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Histone deacetylase inhibitors facilitate partner preference formation in female prairie voles

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Abstract

In the socially monogamous prairie vole (*Microtus ochrogaster*), mating induces enduring pair-bonds initiated by partner preference formation and regulated by a variety of neurotransmitters including oxytocin, vasopressin, and dopamine. Here we examined potential epigenetic mechanisms mediating pair-bond regulation. We show that the histone deacetylase inhibitors sodium butyrate and TrichoStatin A (TSA) facilitate partner preference formation in female prairie voles in the absence of mating. This was associated with a specific up-regulation of oxytocin (OTR) and vasopressin V1a receptors (V1aR) in the nucleus accumbens, through an increase in histone acetylation at their respective promoter. Furthermore, TSA-facilitated partner preference was prevented by OTR or V1aR blockade in the nucleus accumbens. Importantly, mating-induced partner preference triggered the same epigenetic regulation of OTR and V1aR gene promoters as TSA. These observations thus indicate that TSA and mating facilitate partner preference through epigenetic events, providing the first direct evidence for an epigenetic regulation of pair-bonding.

Introduction

Social affiliation is an essential characteristic of human social behaviors and social cognitive deficits are common features in a multitude of neuropsychiatric disorders including schizophrenia and autism spectrum disorders, as well as addiction and depression[1]. The socially monogamous prairie voles (*Microtus ochrogaster*) have emerged as a very interesting model for the investigation of the neurobiological bases of social attachment, as both laboratory and free-living individuals establish long-term pair bonds[2–4], which are first initiated by the formation of selective affiliation behaviors towards the partner, called

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Author contributions

H.W, F.D. and Y.L performed the experiments. H.W. and F.D. analyzed the data. H.W., F.D., Z.W. and M.K designed the study. F.D., Z.W. and M.K. wrote the paper. All of the authors discussed the results and commented on the manuscript.

partner preference[5]. This formation of partner preference involves a variety of neurotransmitters and hormonal systems, including the neuropeptides oxytocin and vasopressin (AVP) and mesolimbic dopamine[5]. In general, partner preference formation is mediated through AVP neurotransmission in the ventral pallidum and lateral septum (LS) in males, and oxytocin neurotransmission in the nucleus accumbens (NAcc) and prelimbic cortex in female prairie voles[6–8]. Typically involved in natural reward such as mating, dopamine also acts as a critical mediator of partner preference in prairie voles. Activation of the dopamine D2-type receptors (D2R) in the NAcc facilitates whereas activation of the dopamine D1-type receptors (D1R) inhibits partner preference formation in both male and female prairie voles[9–11]. Importantly, variations in gene expression of oxytocin and AVP V1a receptors, OTR and V1aR, respectively, can themselves dramatically affect partner preference. In female prairie voles, for instance, the overexpression of OTR in the NAcc facilitates partner preference in the absence of mating[12–14].

Beyond the regulation of pair bonding, oxytocin and AVP are also implicated in a broad range of social behaviors, including social recognition, aggression, and maternal care[15, 16]. Notably, disruptions of the latter behavior in rodents induce long-lasting neuroadaptations through epigenetic mechanisms, including DNA methylation of estrogen receptor alpha[17] and AVP genes[18], as well as histone acetylation of the glucocorticoid receptor promoter[19]. Moreover, histone deacetylase (HDAC) inhibitors, enhancing gene expression through increased histone acetylation in the rodent brain[20], can reverse these alterations[19], and directly influence social behaviors such as sexual receptivity[21]. Importantly, in a lung cancer cell line, the HDAC inhibitor trichostatin A (TSA) directly enhances OTR transcription by locally promoting histone acetylation[22].

Therefore, an epigenetic basis in partner preference formation in prairie voles can be suggested. To test this hypothesis, we first assessed the effects of two HDAC inhibitors, sodium butyrate (NaB) and TSA, on partner preference formation in adult female prairie voles. Thereafter, we investigated the molecular mechanisms mediating the effects of TSA in inducing partner preference in female prairie voles. Finally, we sought to determine whether the epigenetic alterations induced by TSA during cohabitation were also triggered by mating.

Results

TSA treatment facilitates partner preference

Sexually naïve female prairie voles were injected intracerebroventricularly (*i.c.v.*) with CSF or CSF containing 0.08, 0.4 or 4 *n*g of TSA immediately before the 6-hour cohabitation with a male without mating, and their partner preference was then tested. Six hours of cohabitation with a male without mating does not induce partner preference formation in female prairie voles[4] and thus this behavioral paradigm has been extensively used to assess the effects of various drugs on facilitating partner preference formation[5].

CSF-treated animals showed non-selective side-by-side contact with the partner or the stranger following 6 hours of cohabitation without mating (t_{15}= 0.76, P = 0.46; Fig. 1a). However, animals treated with TSA at all doses tested spent preferentially more time with

the partner than with the stranger (t_8= 4.35, P = 0.002 for the TSA 0.08 *n*g group; t_{15}= 3.63, P = 0.002 for the TSA 0.4 *n*g group; and t_8= 2.58, P = 0.03 for the TSA 4 *n*g group). Importantly, no group differences were found in locomotor activity ($F_{3,46}$ = 1.25, P=0.30, Fig. 1b) and no aggressive behavior by the test female towards either the stranger or the partner were observed, demonstrating that the effects of TSA were specific to a social preference, rather than being secondary to an alteration of locomotion or social aversion to the stranger.

To investigate whether TSA enhances histone acetylation in brain structures involved in the formation of partner preference, a separate batch of females was injected with the medium dose of TSA (0.4 *n*g) and cohabited with a male in the absence of mating during 30 min, 2 or 9 hours. Notably, no significant variation in the global histone H3 acetylation (Lys14) levels could be detected at any time-point in both the NAcc and caudate putamen (P > 0.05 for all groups, Fig. 1c & d). This demonstrates that TSA facilitates partner preference formation in the absence of mating, despite not affecting global histone H3 acetylation in the NAcc or the caudate putamen.

Importantly, sodium butyrate also facilitated partner preference in female prairie voles following cohabitation with a male for 6 hours without mating, associated with an increase in global histone H3 acetylation (Lys14) in the NAcc (Supplementary Figure 1). The effects of TSA on partner preference formation could thus be reproduced with another HDAC inhibitor, suggesting the involvement of HDAC inhibition, rather than a non-specific effect of TSA in the facilitation of partner preference. Considering that TSA is a more specific and affine class I/II HDAC inhibitor[23, 24], and that the behavioral effects of TSA were more pronounced than NaB, we chose to use TSA over NaB for investigating the specific molecular correlates in the following parts of the study.

Molecular correlates of TSA-facilitated partner preference

As variations in gene expression levels in the vole NAcc have been associated with different mating strategies between monogamous and non-monogamous voles, and with alteration of partner preference formation in prairie voles in particular[12, 13, 25, 26], we assessed whether TSA-facilitated partner preference formation was associated with variations of gene expression in the NAcc.

