was associated with low

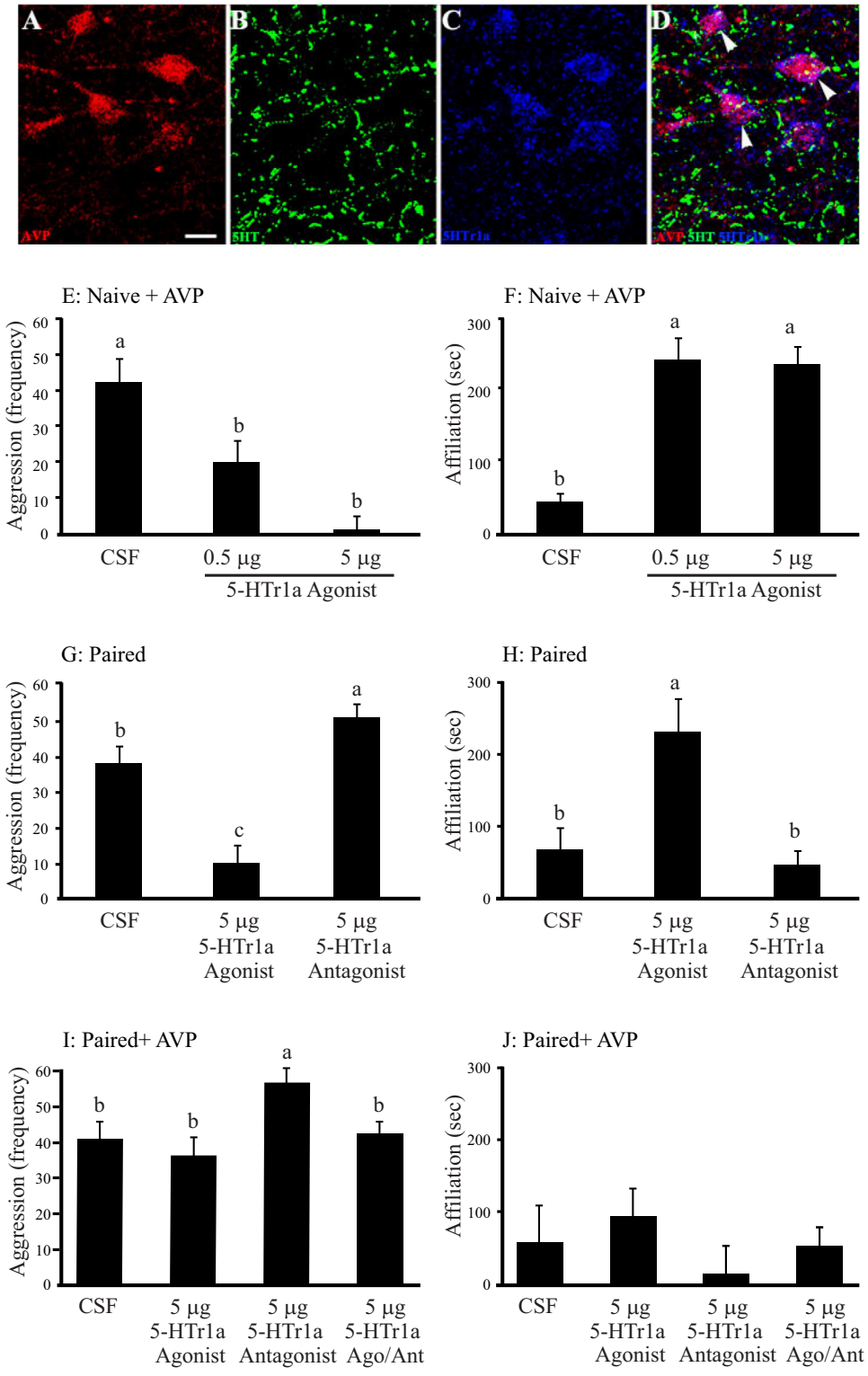

Figure 2. Anterior hypothalamus (AH)-serotonin (5-HT) 1A receptor (5-HTr1a) activation attenuates vasopressin (AVP)- and pair-bonding-induced aggression. **(A)** AVP, **(B)** 5-HT, **(C)** 5-HTr1a, and (**D**) AVP/5-HT/5-HTr1a labeling in the AH. AVP/5-HTr1a double-labeled neurons indicated by white arrowheads. For the behavioral experiments **(E–H)**, sexually naïve male prairie voles received intra-AH injections of AVP (500 ng in 200 nL cerebrospinal fluid [CSF]/side) or AVP with a low (0.5 µg/side) or high (5 µg/side) dose of a 5-HTr1a agonist [R(+)-8-OH-DPAT] followed by a 10-minute resident intruder test toward a novel female subject. Both doses of the 5-HTr1a agonist blocked AH-AVP-induced aggression ($F_{2,18} = 9.23$, $p < .01$) **(E)** and enhanced affiliation ($F_{2,18} = 4.20$, $p < .05$) **(F)** compared with CSF-injected control subjects. Male prairie voles that were pair-bonded with a female subject for 2 weeks were divided into three groups that received intra-AH infusions of CSF (200 nL/side) or CSF containing a 5-HTr1a agonist (5 µg/side) or antagonist [4-(2′-methoxy-phenyl)-1-[2′-(n-2′′-pyridinyl)-p-iodobenzamido]-ethyl-piperazine; 5 µg/side] followed by a 10-minute resident intruder test. **(G, H)** Male subjects treated with the 5-HTr1a agonist displayed a significant decrease in aggression ($F_{2,20} = 40.83$, $p < .001$) and an increase in affiliation ($F_{2,20} = 8.01$, $p < .01$) compared with CSF-injected control subjects. **(G)** In contrast, male subjects treated with the 5-HTr1a antagonist displayed enhanced aggression toward a novel female subject than did CSF-injected control subjects ($F_{2,20} = 40.83$, $p < .001$). Lastly, we tested the