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Inhibition of V1a-like receptors disrupts male-male affiliative relationships formed by triadic cohabitation in large-billed crows

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Competing interests

We declare that we have no competing interests.

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Abstract

Affiliative relationships, such as parent-offspring bonds, male-female pair-bonds, and within-sex coalitions/alliances, are beneficial for group-living animals to increase fitness and reproductive success. Male-male affiliative relationship, such as coalitions/alliances, have been reported in social mammals and birds in situations where three or more individuals interact, suggesting that male-male affiliative relationships can be formed via interactive situations involving three (or more) individuals. However, no experimental studies have tested this hypothesis on social animals. Many studies on mammals and birds have revealed that an affiliative relationship is underpinned by the oxytocin (OT)/vasopressin (VP) system. However, the role of the OT/VP system has been studied only in parent-offspring bonds and pair-bonds and is lacking in male-male affiliative relationships. In this study, we found the 2-week cohabitation of three male crows in an aviary facilitated affiliative relationship formation between two of the three crows (Experiment 1). In Experiment 2, we examined the effects of peripheral administration of vasopressin 1a receptor (V1aR) antagonist peripheral administration on the social behaviour of affiliated males. We found that the V1aR antagonist caused the disappearance of affiliative behaviour and the resurgence of agonistic behaviour in affiliated males. Our findings provide the first evidence for V1aR involvement in male-male affiliative relationships in vertebrates.

Keywords:

affiliative relationship, vasotocin, vasopressin, vasopressin 1a receptor, dominance relationship, birds

1. Introduction

Affiliative relationships, such as parent-offspring bonds [1, 2], male-female pair-bonds [3, 4, 5], and within-sex coalitions/alliances [6], are beneficial for individuals living in groups to increase the fitness of resource acquisition and, ultimately, reproductive success. In particular, an affiliative relationship between same-sex individuals, characterised by exchanging non-sexual affiliative behaviours, including social grooming (allogrooming in mammals and allopreening in birds), huddling, spending time together, and sharing food, is typically called a coalition or alliance which is a form of cooperation in agonistic conflicts to counter dominant individuals [6].

Male-male affiliative relationships such as coalitions/alliances have been reported in various mammals (including humans, *Homo sapiens*, [7, 8, 9, 10]; chimpanzees, *Pan troglodytes*, [11, 12, 13]; baboons, *Papio*, [14, 15]; macaques, *Macaca radiata*, [16]; *M. fuscata*, [17]; cetaceans bottlenose dolphins, *Tursiops truncates*, [18, 19, 20]; spotted dolphins, *Stenella frontalis*, [21]; sperm whales, *Physeter macrocephalus*, [22]; carnivore lions, *Panthera leo*, [23]; cheetahs, *Acinonyx jubatus*, [24, 25]; and birds, corvids, [26]). Most of these studies shared the fact that male-male affiliative relationships were formed in social interactions involving multiple participants, typically two individuals *vs* one individual, in both the wild and in captivity. Theoretical research to explore the social factor of coalition formation predicted that the social interaction among three individuals different in "power" and "activity" should be the minimal group size to produce coalition/alliance [27, 28, 29]. Several previous studies with human participants have examined the combinations of ranks that facilitate the formation of coalitions in game situations [30, 31]. However, to date, no studies have experimentally tested in non-human animals whether social interactions among three individuals, especially males, cause affiliative relationships between two of the three individuals.

Neural mechanisms underlying affiliative relationships have been extensively studied in mammals and birds. Neuropeptides of the arginine vasotocin (AVT) family include arginine vasopressin (VP) in mammals, arginine vasotocin (VT) in non-mammalian vertebrates, and oxytocin (OT). In particular, VP/VT has been shown to crucially modulate sexual behaviour, non-sexual affiliative behaviour, and aggression, particularly in males, based on the affiliative relationship with specific individuals such as partner females of pair-bonds [32, 33]. In mammals, the central infusion of VP in male monogamous prairie voles facilitates affiliative behaviour towards partner females [32]. Vasopressin 1a receptor (V1aR) antagonism of the lateral septum (LS) in male prairie voles selectively violates affiliative body contact behaviour with partner females [34]. The role of VT mediated by V1aR in affiliative relationships in birds is poorly understood. A previous study on monogamous zebra finches reported an increase of V1aR mRNA expression in the auditory pallium

associated with pair-bond formation [35]. In contrast, another study on zebra finches showed that antisense-based VP knockdown had no effect on the formation of pair-bonds in males [36]. In terms of male-female bonds, previous findings on the role of VT mediated by V1aR in affiliative relationships have been inconsistent in birds.