TSA treatment (0.4 *n*g, *i.c.v.*) induced an increase in OTR mRNA levels in the NAcc following 2 hours of cohabitation as compared to CSF-treated controls (t_{10} = 2.38, P = 0.038, [Fig. 2a]), that tended to be sustained after 9 hours of cohabitation (t_9 = 2.17, P = 0.058, Fig. 2b). Although a slight but not significant increase in V1aR mRNA could be observed in the NAcc 2 hours following the TSA injection, no other group differences were detected at either time-point for any of the other mRNAs measured, including D1R or D2R (P > 0.05, Fig. 2a,b). Importantly, no group differences were observed in the caudate putamen at any time-point and for any mRNA measured (P >0.05 for all groups, Fig. 2c,d), suggesting that the increase in OTR mRNA observed in TSA-treated animals was specific to the NAcc. Furthermore, such up-regulation was present only following cohabitation with a male, as OTR and V1aR mRNA levels in the NAcc remained unchanged 2 hours after TSA injection without cohabitation (OTR: 100.0% ± 11.70 for CSF group, 86.7% ± 12.11 for TSA group,

t_{12} = 0.79, P = 0.444; V1aR: 100.0% ± 26.24 for CSF group, 92.3% ± 13.75 for TSA group, t_9 = 0.27, P = 0.791).

In line with higher OTR mRNA levels, TSA-treated animals also exhibited higher OTR protein levels at both time-points in the NAcc (2 hours: t_{10} = 2.34, P = 0.041; 9 hours: t_{10} = 3.16, P = 0.01, Fig. 2e,f), but not caudate putamen (t_{10} = 0.41, P = 0.69, Fig. 2g,h). Interestingly, while no significant alteration of V1aR mRNA levels could be detected in the NAcc at 2 or 9 hours after the TSA injection (Fig. 2a,b), the V1aR protein levels were significantly increased at 9 hours, as compared to CSF-treated animals, in the NAcc (t_9 = 3.46, P = 0.007, Fig. 2f) but not caudate putamen (t_{10} = 0.98, P = 0.35, Fig. 2h). Although with some variations, D1R and D2R protein levels in the NAcc and caudate putamen were not significantly affected by TSA administration (P > 0.05, Fig. 2e-h).

TSA facilitates histone acetylation of oxtr and avpr1a

The increase in both the mRNA and the protein levels for OTR following cohabitation after TSA treatment suggested that TSA likely increased the transcription of *oxtr*, the gene coding for OTR, rather than altering the translation or turnover of the protein. Moreover, V1aR protein levels were higher in the NAcc, associated with a slight but not significant increase in mRNA levels following TSA treatment (Fig. 2). Considering that TSA is a powerful class I and II HDAC inhibitor[23, 24, 27], we hypothesized that TSA increased histone acetylation at the *oxtr* and *avpr1a* promoters in the NAcc, thereafter enhancing their transcription. A new batch of animals received *i.c.v*. injection of TSA (0.4 *n*g) and immediately cohabited with a male without mating for 30 minutes before being sacrificed. The 30-min time window was chosen based on previous work reporting a maximum increase of histone acetylation after local TSA injection in rats and mice[28, 29]. H3K14 acetylation at the *oxtr* and *avpr1a* promoters was then analyzed by chromatin immunoprecipitation.

In line with the increase in OTR mRNA and protein levels previously observed, TSA-treated animals exhibited a very high increase (+460%) in histone H3 acetylation at the *oxtr* gene promoter, as compared to CSF-treated controls, in the NAcc (t_{10} = 5.88, P = 0.0002), but not caudate putamen (t_9=0.31, P = 0.76, [Fig. 3a]). Moreover, histone H3 acetylation at the *avpr1a* promoter was significantly elevated 30 min following TSA administration (+196%) in the NAcc (t_{10} = 3.12, P = 0.01) but not caudate putamen (t_9=0.38, P = 0.71), as compared to CSF-treated controls (Fig. 3b). Therefore, TSA increased histone acetylation site specifically in the NAcc as early as 30 minutes after the beginning of the cohabitation with a male.

TSA facilitates partner preference via OTR and V1aR

From the previous set of experiments, a molecular model of action emerges, where during cohabitation, TSA potentiates histone acetylation at the *oxtr* and *avpr1a* promoters, thereafter enhancing their transcription and resulting in higher OTR and V1aR protein levels up to 9 hours after the beginning of the cohabitation period. Importantly, this TSA effect is site-specific as the caudate putamen remains unaffected. Here we tested whether this TSA-induced increase in OTR and V1aR is related to the facilitation of partner preference formation. Female prairie voles received an intra-NAcc injection of TSA (0.04*n*g per side) with or without prior injection (30minutes before TSA injection, 0.5*n*g per side) of CSF or

CSF containing one of two different OTR antagonists, OTA(B) and OTA(T), or a V1aR antagonist (V1aRA). Immediately after the TSA injection, the females were cohabited with a male for 6 hours without mating, followed by a partner preference test.

CSF-treated animals did not show a partner preference (t_5 = 0.17, P = 0.87, Fig. 4a). However, TSA-treated animals spent significantly more time in side-by-side contacts with the "partner" than with the "stranger", suggesting that a single TSA injection directly into the NAcc is sufficient to facilitate partner preference formation without mating (t_5 = 7.04, P = 0.0009). Interestingly, blockade of either OTR or V1aR by pre-treatment with OTA(B), OTA(T), or V1aRA prevented the effects of TSA (P >0.05 for all groups). As no group differences were found in locomotor activity ($F_{4,32}$ = 1.89, P = 0.14, Fig. 4b), these data suggest that TSA in the NAcc facilitates partner preference formation via OTR- and V1aR-mediated mechanisms in a behavior-specific manner.

Mating induces similar neuroadaptations as TSA

Following our previous observations, we established that the epigenetic potentiation of oxytocin and vasopressin neurotransmission in the female NAcc was sufficient to facilitate partner preference formation in the absence of mating. To investigate whether these neuroadaptations also occur during natural formation of partner preference, female prairie voles were cohabited with a male during 24 hours in the presence of mating, which induces partner preference4, and sacrificed. We observed an increase in both OTR and V1aR mRNA and protein levels in the NAcc, as compared to sexually naïve females (OTR: +38%, t_{10} = 2.68, P = 0.02 for mRNA, and +58%, t_8 = 3.05, P = 0.01 for protein; V1aR: +89%, t_{14}=2.53, P = 0.02 for mRNA, and +26%, t_{20} = 2.23, P = 0.037 for protein, Fig. 5a,b).