behavioral consequences of AVP administration in the AH of pair-bonded male subjects treated with a combination of 5-HTr1a agonist/antagonist infusions. Overall, male subjects displayed significantly higher levels of offensive aggression and low affiliation toward a sexually receptive female subject **(I, J)**. Male subjects treated with the 5-HTr1a 5-µg antagonist exhibited significantly higher levels of offensive aggression ($F_{2,20} = 5.39$, $p < .05$) **(I)**. The effect of enhancing aggressive responding by blocking AH-5-HTr1a in intra-AH-AVP infused sexually naïve male subjects **(E–H)** is abolished in pair-bonded intra-AH-AVP treated male subjects, while 5-HTr1a antagonist treatment in these male subjects enhances offensive aggression **(I)**. Thus, the effect of 5-HTr1a activation on aggression can be blocked by AVP, whereas the effect of AVP cannot be blocked by 5-HT antagonists. Bars indicate means ± standard error of the mean. Bars with different alphabetical letters differ significantly from each other. Scale bar = 10 µm.

Table 2. AH Double-Label Immunofluorescence Neurochemical Profiling (No. Neurons/mm^2 Brain Region Volume)

No. Animals	No. Sections	Neurochemical Markers					Neurochemical Double-Labeling
		AVP	CRF	5-HTr1a	CRFR2	V1aR	
8	16	179.8 ± 19.1[a] (19.76%)[b]		104.5 ± 13.8 (33.99%)[b]			35.5 ± 9.4 (AVP/5-HTr1a)
6	12		439.3 ± 35.2 (16.55%)[b]	122.9 ± 15.5 (59.13%)[b]			72.7 ± 13.7 (CRF/5-HTr1a)
7	14	142.6 ± 16.0 (44.99%)[b]			158.3 ± 29.6 (40.53%)[b]		64.2 ± 8.4 (AVP/CRFR2)
8	16		411.1 ± 28.9 (14.49%)[b]			348.6 ± 37.8 (25.10%)[b]	87.4 ± 14.3 (CRF/V1aR)

AH, anterior hypothalamus; AVP, vasopressin; CRF, corticotropin-releasing factor; CRFR2, CRF_2 receptors; 5-HTr1a, $5\text{-}HT_{1A}$ receptors; V1aR, AVP 1A receptors.