The role of VT-mediated V1aR in inter-individual affiliation, other than pair-bonds, has been suggested by species-comparison studies in estrildid finches with different gregariousness. Goodson and Wang [37] found that VT neurons in the medial bed nucleus of the stria terminals (BSTm) were activated, in terms of immediate early gene Fos expression, in response to the exposure of same-sex conspecifics in gregarious species but did not in asocial ones. It has also been found that the activation of VT neurons is specifically induced by social stimuli with a positive valence to enhance affiliative behaviour [37]. Antisense-based knockdown of VT production in the BSTm of male zebra finches, a gregarious species, increased aggressive behaviour and reduced courtship in colony housing situations [38]. The relevance of social affiliation and VT signals, specifically in males of gregarious birds, has been suggested to be mediated by V1aR. A previous study reported that the density of V1aR was higher in gregarious species than in asocial territorial species [37]. V1aR antagonism in the LS of male zebra finches was found to reduce flock size in housing conditions [39]. These previous findings suggest a role for VT and V1aR in the social affiliation of males, raising the hypothesis that VT is mediated by V1aR in the affiliative relationships between males. However, the involvement of neuroendocrine mechanisms, such as V1aR-mediated VT signals, in male-male affiliative relationships in birds and mammals is yet to be reported.

Large-billed crows (*Corvus macrorhynchos*) are ideal models for investigating male-male affiliative relationships. Non-breeder individuals form fission-fusion aggregations in a wide range of areas, behaving as free floaters, akin to other *Corvus* species [40, 41, 42]. Such social dynamics in *Corvus* species provide an ecological basis for the formation of affiliative relationships among males as counter-tactics against dominant birds. Indeed, in various *Corvus* species, affiliative relationships are formed by exchanging affiliative behaviours such as allopreening [26, 43, 44, 45, 46, 47]. Similar to other *Corvus* species, large-billed crows, mostly in captive non-breeder groups, have been found to strictly form a linear dominance hierarchy in males [40, 48, 49, 50] and allopreening often occurs between males. Allopreening is primarily initiated by dominants but also occurs from subordinates [50].

Given the triadic interactive situation as an ecological factor that facilitates the formation of and involvement of V1aR in affiliative relationships, we hypothesised that cohabitation in male triads should facilitate the formation of male-male affiliative relationships in a dyad of non-breeder large-billed crows and

that V1aR should be involved in the maintenance of male-male affiliative relationships. We conducted two experiments to test our hypotheses. In Experiment 1, we introduced three male crows, those which had formed dominance relationships but not affiliative relationships prior to this study, to a triadic cohabitation in an aviary for two weeks and examined the formation of an affiliative relationship in one of the three possible dyads by comparing the occurrence of reciprocal allopreening before and after cohabitation. In Experiment 2, if an affiliated relationship was formed in certain male-male dyads in Experiment 1, we examined the effects of peripheral administration of a V1aR antagonist on social behaviour. We examined whether allopreening was reduced and, aggression by dominants and submission by subordinates increased between males with affiliated relationships.