As both mRNA and protein levels for OTR and V1aR were increased by cohabitation with mating, we next investigated whether this up-regulation was associated with an epigenetic enhancement of *oxtr* and *avpr1a* genes transcription. A new batch of females was thus cohabited with a male for 6 hours with mating and H3K14 acetylation at the *oxtr* and *avpr1a* promoters measured by chromatin immunoprecipitation. In line with OTR and V1aR mRNA and protein levels, female prairie voles exhibit higher H3K14 acetylation at the *oxtr* and *avpr1a* promoters in the NAcc, as compared to sexually naïve females (*oxtr*: t_9 = 2.64, P = 0.02; *avpr1a*: t_9 = 2.91, P = 0.017 Fig. 5c,d). These data suggest that cohabitation paradigms that reliably induce partner preference in female prairie voles trigger an up-regulation of OTR and V1aR expression in the NAcc through epigenetic mechanisms, as observed after TSA treatment.

Discussion

In the present study, we report for the first time an epigenetic regulation of partner preference formation. First, we demonstrated that increasing histone acetylation in the NAcc by administration of an HDAC inhibitor facilitates partner preference formation in adult female prairie voles in the absence of mating. Then, we unveiled direct evidence that partner preference formation in females is epigenetically driven, as cohabitation and mating with a male increased *oxtr* and *avpr1a* genes expression through enhanced histone acetylation in the NAcc. TSA administration in the NAcc induced partner preference and led to higher

levels of OTR mRNA and proteins in the NAcc. Moreover, although the global histone H3 acetylation was unaffected in TSA-treated females, a marked enrichment of histone acetylation at the *oxtr* promoter in the NAcc was observed as early as 30 minutes following TSA administration. Finally, blocking OTR in the NAcc was sufficient to prevent the TSA-facilitated partner preference. Since similar epigenetically-driven modifications were detected following cohabitation with mating, under procedures known to induce partner preference, our data put forward a model for an epigenetic regulation of social behavior. During cohabitation with a male, TSA, or mating, rapidly induces a specific histone H3 acetylation at the *oxtr* promoter in the NAcc that enhances its transcription, resulting in higher OTR mRNA and protein levels, which thereafter facilitate partner preference formation.

In female prairie voles, 6 hours of cohabitation with a male without mating does not induce partner preference formation[4], and this behavioral paradigm has been used to examine the effects of pharmacological manipulations on the induction of partner preference[5]. In our study, while saline- or CSF-treated controls did not develop partner preference, female prairie voles treated with NaB or TSA did. As neither NaB nor TSA affected the overall locomotion, their effects on partner preference seemed to be behavior-specific rather than secondary effects on locomotion. This specific effect of TSA was further confirmed by our molecular observations. Indeed, although administered *i.c.v.*, we were able to detect a specific alteration of gene expression in the NAcc but not in an adjacent structure, the caudate putamen. In addition, even within the NAcc, D1R and D2R mRNA and protein levels remained unaffected. Such specificity appears to be surprising for a broad HDAC inhibitor like TSA which affects both class I and II HDACs. Nevertheless, TSA has been reported to affect the expression of only a small subset of genes in the mammalian genome[30–32], including in mice[20].

We demonstrated here that acetylation of histone H3 on Lys14 at the *oxtr* promoter, a modification associated with enhanced gene transcription including during cerebral plasticity[33, 34], underlines higher OTR mRNA and protein levels. In response to TSA, histone acetylation at the *oxtr* promoter increases and facilitates activation of its transcription in human cell line[22], supporting our finding that *oxtr* can be regulated epigenetically. Since a local blockade of OTR in the NAcc was sufficient to prevent the behavioral effects of TSA, our data suggest that the TSA-induced expression of OTR in the NAcc during cohabitation mediated the facilitation of partner preference formation. Moreover, 24 hours of cohabitation with mating, a procedure known to reliably induce partner preference in female prairie voles, induced a similar increase in OTR expression in the female NAcc . This is in complete agreement with the known involvement of oxytocin and its receptor in the neurobiology of partner preference formation in female voles. Mating induces an increase in extracellular oxytocin levels in the NAcc[25], and local infusion of oxytocin into the NAcc facilitates partner preference formation in the absence of mating[8]. Moreover, OTR antagonists block partner preference formation induced by oxytocin administration or mating[8, 35]. Importantly, the viral-mediated overexpression of OTR in the female NAcc is sufficient to facilitate partner preference formation[12, 13]. In addition to strengthening the role of OTR, our results also provide evidence for an activation of OTR

gene expression through epigenetic mechanisms during cohabitation with a male in the absence of mating. Indeed, although insufficient to induce partner preference, such cohabitation without mating for short periods of time activates the neurobiological processes underlying partner preference formation. For instance, two hours of free exposure to a male induce slight but non-significant elevations in oxytocin release in the female NAcc[25]. It can therefore be proposed that TSA or NaB potentiate the neuroadaptations induced by cohabitation with a male, facilitating the development of partner preference. Interestingly, such potentiation has already been reported in rodents where class I and II HDAC inhibitors, including NaB and TSA, facilitate consolidation of a learning event that does not result in long-term memory formation in control animals[36, 37]. In support of this notion, longer periods of cohabitation (*e.g.*, 48hours) can induce partner preference even in the absence of mating[4]. It is also important to note that cohabitation with mating triggered in the female NAcc an up-regulation of OTR and V1aR expression through the same epigenetic mechanisms as those observed after cohabitation with TSA treatment, which demonstrate that TSA and mating affect the same pathways to promote partner preference formation. Importantly, TSA does not induce an upregulation of OTR and V1aR in the female NAcc in the absence of cohabitation with a male. Altogether, this supports the hypothesis that TSA facilitates the formation of partner preference through the potentiation of endogenous neuroadaptations naturally triggered by the cohabitation with a male, rather than activating on its own these or different neuroadaptations.

Our study also highlights a critical role of the NAcc V1aR in female's partner preference formation, as TSA-treated animals exhibit higher V1aR levels, whose blockade prevented TSA-facilitated partner preference formation. Moreover, these effects were associated with higher histone acetylation at the *avpr1a* promoter, despite no significant elevation of V1aR mRNA likely due to a non-optimal time-point. Although we cannot rule out a regulation of protein stability by TSA through acetylation of non-histone proteins[38], this finding suggests that, similar to the *oxtr* promoter, TSA might promote *avpr1a* transcription through local histone acetylation. While the contribution of AVP in male's pair bonding has been described[5], its role in the female's behavior is still controversial. On one hand, an *i.c.v.* AVP injection facilitates partner preference formation in both male and female voles, which is prevented by blockade of either V1aR or OTR[39]. On the other hand, an *i.c.v.* injection of the V1aR antagonist blocks the mating-induced partner preference in male, but not in female, prairie voles40. However, all these studies used *i.c.v.* injections, preventing any further insight into the structures involved. Here, we provide the first evidence that AVP neurotransmission within the NAcc can be involved in partner preference formation in the female voles, while most of the literature describes its involvement in different areas such as the ventral pallidum, lateral septum, the bed nucleus of the stria terminalis, and amygdala in males[5]. It therefore becomes interesting to note that the blockade of either OTR or V1aR in the female's NAcc was sufficient to prevent partner preference formation following TSA treatment, suggesting that partner preference formation requires the activation of both V1aR and OTR. This finding is in line with, and supports, an earlier observation in male prairie voles that a concurrent access to both OTR and V1aR in the LS is essential for AVP-induced partner preference[6]. Moreover, the observation of a specific increase in both OTR and V1aR

levels in TSA-treated animals further supports the requirement of a simultaneous activation of the AVP and oxytocin neurotransmissions for pair bonding.