[a]Data are presented as mean ± standard error.

[b]Percent marker coexpressing the additional label on the same row.

aggression and high affiliation (Figure 4D–G). Conversely, a reverse neurotransmitter release pattern was associated with aggression toward a novel female subject (Figure 4D). These release findings were also observed by activating V1aR or CRFR2 in the AH (Figure 4E, F), which induced aggression and impaired affiliation (Figure 4B, C). Diminished aggression and enhanced affiliation (Figure 4B, C) toward novel female subjects, by V1aR or CRFR2 blockade or by 5-HTr1a activation, were associated with an inhibition in AVP and CRF release (Figure 4E–G). Finally, impaired partner affiliation, by 5-HTr1a antagonism in the AH, was associated with the disappearance of high 5-HT and low AVP/CRF release (Figure 4G).

DISCUSSION

Healthy social relationships are necessary for maintaining human mental health, yet we know little regarding interconnections of brain regions and neurochemical interactions underlying the formation and maintenance of sociality. Using

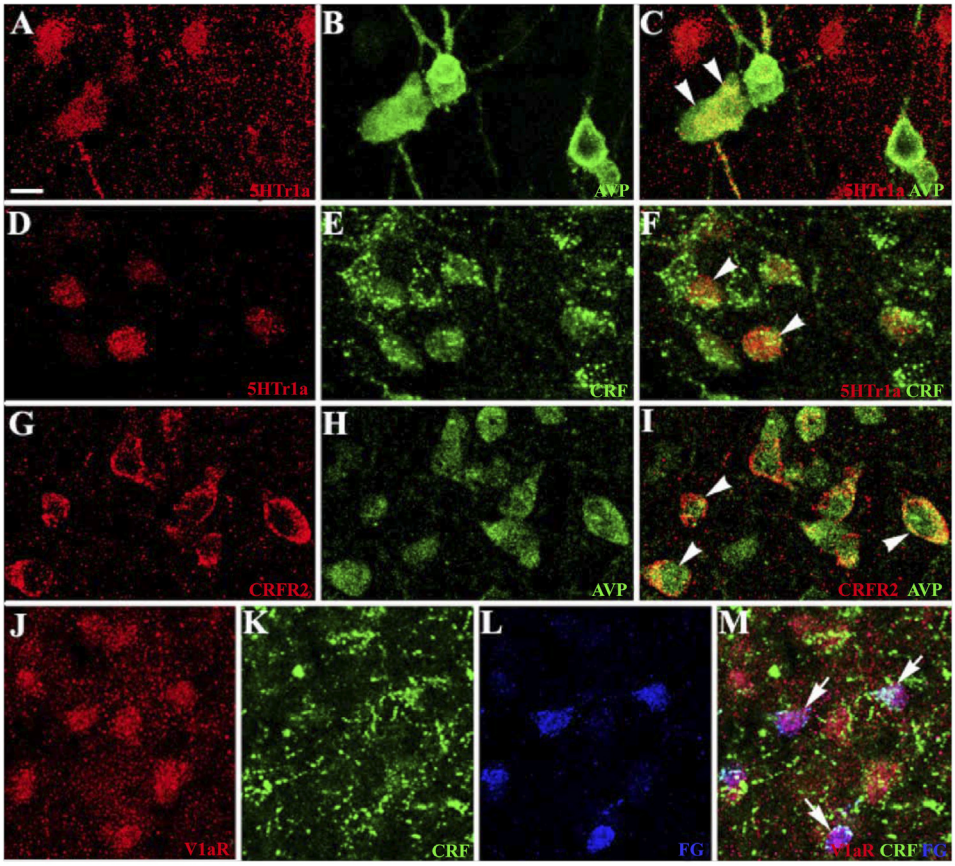

Figure 3. Serotonin (5-HT)-, corticotropin-releasing factor (CRF)-, and vasopressin (AVP)-expressing neurons/receptors colocalize in the anterior hypothalamus. Photomicrographs displaying cytochemical marker fluorescence histochemistry in the anterior hypothalamus. **(A)** $5\text{-}HT_{1A}$ receptors (5-HTr1a), **(B)** AVP, and **(C)** 5-HTr1a/AVP double-labeled neurons indicated by white arrowheads. **(D)** 5-HTr1a, **(E)** CRF, and **(F)** 5-HTr1a/CRF double-labeled neurons indicated by white arrowheads. **(G)** CRF_2 receptors (CRFR2), **(H)** AVP, and **(I)** CRFR2/AVP double-labeled neurons indicated by white arrowheads. **(J)** AVP 1A receptors (V1aR), **(K)** CRF, **(L)** Fluoro-Gold (FG) (injected into the lateral septum), and **(M)** V1aR/CRF/FG triple-labeled neurons indicated by white arrows. Scale bar = 10 μm.