2. Methods

Subjects and housing

We used 16 adult male crows aged between 4 and 7 years with body weights ranging from 640 to 790 g. Sex was determined using DNA from blood samples [51]. The birds were caught as yearlings in Tokyo and neighbouring areas during 2017–2019 with permission from the Ministry of the Environment Government of Japan (authorisation nos. 29030001, 30081482, 30030019, and 30224001). After being captured, the subject crows were housed in one of three different groups with 5–10 other crows, which were not used in this study (Table S1), in outdoor aviaries (W 5 m × D 10 m × H 3 m). Three to six months before the start of this study, the crows were transported to individual steel-mesh cages (W 57 cm × D 93 cm × H 63 cm) in an animal housing room to prevent the formation of dominance or affiliative relationships through physical interactions. Individual cages were placed side by side on shelves in the room. The birds were visible and audible to each other but without physical contact to reduce physical and social stress due to individual housing. Room temperature was set at 21 ± 2°C, and light/dark cycles were 13-h light and 11-h dark with light onset at 0800. Food (dried dog food and other supplements) and water were freely available in the cages. The experiments were conducted from Oct 2019 to Sep 2022. Owing to the accidental death of some birds and the restricted use of the experimental facility during the pandemic situation of COVID-19, a part of the planned experiments were not performed, and the data were unbalanced. The experimental and housing protocols adhered to the Japanese National Regulations for Animal Welfare and were approved by the Animal Care and Use Committee of Keio University (#20036, #A2022-055).

Determination of relative dominance in dyads

Seventeen birds were assigned to eight triads for the cohabitation experiment (Table 1). For the triad group assignment, the relative dominance between each of the two birds was determined through three trials of dyadic social encounters in an indoor aviary (W 2 m × D 2 m × H 2 m) using a procedure similar to that reported in previous studies [48, 52]. In each trial, two birds were given a 5-min opportunity to freely interact, and the winner and loser were determined according to the behavioural criteria used in previous studies [40, 48, 52]. Specifically, the loser was identified as an individual exhibiting submissive behaviour (i.e., submissive begging vocalisation and/or avoidance) in response to aggressive behaviour (i.e., jab, peck, aggressive vocalisation, and approach). Within the dyad, the individual who won all three trials was defined as dominant, and the other were defined as subordinates. All 26 dyads showed an apparent asymmetry of win/loss numbers in the three trials and were considered to have formed a dominance relationship. Therefore, 26 dyads with clear dominance relationships were used in Experiment 1. The social encounter trials were conducted between 10:00 and 15:00, with a 2-day interval between consecutive trials involving the same dyads. No subjects showed allopreening in any trial, indicating that no affiliative relationship was formed in any dyad at this stage.

Experiment 1: Formation of affiliative relationship through the triadic cohabitation

After determining the relative dominance positions of the individuals in the dyads of each triad, we tested whether a 2-week cohabitation in triads facilitated the formation of affiliative relationships between two specific birds in the triad. The formation of an affiliative relationship was evaluated based on the emergence and increase in reciprocal allopreening exclusively in two specific birds in each triad. To examine the increase in allopreening frequency of dyads, all possible dyads of each triad (i.e., three dyads) were housed together in the experimental aviary on separate occasions for three days before the start and after the end of the 2-week triadic cohabitation (hereafter referred to as pre-test and post-test, respectively). In the pre- and post-tests, each of the two birds was transferred from its individual home cage into the aviary at 8:00 am, and after 72 h, it was returned to the home cage at 8:00 am. On the day following the end of the pre-test period, the three birds were introduced into the experimental aviary and housed together for two weeks. At the end of the triadic cohabitation, the birds were transferred back to their individual home cages. At 8:00 am the following day, each pair of the triad was again transferred into the aviary for 3 days as a post-test on separate occasions.

The behaviour of the birds during the pre-/post-tests and triadic cohabitation was video-recorded using a camera (EX-ZR4100, CASIO, Japan) on the ceiling of the aviary. The recording was conducted for 30 min

randomly between 9:00 and 17:00 every day in the pre- and post-tests and for the early (1−3 day), middle (7−9 day), and last (12−14 day) three days of triadic cohabitation. Therefore, 90-min video data (30 min × 3 days) were used to analyse social interactions in each test, as well as 3 × 3-day blocks in the triadic cohabitation period. In the aviary, three wooden perches were placed in parallel with 30 cm intervals at a hight of 80 cm above the floor. Food, water, and a bathing tub were consistently provided, except during the 30-min video recording session.