In combination with oxytocin and AVP, the dopamine neurotransmission in the NAcc modulates in partner preference formation in the female voles[9]. Although mating induces dopamine release in the NAcc[9], variations in receptor levels are observed only after an extended period -longer than 24h- of cohabitation with mating, important to the maintenance of pair bonding[10]. In line with these observations, female prairie voles treated with TSA did exhibit partner preference without significant variation in the dopamine D1R and D2R receptors. Therefore, this absence of dopamine receptor regulation provides another proof for the specificity of TSA.

Our data report for the first time an epigenetic component in the neurobiology of pair-bonding, and suggest that TSA induces a “permissive state” in female prairie voles, potentiating the natural molecular response to the cohabitation, and promoting the formation of stronger social interactions leading to partner preference. It is therefore tempting to hypothesize that a TSA-facilitated partner preference could be further strengthened and lead to persistent bond. Although the specific HDACs involved remain to be identified, it would thus be interesting to further investigate the effects of TSA on other behaviors associated with the monogamous life strategy in prairie voles, such as selective aggression and bi-parental care. Considering the relevance of the prairie voles in modeling the neurobiological mechanisms of pair bonding in humans[5], and the promising HDAC inhibitors already in clinical trials[24, 41, 42], our data pave the way for new pharmacological possibilities to influence social behaviors.

Methods

Subjects

Sexually naïve female prairie voles (*Microtus ochrogaster*) from a laboratory breeding colony were weaned at 21 days of age and housed in same-sex sibling pairs in plastic cages (12 × 28 × 16 cm) with water and food provided *ad libitum*. All cages were maintained under a 14:10 h light-dark cycle, and the temperature was approximately 20°C. All animals were randomly assigned into experimental groups when they reached 70–90 days of age. The number of animals used was based on previous studies in the field by our group and others, combined with a power analysis. Experimental procedures were approved by the Institutional Animal Care and Use Committee at Florida State University.

Drugs

Sodium butyrate (NaB), dissolved in saline, and Trichostatin A (TSA), dissolved in artificial cerebrospinal fluid carrier (CSF, BioFluids, Rockville, MD) were both purchased from Sigma-Aldrich (St Louis, MO). NaB was injected intraperitoneally (*i.p.)* at a dose of 600 mg/kg, which is known to induce histone acetylation in several brain structures in mice[43, 44]. Similarly, the dose-range used for TSA was based on previous work determining its effectiveness in inducing local histone acetylation events and variations in gene expression in rodents[19, 20]. The selective V1aR receptor antagonist V1aRA, *d*$(CH_2)_5$[Tyr(Me)]AVP,

and the OTR antagonist OTA(B), [$d(CH_2)_5$, Tyr(Me)^2, Thr^4, Tyr-NH_2^{9}]-OVT), were obtained from Bachem (Torrance, CA). A second, more selective OTR antagonist, OTA(T), *d*Gly-NH_2-$d(CH_2)_5$ [Tyr(Me)^{2,Thr4}]OVT45, was kindly provided by Dr. Maurice Manning (University of Toledo, OH). These antagonists and doses used have been chosen based on previous studies demonstrating their selectivity for either V1aR or OTR, respectively[35, 39, 46–49].

Stereotaxic cannulation and microinjection

Females were anesthetized with sodium pentobarbital (1mg/10g body weight), and 26 gauge stainless steel guide cannulae (Plastics One, Roanoke, VA) were stereotaxically implanted, aimed to the lateral ventricle (unilaterally; nose bar at −2.5 mm, 0.6 mm rostral, 1.0 mm lateral, and 2.6 mm ventral to bregma) or site-specifically to the NAcc (bilaterally; nose bar at −2.5 mm, 1.7 mm rostral, ±1.0 mm bilateral, and 4.5 mm ventral to bregma). After 3 days of recovery, subjects received microinjections of either CSF or CSF containing different concentrations of TSA. When selective antagonists for OTR or V1aR were used, they were injected 30 minutes prior to TSA. Injections were made with a 33 gauge needle that extended 1 mm below the guide cannula into the target area, in an injection volume of 500 nL into the lateral ventricle (*i.c.v.*) or 200 nL per side into the NAcc. The needle was connected to a Hamilton Syringe (Hamilton, Reno, NV) through polyethylene-20 tubing and plunger depression was performed slowly, requiring 1 minute per injection. At the end of the experiment, all subjects were sacrificed by quick decapitation and the brains extracted to verify cannulae placement by an observer blind to experimental conditions. Subjects with misplaced cannulae were excluded from data analysis.

Cohabitation and partner preference test

Immediately following *i.p.*, *i.c.v.*, or intra-NAcc injections of drugs, the females were cohabited with a male for 6 hours without mating. The absence of mating was verified by examining the videotaped behavior. For the investigation of the neuroadaptations triggered by cohabitation with mating, estrogen-primed females (2μg per day, *i.p.*, for 3 days) were cohabitated with a male during 6 or 24 hours, and the presence of mating was verified *a posteriori* on videotape (ranging from 6 to 11 bouts during the first 6 hours of cohabitation).

The partner preference test was performed immediately following the 6-hour cohabitation, as previously described[11]. Briefly, the three-chamber testing apparatus consisted of a neutral cage connected to two parallel identical cages, each housing a stimulus animal - an unfamiliar male “stranger” or a familiar male “partner” used during the cohabitation period. Female subjects were free to move throughout the apparatus during the 3-hour testing, and the stimulus males were tethered within their cages, allowing no direct contact with each other. The entire session was videotaped and the duration of the subject’s side-by-side contact with either the partner or stranger was later quantified by a trained experimenter unaware of the biological groups. A partner preference was defined as subjects spending significantly more time in body contact with the partner versus stranger, as determined by a paired, two-tailed *t*-test. In addition, the three-chamber apparatus was equipped with photobeam-sensors, allowing the determination of locomotor activity indicated by the number of entries of the female into the stimulus chambers. This locomotor score thus

allows us to control for putative secondary effects of the drugs on the females' behavior, such as general activity, anxiety, or altered exploration of a novel environment, as commonly used by our group and others[12].

RNA and proteins extraction

Females were sacrificed by rapid decapitation, and brains were immediately extracted and frozen on dry ice. Coronal sections (200 μm) were cut on a cryostat and frost mounted onto microscope slides. Bilateral tissue punches with a 1 mm diameter were taken from the entire NAcc and caudate putamen, the latter being a control area, and stored at −80°C until processed. Total RNA and proteins were extracted using the TRI-Reagent protocol according to manufacturer's instructions (Molecular Research Center, Cincinnati, OH).