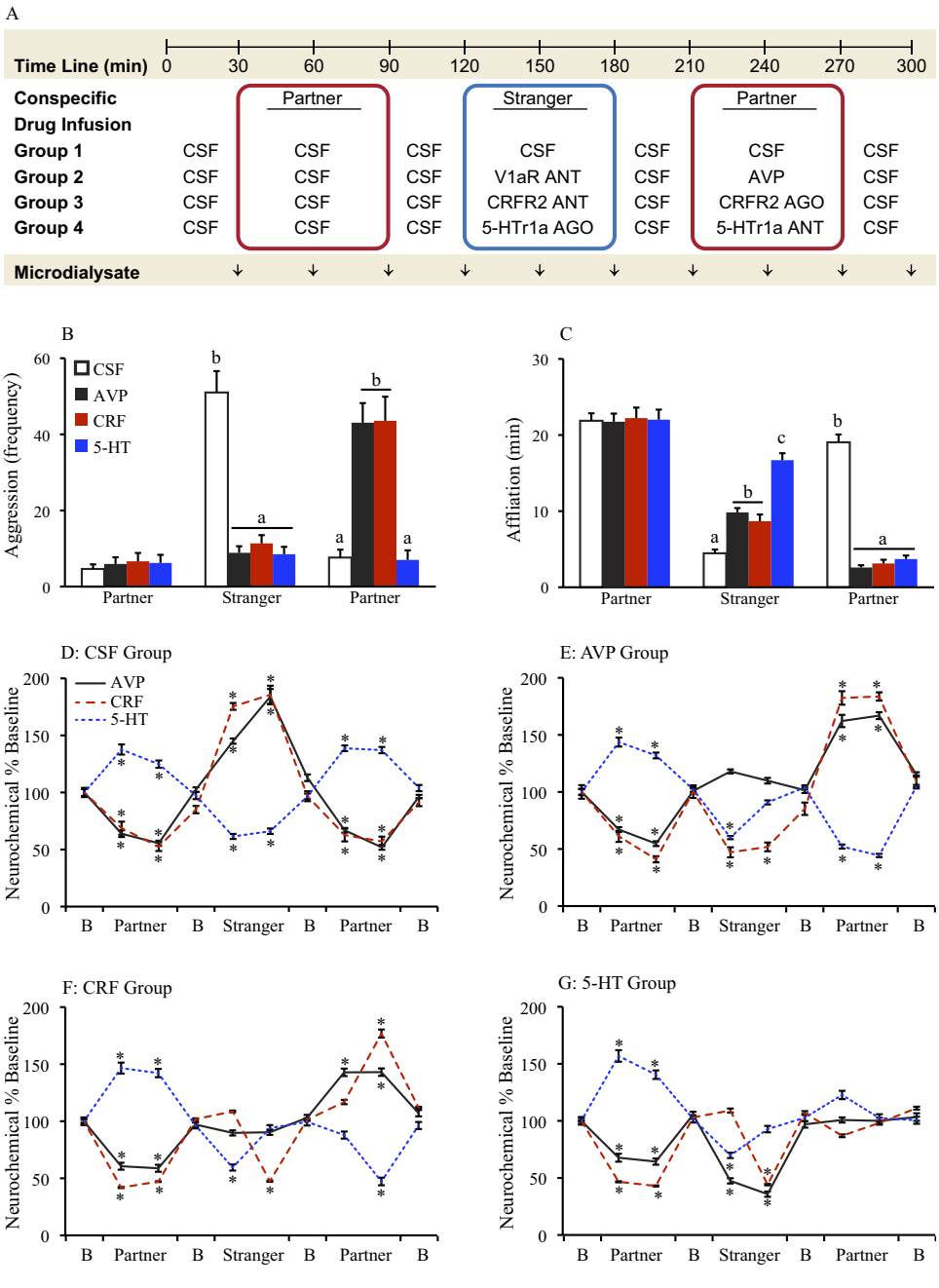

Figure 4. Behaviorally and pharmacologically evoked neurotransmitter release in the anterior hypothalamus (AH) reveals dynamic regulation of behavioral switches. **(A)** Real-time in vivo brain microdialysis paradigm. Pair-bonded male subjects were stereotaxically implanted with a microdialysis probe aimed at the AH and divided into four pharmacologic treatment groups for vehicle (cerebrospinal fluid [CSF]) infusions with manipulations of vasopressin (AVP), corticotropin-releasing factor (CRF), and serotonin (5-HT) systems, respectively. Microdialysate samples were collected every 30 minutes over a 5-hour period in which male subjects were reunited with their female partner, introduced to a novel female subject, and then reexposed to their female partner again. Reverse dialysis of pharmacologic compounds were infused during exposure to novel female subjects and reexposure to their female partner. **(B, C)** Blockade of AVP 1A receptors (V1aR) or CRF_2 receptors (CRFR2) or activation of $5\text{-}HT_{1A}$ receptors (5-HTr1a) in the AH abolished aggression ($F_{3,28} = 56.25$, $p < .001$) **(B)** and facilitated affiliation ($F_{3,28} = 41.61$, $p < .001$) **(C)** toward novel female subjects. Activation of either V1aR or CRFR2 induced aggression ($F_{3,28} = 18.26$, $p < .01$) **(B)** and reduced affiliation ($F_{3,28} = 15.92$, $p < .01$) **(C)** toward their female partner, while blockade of 5-HTr1a decreased affiliation ($F_{3,28} = 63.74$, $p < .001$) **(C)** but did not induce aggression toward their