Experiment 2: Effects of V1a receptor antagonism on affiliative relationship

To investigate the involvement of V1a-like receptors in the maintenance of male-male affiliative relationships, the birds forming affiliated relationships in Experiment 1 were systematically administered V1aR antagonist (SR 49059, Sigma-Aldrich, MO, USA) to examine the effects on social behaviour towards the affiliated counterpart and the unaffiliated male. This V1aR antagonist was previously shown to be an effective antagonist of the VT4 receptor in birds [53]. The effect of V1aR antagonist was tested with high dose (0.3 mL × 1 mg/mL vehicle solution) and low dose (0.3 mL × 0.1 mg/mL vehicle solution), compared with 0.3 mL vehicle solution as control, via intramuscular injection onto the chest of subject crows. The vehicle solution contained 10% dimethyl sulfoxide in a saline solution.

In each trial, the V1aR antagonist (high or low dose) or vehicle was administered to either the dominant or subordinate. Both the dominant and subordinate of each affiliated dyad were tested in 8 trials: 3 trials with the high-dose condition, 3 trials with the low-dose condition, and 2 trials with the vehicle control condition. The trial order of the dominance position of the focal individual (dominant or subordinate) and the drug conditions were counterbalanced within and between dyads. After the affiliated dyad test, the birds underwent three additional trials of dyadic interactions without drug injection to ensure the maintenance or recovery of the relationship. At least one week after this recovery trial, both birds of affiliated dyads received another set of five trials with the unaffiliated individual (the third bird in the triadic cohabitation in Experiment 1) as an unaffiliated dyad test with the same procedure as that used in the affiliated dyad test.

For each trial, either the dominant or the subordinate individual received an injection 1-h before the start of the trial (hereafter referred to as the focal individual). During the 1-h period, the focal bird was isolated from other birds in an individual cage. A trial was started soon after the focal individual was introduced into the indoor aviary (W 2 m × D 2 m × H 2 m), to which another individual was introduced 1 min beforehand. The trial procedure was identical to that of Experiment 1, and the birds were allowed to interact freely for 30 min. Given the typical social behaviour in the dyad and interactions between dominant and subordinate

large-billed crows [40, 48, 52], we coded allopreening; aggressive behaviour such as peck, jab, and kick; and submissive begging vocalisations from video-recorded data for offline analysis. The inter-trial interval was three and five days. Each bird participated in one daily trial. All trials were conducted between 8:30 and 12:00.

3. Analyses

Experiment 1: Formation of affiliative relationship through the triadic cohabitation

We defined an affiliative relationship as a dyad which exchanged allopreening reciprocally, but not unidirectionally, between two birds, independent of the dominance relationship. This definition meets the behavioural characteristics of affiliative relationships in primates and other social animals, such as maintaining close proximity and exchanging affiliative behaviour [54]. An allopreening bout was counted when a bird passed its bill through the feathers of another bird for longer than 2 s [51]. Given the absence of allopreening in any dyad before triadic cohabitation, we defined a dyad as having an affiliative relationship in which both individuals initiated allopreening in 10 or more bouts per 30 min in either of 3-day block in the triadic cohabitation. A dyad that did not meet this behavioural criterion was treated as having not formed affiliative relationship. To confirm the exclusive formation of affiliative relationships through triadic cohabitation, a discriminant analysis was performed to examine whether the numbers of dominant-initiated and subordinate-initiated allopreening in the pre- and post-triadic cohabitation tests could distinguish affiliated dyads from those before forming their affiliative relationships and from unaffiliated dyads. Leave-one-out cross validation was used to calculate the correct discrimination rate. The discriminant analysis was performed using SPSS Statistics version 29. Allopreening was coded using BORIS v.7 [55].

Experiment 2: Effects of V1a receptor antagonism on affiliative relationship

To compare the number of social behaviours in the test trials among the drug conditions and between dominants and subordinates, we applied generalised linear mixed models (GLMMs) with Poisson error distribution and the log link function separately for allopreening behaviours, aggressive behaviours, and submissive vocalisations. The model included the number of behaviours per 30 min as the response variable; *dominance* (dominant or subordinate), *drug condition* [V1aR antagonist (V1aRA) high-dose, V1aRA low-dose, or vehicle], and their interaction as explanatory variables; and *trial number* and *identity of focal individual* as random factors. If the interaction was significant, we stratified the dataset between the dominant and subordinate GLMMs to examine *drug* effects. The significance of variables in each model was

evaluated based on the Wald χ^2 statistics. All statistical analyses were performed using R version 4.2.2. with the package 'lme4' [56] for fitting GLMMs to the data. The significance level was set at 5%.