Protein expression analysis by Western-blot

Following separation on a 10% polyacrylamide gel (15% for histones), proteins were transferred to a nitrocellulose membranes and incubated with the following primary antibodies: anti-OTR (sc-8102, 1:1000), -V1aR (sc-18096, 1:500), -D1R (sc-33660, 1:1000), -D2R (sc-9113, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), -actin (A2066, 1:1000, Sigma Aldrich. St Louis, MO), or anti-acetyl histone H3 (Lys14, #06–911, 1:1000) and total H3 (#05–928, 1:1000, Millipore, Temecula, CA). All antibodies are validated for their use in humans, rats, and mice, with which prairie voles share high percentages of homology (ranging from 81 to 96%). After hybridization with an HRP-conjugated secondary antibody, membranes were revealed with ECL (ECL SuperSignal West Dura substrate, Pierce Biotechnologies, Rockford, IL) and exposed on Fuji XAR film (Fuji Film, Tokyo, Japan). Quantification was performed using AIS 6.0 Image software (Imaging Research, St. Catharines, Ontario, Canada), and all signals were normalized within the same membrane to actin, except for the acetyl-H3 signal which was normalized to the total histone H3 signal. Normalized data are then expressed as percentage of CSF-treated animals.

Semi-quantitative real-time polymerase chain reaction (RT-PCR)

0.5 μg of total RNA was processed for complementary DNA synthesis, and then analyzed as previously described[50] with normalization to the nicotinamide adenine dinucleotide dehydrogenase (NADH) gene. All reactions were done in triplicates and their specificity verified by melting curve analysis and separation on a 2% Agarose gel. The primers sequences used were as follows: 5'-TCCAAGGCCAAAATCCGCACGG-3' (Fwd) and 5'-GGCAGAAGCTTCCTTGGGCGC-3' (Rev) for OTR, 5'-GAGGTGAACAATGGCACTAAAACC-3' (For) and 5'-CCAGATGTGGTAGCAGATGAAGC-3' (Rev) for AVP1aR, 5'-TTAACAACAATGGGGCTGTG-3' (For) and 5'-GGCATGAGGGATCAGGTAAA-3' (Rev) for D1R, 5'-GTGAAGGCGCTGTAGAGGAC-3' (For) and 5'-CGGTGTGTTCATCATCTGCT-3' (Rev) for D2R, and 5'-CTATTAATCCCCGCCTGACC-3' (For) and 5'-GGAGCTCGATTTGTTTCTGC-3' (Rev) for NADH. The normalized data are expressed as a percentage of CSF-treated animals.

Chromatin Immunoprecipitation

Histone H3 acetylation (Lys14) in NAcc and caudate putamen tissue punches was analyzed by using the Magna ChIP protein G Tissue Kit (Millipore, Temecula,CA) following the manufacturer's instructions. Briefly, after crosslinking with 1% formaldehyde, chromatin was sheared using a Misonix XL-2000 to fragments of 200–600 bp. Immunoprecipitation of acetylated histone H3 (Lys14) was then realized with 10μg of anti acetyl-H3(Lys14) antibody (Millipore) overnight at 4°C. After washes, elution from beads and reversal of cross-link, immunoprecipitated DNA was purified and analyzed in triplicates by RT-PCR on an iCycler platform (see above) with an internal standard curve made from pooled INPUT samples. The primers were designed to amplify a 236 bp-long region located 128 bp upstream of the first exon coding for the prairie vole OTR (*oxtr*, Genbank accession #AF079980), or 192 bp-long region located 141 bp upstream of the first exon coding for the prairie vole V1aR (*avpr1a*, Genbank accession #AF069304). The sequences were as follow: 5'-CTCCGGAGCCGGGGCTAAGT-3' (Fwd) and 5'-ACCGCTTCCCCGAGAGTAGGG-3' (Rev) for *oxtr*, and 5'-GGTGGACCAGCCAGACCCCA-3' (Fwd) and 5'-TGCAGAGCCAGGCGCTTTCC-3' (Rev) for *avpr1a*. Each sample was normalized by the respective INPUT value, and data are then expressed as a percentage of CSF-treated animals.

Statistical analyses and data processing

For analyses of partner preference, animals that displayed mating behaviors during the cohabitation period or with misplaced cannulae were excluded. For all other molecular analyses, a maximum of one data point per biological group was excluded when identified as outlier. Most of the experiments were replicated, except when the results were very clear. The time spent in side-by-side contact with either stimulus animal during the partner preference test was analyzed with a two-tailed paired *t*-test. The locomotion scores were analyzed using a two-tailed *t*-test (for two groups) or a one-way ANOVA (for more than two groups), and when appropriate, Fischer's PLSD *post-hoc* tests were conducted with a significance threshold of $P < 0.05$. After verification of normality, all other data were analyzed with a two-tailed *t*-test assuming equal or unequal variances tested beforehand. All statistical analyses were performed using the StatView software (SAS Institute). When data are standardized to their respective controls (% of CSF, Saline or Mating groups), the statistical analyses were conducted on the raw data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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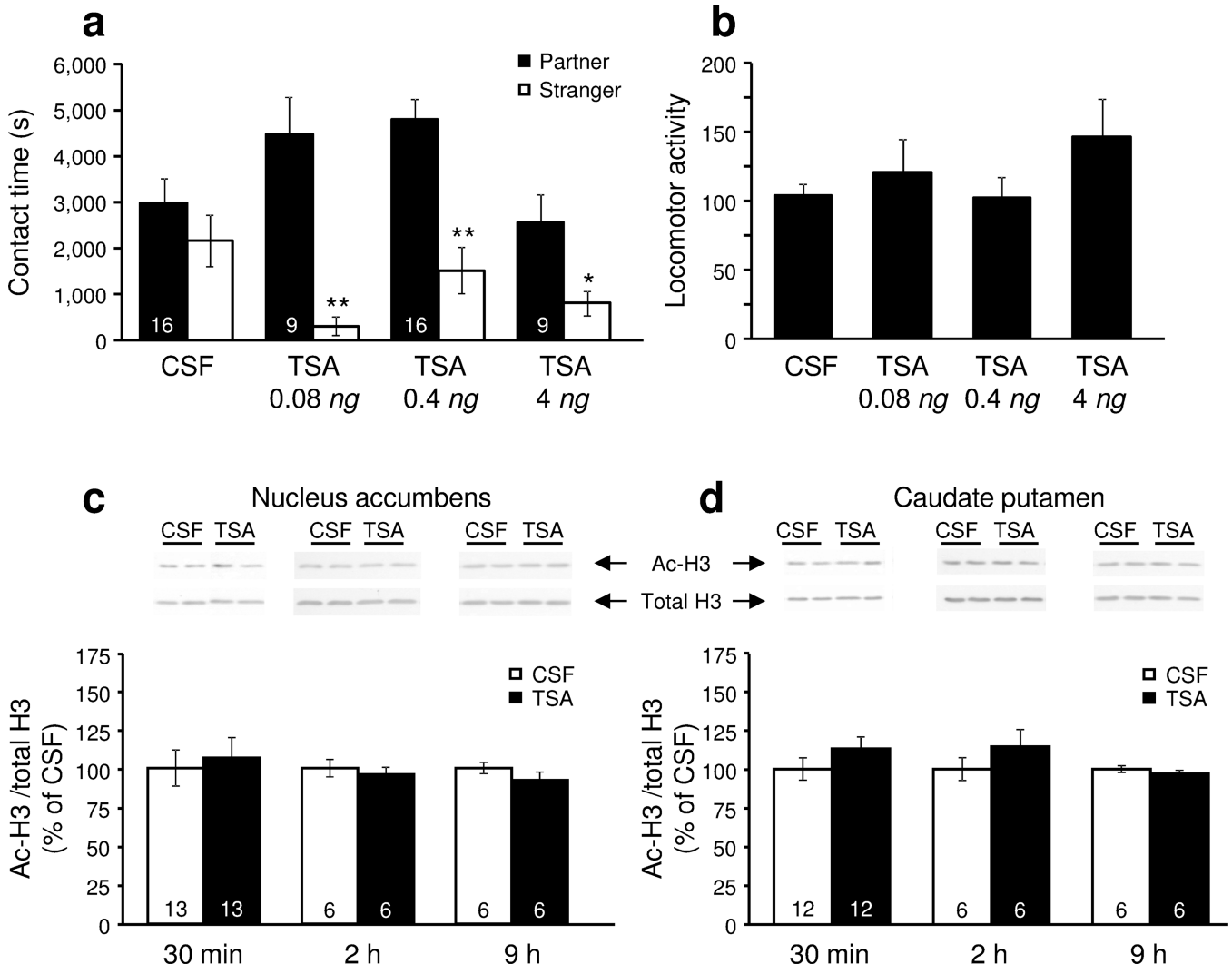