female partner ($F_{3,28} = 2.91$, nonsignificant) **(B)**. **(D)** In CSF control male subjects, 5-HT release increased while AVP/CRF release decreased when male subjects were either reunited or reexposed ($F_{3,28} = 14.52$, $p < .01$) to their female partner. A reverse pattern of neurotransmitter release was found when male subjects were fighting novel female subjects ($F_{3,28} = 17.49$, $p < .01$). **(E)** Blockade of V1aR in the AH attenuated AVP/CRF release associated with exposure to novel female subjects ($F_{3,28} = 13.46$, $p < .01$), while activation of V1aR facilitated AVP/CRF release and decreased 5-HT release in the AH during partner reexposure ($F_{3,28} = 15.82$, $p < .01$). **(F)** Blockade of CRFR2 also diminished AVP/CRF release associated with exposure to novel female subjects ($F_{3,28} = 14.88$, $p < .01$), while activation of CRFR2 enhanced AVP/CRF release and decreased 5-HT release during partner reexposure ($F_{3,28} = 13.37$, $p < .01$). **(G)** Activation of 5-HTr1a attenuated AVP/CRF release associated with exposure to novel female subjects ($F_{3,28} = 18.59$, $p < .01$), while blockade of 5-HTr1a diminished increased 5-HT release and decreased AVP/CRF release during partner reexposure. Bars indicate means ± standard error of the mean. Bars with different alphabetical letters differ significantly from each other at $p < .01$. Line time points indicate percent change from baseline ± standard error of the mean. *$p < .01$. ANT, antagonist; AGO, agonist; B, baseline (with CSF infusions). See also Supplemental Figure S1D.