4. Results

Experiment 1: Formation of affiliative relationship through the triadic cohabitation

In six of the eight triads, the 2-week cohabitation resulted in the formation of an affiliative relationship exclusively in one dyad but not in the other dyads (Figure 1). A specific dyad in each triad (#1 – #6), which showed no allopreening in the pre-test, exhibited exclusively increased allopreening through the 2-week triadic cohabitation. In the post-test of these six dyads, allopreening was initiated by both dominants and subordinates, whereas they did not exhibit reciprocal allopreening in the pre-test, and no such allopreening emerged in any other dyads in either test (Figure 2). Discriminant analysis based on the number of allopreening initiated by dominants and subordinates within the dyads yielded two discriminant functions. Using the leave-one-out method, the two discriminant functions could distinguish the affiliated dyads in the post-test from themselves in the pre-test and the other unaffiliated dyads with an 83.3% correct rate (i.e., five of six dyads were classified as affiliated dyads; see the outputs for Table S2). These results indicate that the 2-week cohabitation in the triad facilitated the formation of the affiliative relationship exclusively in two of the three birds characterised by the exchange of allopreening.

Because one bird with a physical injury was discarded in the subsequent experiment, five of the six dyads forming an affiliated relationship were used for the test in Experiment 2.

Experiment 2: Effects of V1a receptor antagonism on affiliative relationship

Allopreening in the affiliated dyads was severely reduced by the administration of the V1aR antagonist to the dominants and subordinates (Figure 3A). The best model of the GLMM analysis on the allopreening in the affiliated dyads showed a significant effect of the *drug condition*, namely the *vehicle* condition with a positive coefficient (estimate ± s.e. = 1.639 ± 0.217, $p < 0.001$) and the *high dose* condition with a negative coefficient (estimate ± s.e. = -0.724 ± 0.313, $p < 0.05$; Table 2) but no significance of the dominance and the interaction factors. These results indicate that the numbers of allopreening decreased dose-dependently among the three conditions, irrespective of the relative dominance position in the affiliated dyads. In contrast, no effect was found on allopreening by V1aR antagonist administration in any birds of the unaffiliated dyads (Figure 3B). The GLMM analysis was not performed on unaffiliated dyads because no allopreening occurred under any condition or with any bird.

Administration of the V1aR antagonist in the affiliated dyads elevated aggression from dominants to subordinates, but not from subordinates to dominants (Figure 4A). The best model of the GLMM analysis on the aggressive behaviours in the affiliated dyads showed a significant effect of the *drug condition*, specifically the high dose condition with a positive coefficient (estimate ± s.e. = 0.791 ± 0.251, $p < 0.01$) but no significance of the vehicle condition ($p = 0.54$; Table 3), indicating the significant increase of dominants' aggression upon the administration of a high dose of the V1aR antagonist. However, no such increase in aggression by the V1aR antagonist was found in subordinates (Figure 4A; GLMM was not performed owing to no aggression in any condition or with any bird). Administration of the V1aR antagonist did not increase aggression in unaffiliated dyads (*Vehicle*, $p = 0.929$; *V1aRA high-dose*, $p = 0.89$; Figure 4B and Table 4).

Administration of the V1aR antagonist in the affiliated dyads increased the submissive begging vocalisation of the subordinates but not the dominants in the affiliated dyads (Figure 5A). The best model of the GLMM analysis of the submissive vocalisations in affiliated dyads produced a significant effect of the *drug condition*, namely the high dose condition with a positive coefficient (estimate ± s.e. = 1.929 ± 0.253, $p < 0.001$) but no significance of the vehicle condition ($p = 0.455$; Table 5). In contrast, no such increase in submissive vocalisation was found in any condition in the unaffiliated dyads (*Vehicle*, $p = 0.791$; *V1aRA high-dose*, $p = 0.777$; Figure 5B and Table 6).