Figure 1.

An acute injection of trichostatin A (TSA) facilitates partner preference formation in female prairie voles in the absence of mating. (a) Cerebrospinal fluid (CSF)-treated females exposed to a male for 6 hours in the absence of mating showed non-selective side-by-side contact during the partner preference test, while females injected with 0.08, 0.4 or 4 *n*g of TSA spent more time with the partner than with the stranger. (b) The TSA injection did not influence the locomotion of the animals, at any of the doses used. (c,d) At 0.4 *n*g, TSA does not significantly alter the global histone H3 acetylation (Lys14) levels in the NAcc, or the caudate putamen, as measured 30 minutes, 2 and 9 hours after the beginning of the cohabitation period. (c,d) Typical blots of CSF-, or TSA-treated animals are shown above the figures and full-length blots are presented in the Supplementary Figure 2. The number of animals is indicated within columns. **P* < 0.05, ***p* < 0.01 versus *Partner* (a), unpaired two-tailed paired *t*-test. Data are represented as mean ± SEM.

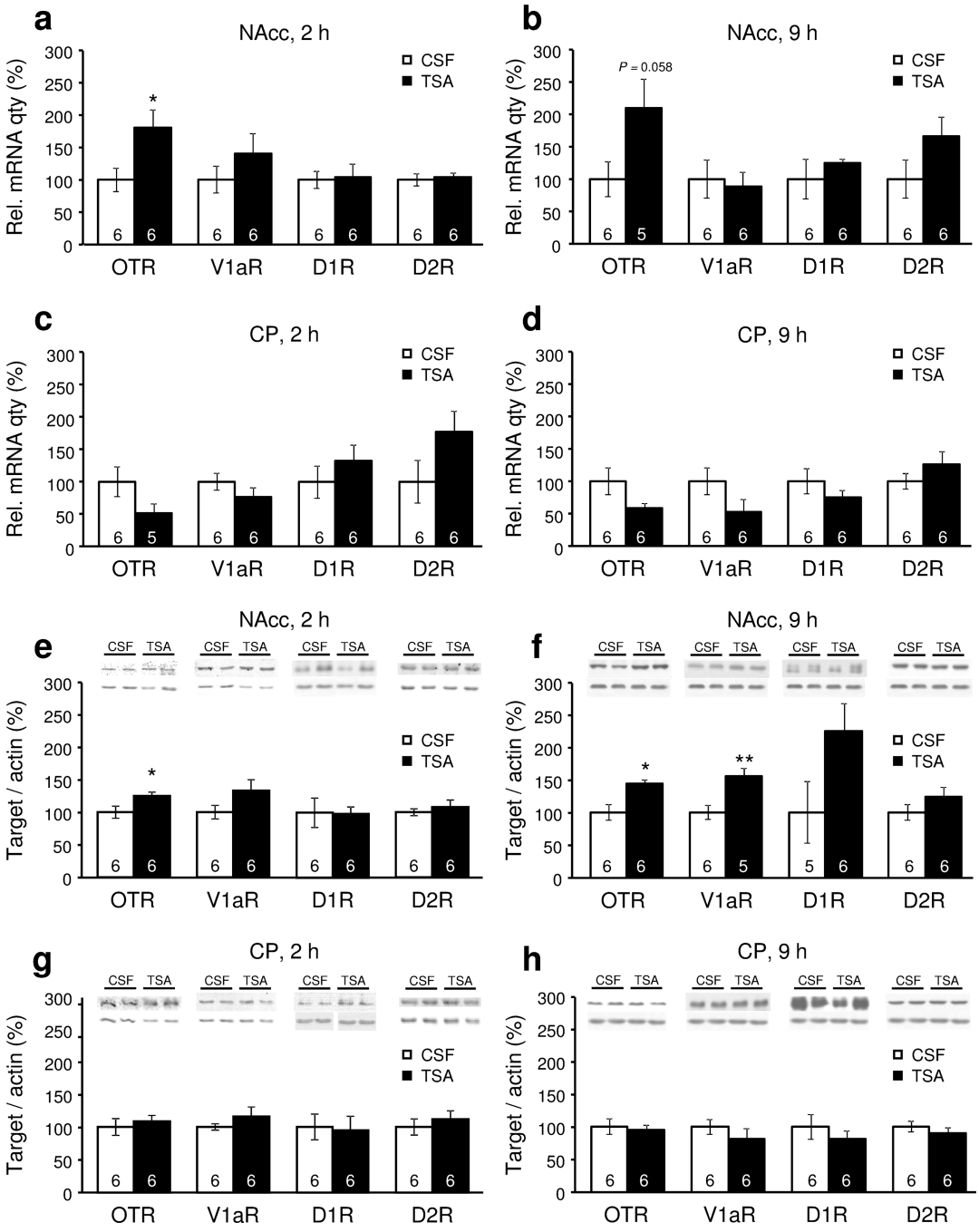

Figure 2.