the socially monogamous prairie vole, we provide data, for the first time, illustrating a novel limbic network wherein several neurochemical systems converge to regulate ethologically important behaviors critical for male-female pair-bonding.

Our data indicate that the display of aggression by male voles was associated with activation of a subpopulation of neurons in the MeAPD projecting to the AH and ones in the AH projecting to the LS, and those projection neurons in the MeAPD-AH-LS circuit expressed CRF. Male voles are

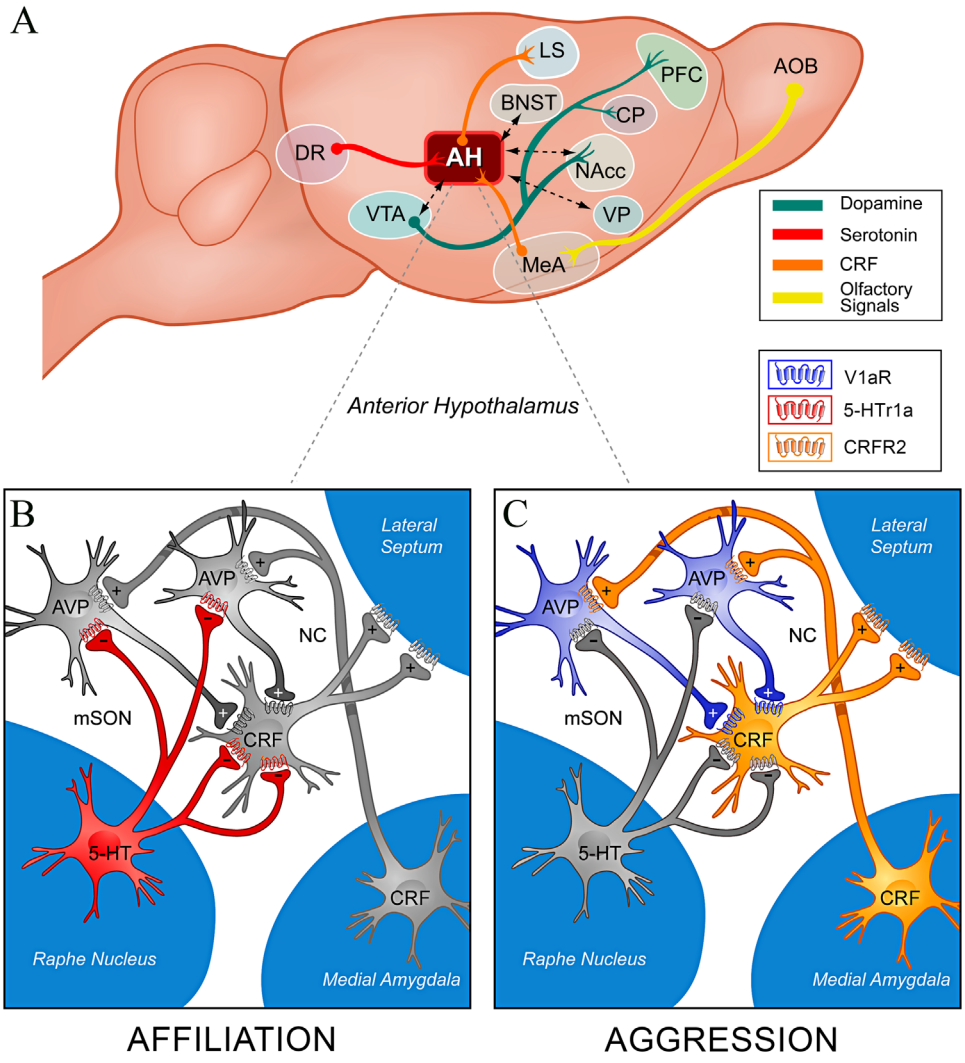

Figure 5. Microcircuit switch mechanism programming behavioral state. **(A)** Schematic illustrates the neurocircuitry and neurotransmitter circuit phenotypes summarized from monosynaptic neuronal tract-tracing and histochemical experiments. Black arrows indicate anatomical connections and neurochemical projections are color-coded. The anterior hypothalamus (AH) projects to forebrain areas ventral pallidum (VP) and bed nucleus of the stria terminalis (BNST), which are involved in pair-bonding behavior; intersects several dopaminergic regions, including the ventral tegmental area (VTA), nucleus accumbens (NAcc), caudate putamen (CP), and prefrontal cortex (PFC); integrates olfactory and pheromonal information from the vomeronasal organ through the accessory olfactory bulb (AOB) via the posterior dorsal medial amygdala (MeA); receives corticotropin-releasing factor (CRF) projections from the posterior dorsal MeA and serotonergic input from the dorsal raphe (DR); and sends CRF projections to the lateral septum (LS). **(B)** During affiliation, a subset of serotonin (5-HT) neurons in the DR project axonal collaterals and release 5-HT in the AH. Released 5-HT acts on postsynaptic 5-HT_{1A} receptors (5-HTr1a) coexpressed on vasopressin (AVP)- and CRF-containing interneurons, in the medial supraoptic nucleus (mSON) and