5. Discussion

The present study suggested that in Experiment 1, triadic cohabitation facilitated the formation of an affiliative relationship exclusively between two specific birds of each triad and that in Experiment 2, the peripheral administration of a V1aR antagonist destroyed the affiliated relationship by the resurgence of aggressive and submissive/avoiding behaviour depending on the relative dominance but had no effect on social behaviour within unaffiliated dyads. These findings represent the first evidence not only in birds but also in mammals and other taxa, suggesting the facilitative role of the triadically interactive environment in the formation of male-male affiliative relationships and the crucial involvement of V1aR in the maintenance of male-male affiliation as has been suggested in mother-infant bonds and pair-bonds.

Formation of affiliative relationship through the triadic cohabitation

The results of Experiment 1 suggest that the triadically interactive situation plays a crucial role in facilitating the formation of male–male affiliative relationships exclusively between two of the three in the triad. Our finding is in accordance with the ideas of previous theoretical studies [27-29], which explained

that the triad, including individuals with different powers, is the minimal social group that promotes the formation of a coalition in the form of two against one. This two-vs-one form has been confirmed in the previous behavioural observation studies regarding the same-sex alliance and coalition of primates, cetaceans, and corvids both in the wild and captivity [7-26]. Our findings in Experiment 1 provide the first experimental evidence that a triadically interactive situation, such as 2-week cohabitation in a triad, indeed facilitates the formation of a male-male affiliative relationship, which fills the gap between previous theoretical and behavioural observation studies.

Two of the eight triads tested in the present study did not form affiliative relationships, even after 2-week cohabitation. As shown in Figure 1, allopreening occurred slightly in two males of the two triads during the cohabitation period but finally disappeared. According to the video observation of behaviour during the cohabitation period, one possible cause for the failure of affiliative relationship formation in those triads is the avoidance response of one male to the approach to initiate allopreening by the other. Another possible cause is intervention by the third-party male in attempts of socially affiliative interactions between two males. Third-party individual's intervention in affiliative interactions between individuals was found in a captive group of ravens [57]. There is still an open question as to what social characteristics of individuals in dyads could facilitate or inhibit allopreening and what individual and/or social factors could drive the intervention of others' affiliative behaviour in birds and mammals. An in-depth analysis of social behaviour using the triadic cohabitation paradigm used in this study may allow us to elucidate the behavioural and psychological mechanisms for forming male-male affiliative relationships.

Effects of V1a receptor antagonism on affiliative relationship

Systemic injection of a V1aR antagonist to males with affiliative relationships caused a dose-dependent decrease in allopreening and the resurgence of agonistic behaviour associated with relative dominance, such as aggressive behaviour of the dominant and submissive behaviour (i.e., avoiding and/or submissive begging calls) of the subordinate. In contrast, dominant and subordinate males without an affiliated relationship showed no behavioural effects by V1aR blocking. These results suggest that V1aR may be involved in the suppression of dominance-dependent aggressive/submissive behaviour and facilitate affiliative behaviour, thus playing a crucial role in the formation and maintenance of affiliated relationships between males.

Given the peripheral administration of the drug in this study, the behavioural effects found in affiliated dyads by V1aR antagonists do not necessarily reflect the role of V1aR in the brain. Peripheral nonapeptide receptors have been suggested to influence a variety of homeostatic functions such as hydromineral balance