TSA treatment (0.4 *n*g) up-regulates oxytocin (OTR) and vasopressin (V1aR) receptors in female prairie voles during cohabitation with a male in the absence of mating. OTR mRNA (a,b) and protein (e,f) levels were up-regulated following 2 (a,e) and 9 hours (b,f) of cohabitation without mating in the nucleus accumbens of TSA-treated females. Similarly, V1aR protein levels were increased following 9 hours of cohabitation (f), while D1R and D2R remained unaffected at all time-points. (c,d,g,h) No variations were observed for any target in the caudate putamen. In (e-h), representative blots for each target protein (top line)

and actin (bottom line) are shown above their respective columns, and full-length blots are presented in the Supplementary Figure 3. The number of animals is indicated within columns. *$P < 0.05$, **$P < 0.01$ versus CSF, two-tailed unpaired *t*-test. Data are represented as mean ± SEM. NAcc: nucleus accumbens, CP: caudate putamen.

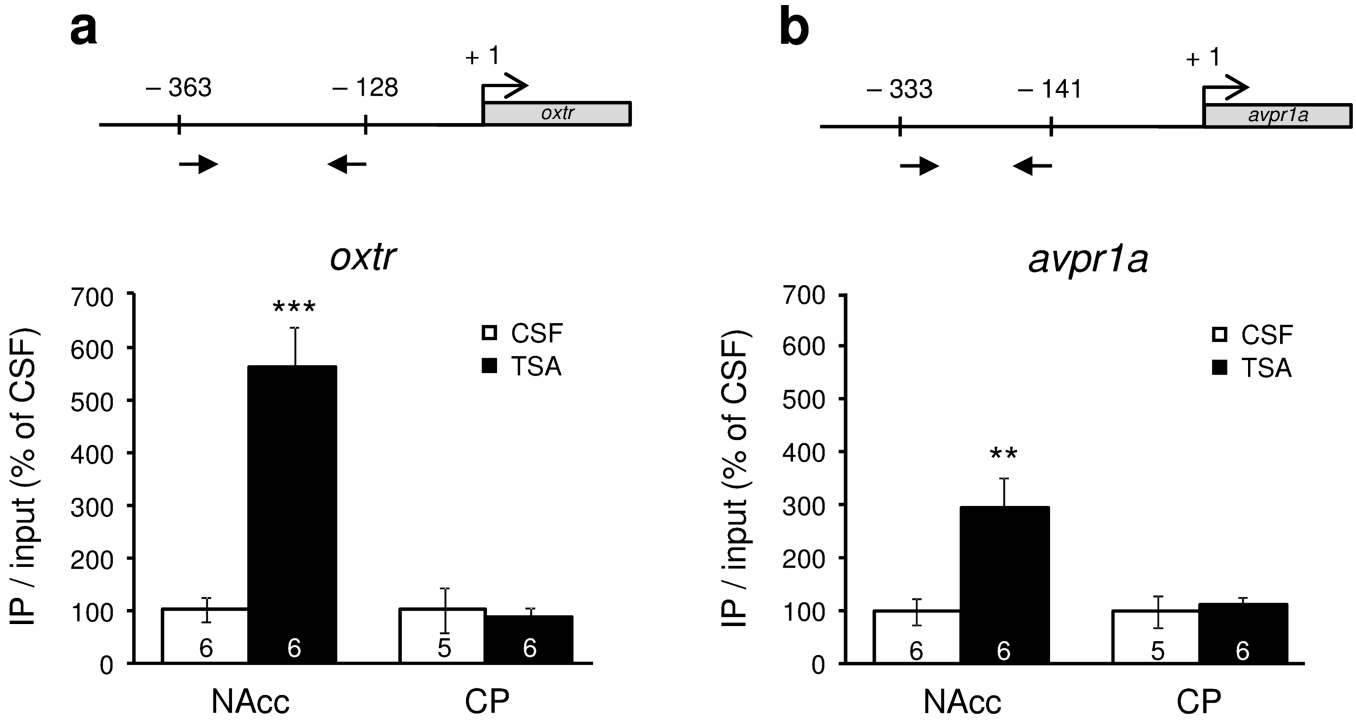

Figure 3.

TSA treatment enhances histone acetylation of *oxtr* and *avpr1a* promoters during cohabitation with a male in the absence of mating. Histone H3 acetylation (Lys14) at *oxtr* (a) and *avpr1a* (b) promoters was increased in the nucleus accumbens (NAcc) but not caudate putamen (CP) of females prairie voles treated with TSA (0.4 ng) following 30min of cohabitation with a male in the absence of mating. A schematic map of each promoter is shown above each figure with the respective primers used (arrows) and their position relative to the transcription start site (+1 site). The number of animals is indicated within columns. $^{**}P < 0.01$, $^{***}P < 0.001$ versus CSF, two-tailed unpaired *t*-test. Data are represented as mean±SEM.