nucleus circularis (NC), to suppress local AVP/CRF release, enhance affiliation, and inhibit aggression. **(C)** During aggression, a subpopulation of CRF neurons in the posterior dorsal MeA project dendritic arbors to and release CRF in the AH. Released CRF binds to postsynaptic CRF_2 receptors (CRFR2) coexpressed on the membrane surface of AVP interneurons, in the AH, to facilitate local AVP release. Released AVP then acts on postsynaptic AVP 1A receptors (V1aR) coexpressed on a subset of CRF neurons projecting to the LS, where CRF is released and activates CRFR2-expressing neurons to escalate aggression.

physiologically stressed when separated from their partner (54,55), and when presented with a novel conspecific, this extra-hypothalamic CRF stress circuit is engaged to facilitate aggression (16–18). Released CRF in the AH acts on CRFR2 expressed on AVP neurons. CRFR2 are coupled to a stimulatory G-protein signaling cascade (56), activating adenylate cyclase and increasing cyclic adenosine monophosphate and intracellular ionized calcium (57,58). CRFR2 activation may lead to membrane depolarization, facilitating AVP and CRF release in the AH to enhance aggression. On the other hand, released AVP can bind to V1aR expressed on CRF neurons. V1aR are also coupled to stimulatory G-proteins (59,60), and their activation enhances adenylate cyclase activity and increases cyclic adenosine monophosphate and intracellular ionized calcium (61,62) within CRF neurons projecting to the LS, where CRF is released and acts on CRFR2 (63,64) to escalate aggression (65,66). Thus, AH-AVP microinfusion can increase CRF levels and may reduce 5-HT inputs to the AH by stimulating gamma-aminobutyric acidergic projections synapsing onto 5-HT neurons in the DR-AH pathway. Furthermore, released AVP in the AH can act directly on local V1aR-expressing neurons to regulate selective aggression (17).