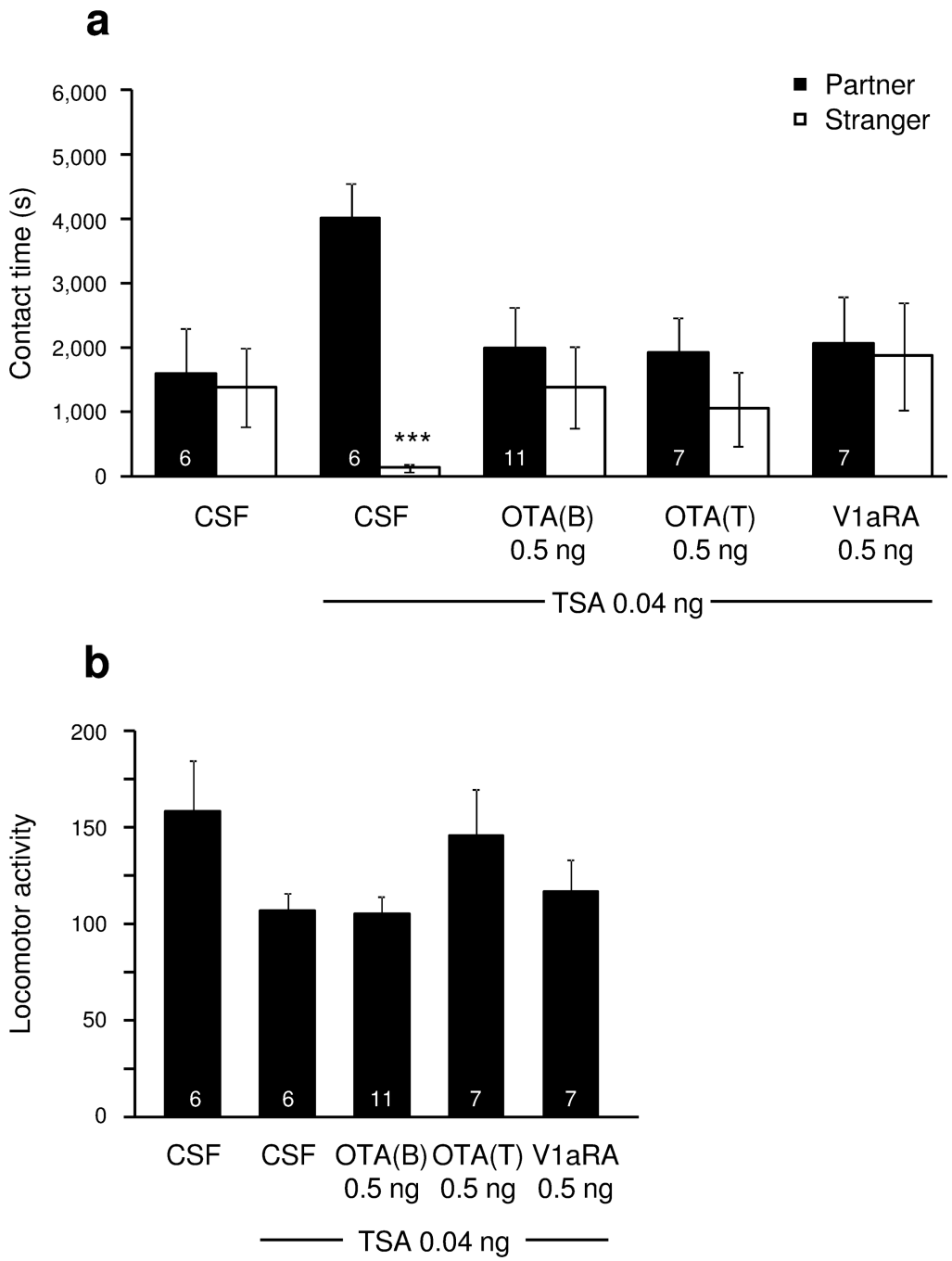

Figure 4.

TSA-facilitated partner preference requires the oxytocin- (OTR) and vasopressin (V1aR) receptors-mediated neurotransmission in the female nucleus accumbens. (a) TSA facilitates partner preference when infused into the nucleus accumbens (0.04 *n*g per side), but its effects are prevented by pre-administration of OTR or V1aR antagonists 30 minutes prior. (b) The locomotion remained unaffected by any of the treatments. The number of animals is indicated within columns. ***$P < 0.001$ versus *partner*, two-tailed paired*t*-test. Data are represented as mean ± SEM.

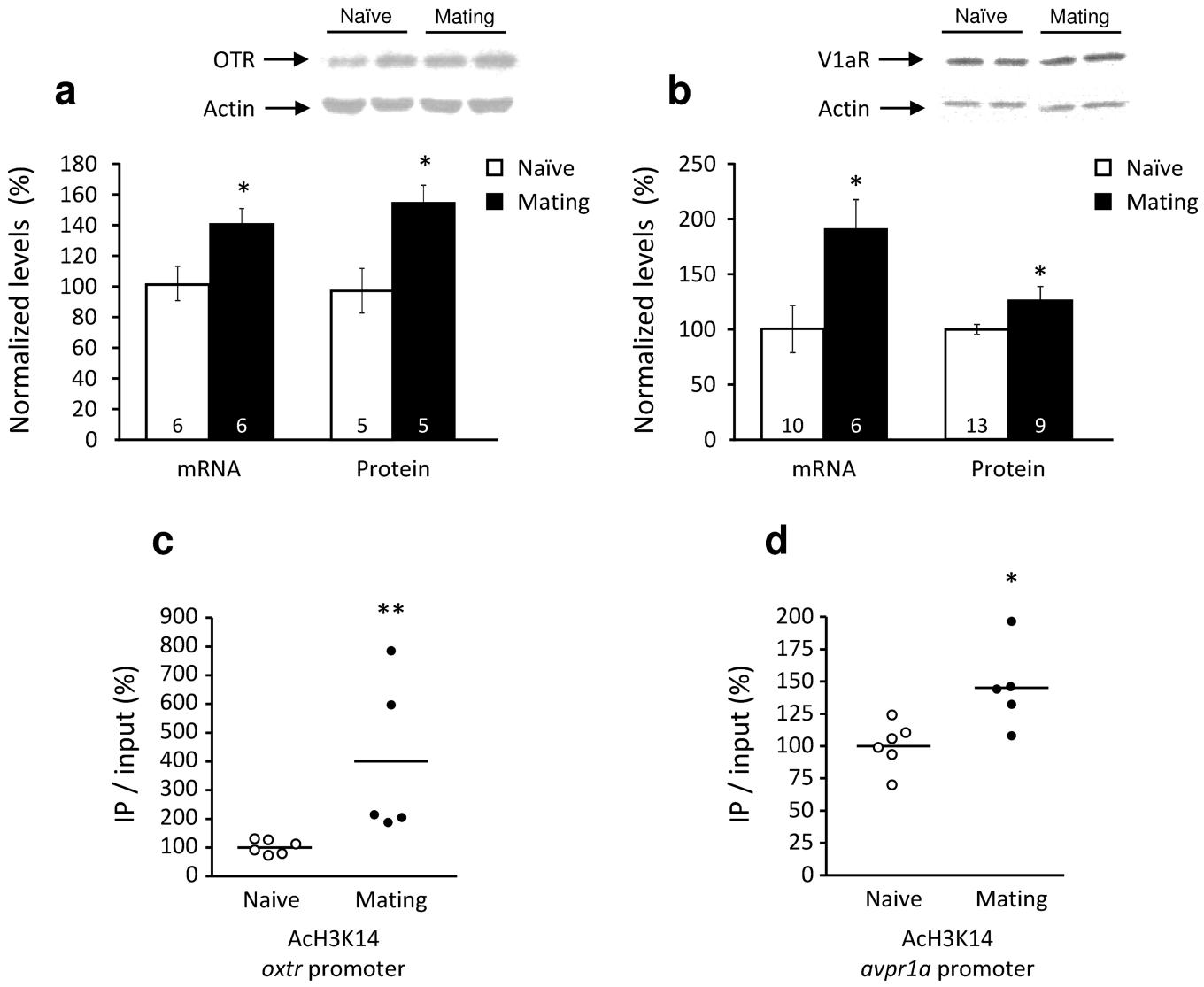

Figure 5.

Cohabitation with mating induces an upregulation of the oxytocin (OTR) and vasopressin (V1aR) receptors in the nucleus accumbens (NAcc) of female prairie voles. 24h Cohabitation with a male with mating up-regulates OTR (a) and V1aR (b) mRNA and protein levels. Accordingly, histone H3 (Lys14) acetylation in the nucleus accumbens at the *oxtr* (c) and *avpr1a* (d) promoters was increased following 6 hours of cohabitation with mating. In (a,b), typical blots of "Naïve" and "Mating" groups are shown above the figure and full-length blots are presented in the Supplementary Figure 4, and the number of animals is indicated within columns. **P* < 0.05, ***P* < 0.01 versus naïve,two-tailed unpaired *t*-test. Data are represented as mean ± SEM in (a,b), or as individual data points with mean (black bar) in (c,d).