Conversely, when male voles were reunited with their partner, a subset of neurons in the DR that expresses 5-HT and projects to the AH was activated. Released 5-HT in the AH acts on 5-HTr1a expressed on both AVP and CRF neurons. 5-HTr1a are coupled to an inhibitory G-protein signaling cascade (67), and their activation results in decreases in adenylate cyclase activity, intracellular Ca^{2+}, and cellular depolarization (68,69), leading to a decrease in AVP/CRF release in the AH. Involvement of a serotonergic microcircuit in pair-bonding behavior is further supported by

our data showing that acute treatment of fluoxetine suppressed AVP-induced aggression, reduced AH 5-HT turnover, and enhanced social affiliation in sexually naïve male subjects. Indeed, fluoxetine has been found to block agonistic behavior in several species including humans (42,70–72) and to abolish AVP-induced aggression in rodents (44,47) by decreasing levels of AVP in the AH (71,73).

5-HT has long been considered an important neurotransmitter in the regulation of impulsive aggressive behavior. Patients with a history of physical violence exhibit low CSF levels of 5-HIAA (74,75). Low 5-HIAA typically indicates decreased 5-HT release and correlates with aggressive behavior (76) and alcohol-related forcefulness (77) in adults, as well as impulsive violent behavior in children (78). In free-ranging rhesus monkeys, low levels of CSF 5-HIAA correlate with increased aggression and risk taking (79,80). In talapoin monkeys with an established social hierarchy, high levels of 5-HIAA in subordinates are related to their low social status characterized by high levels of withdrawal and diminished aggression (81). Human imaging work has shown that a DR 5-HT brainstem microcircuit is activated when subjects are presented with images of their long-term partner (82).

The notion that 5-HT may inhibit AVP release in the human brain is supported by clinical findings that patients presenting with a personality disorder and exhibiting a lifetime history of fighting and assault showed a positive correlation between CSF levels of AVP and aggression (83) with a hyporeactive 5-HT system as assessed by fenfluramine challenge (84). Fenfluramine is a 5-HT-releasing drug that normally stimulates prolactin release as a neuroendocrine measure of central 5-HT activity. Patients with a lifetime history of escalated aggression and violence show blunted prolactin release in response to fenfluramine (85,86). This is also true in macaque monkeys, which show increased aggressive responding that negatively correlates with diminished prolactin release in response to fenfluramine challenge (87). Interactions between AVP and 5-HT have also been implicated in pathologic aggression in patients with borderline personality disorders (83) who exhibit impairment in bonding with mates (88,89) because of partner-directed violence (90). Interactions between CRF and 5-HT also underlie aggression toward offspring in abusive rhesus macaque mothers (91). Because most neuromodulators released by amine- and peptide-containing neurons show remarkable preservation of structure and function within the animal kingdom (92), this evidence in humans and nonhuman primates, coupled with our results in prairie voles, suggests that prosocial behavior associated with pair-bonds may be subserved by evolutionarily conserved neurochemical circuitry facilitating affiliation.

In summary, our data establish the medial extended amygdala as a critical neural node in which neurochemicals interact in programming naturally occurring social behaviors associated with pair-bonding (Figure 5). These data illustrate the spatiotemporal precision of neurotransmitter release required for the expression of partner affiliation, the reversible capacity of these neuromodulatory circuits in response to changes in social stimuli, and the great utility of the prairie vole model to study neurotransmitter microcircuits in mediating and optimizing decision making and behavioral switch under specific environmental contexts.

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KLG conceived the project, conducted the experiments, contributed to data collection/analysis, and wrote the manuscript. KLG and XJ performed immunofluorescence histochemistry and confocal microscope analysis. KLG and YL performed in vivo brain microdialysis experiments. ZXW oversaw experimental design, interpretations of all acquired data, and manuscript writing.

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