306 [58] and thermoregulation [59]. For example, maternal crouching in rodents serves a thermoregulatory
307 function [60] and, is regulated by V1aR [62]. V1aR has also been suggested to be involved in peripheral
308 thermoregulation [62, 63, 64]. Thus, the effects of maternal crouching by V1aR blocking in rodents may be
309 indirectly caused by thermoregulation via V1aR antagonism. Similarly, maternal licking and grooming in
310 rodents may indirectly involve peripheral V1aR [62]. Given the fact that during maternal licking and
311 grooming, mothers often ingest water to maintain the hydromineral balance [65] based on peripheral V1aR
312 [66], maternal licking and grooming may be regulated indirectly by peripheral V1aR. Although the
313 distribution of V1aR (i.e., VT4R) has been examined in the brain and pituitary gland [67, 68] but not in
314 peripheral organs in birds, previous studies reporting the involvement of VT in renal osmoregulation [58, 69]
315 suggest the presence of V1aR in the periphery of birds. Thus, we cannot exclude the possibility that
316 peripheral injection of a V1aR antagonist affected osmoregulation and secondary social behaviour effects.
317 Although we did not measure the effects of body temperature and the amount of drinking/evacuation
318 following V1aR antagonist injection, such homeostatic effects on the periphery by V1aR blocking are
319 unlikely to occur selectively in affiliated dyads. To determine the involvement of the central V1aR in the
320 social behaviour of affiliated males, it will be necessary to examine the effect of central infusion of V1aR
321 antagonists in future studies.
322 The present findings on the involvement of V1aR in promoting inter-individual affiliation, such as male-
323 female pair-bonds and group formation, are consistent with earlier findings in in mammals and birds. In
324 monogamous prairie voles, the injection of V1aR antagonism into the lateral septum of males selectively
325 reduced body-contact affiliation to partner females compared to stranger females [34]. In zebra finches, the
326 formation of pair bonds was found to be accompanied by increased activity in terms of immediate early gene
327 ZENK expression in neurons with V1aR mRNA expression in the auditory pallium [35]. Regarding group
328 formation in gregarious finch species, VT neurons in males and females are activated in response to
329 exposure to a conspecific stimulus [37]. It has also been found that intraseptal infusions of a V1aR antagonist
330 reduced gregariousness in male zebra finches [39]. Although these previous studies did not reveal the
331 modulatory role of V1aR in specific affiliative behaviours as a facilitative factor for the promotion of male-
332 conspecific affiliation, our finding of male-male affiliation in crows could support the role of V1aR in the
333 formation and/or facilitation of inter-individual affiliation.
334 Given the results of Experiment 2, the role of V1aR in the inter-individual affiliation might be effective
335 in inhibiting agonistic behaviour (i.e., aggression and submission), specifically in affiliated males and not in
336 unaffiliated ones. Similar context-specific V1aR involvement in the control of aggressive behaviour in males

has been suggested in previous studies on birds. A previous study on violet-eared waxbills (*Uraeginthus granatina*) found that peripheral injection of a V1aR antagonist disinhibited and increased the aggression of subordinate males in a territorial challenge context, although V1aR decreased the aggression of a dominant male toward rival males in a mate competition context [70]. To clarify the role of V1aR and the relevant VP/VT signals in facilitating affiliative behaviour and/or inhibiting agonistic behaviour for the male-male affiliation, central infusion of a V1aR antagonist and assessment of V1aR-/VT-neuron activities should be investigated in future studies using crows.

In summary, the present study suggests, for the first time in social vertebrates, that male-male affiliative relationships share neural underpinnings, such as the VP/VT systems, with inter-individual social affiliations, such as pair and mother-infant bonds, which have been extensively studied in mammals and birds. Future research should be directed to investigate the role of VP/VT as well as OT/MT and their receptor subtypes in social affiliation between individuals of the same sex, which will allow us to understand inter-individual affiliation in the social lives of animals, including humans.

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545 **Figure captions**

546

547 Figure 1. Total number of allopreening behaviours in each dyad across the experimental periods; pre-tests, 2-week
548 triadic cohabitation (early, middle, and last), and post-tests. Data plots are presented with mean ± 1 s.e. Panels
549 indicate triads (#1 − #8). Bright-coloured plots (pink, green, purple, blue, orange, and red) in the #1 − #6 triads
550 represent dyads that showed the formation of affiliated relationships. Darker plots denote the dyads that did not
551 form affiliative relationships.

552

553 Figure 2. Scatter plots of allopreening behaviours initiated by dominants (x-axis) versus subordinates (y-axis) of
554 dyads in the pre-tests (opened) and the post-tests (shaded). Coloured plots represent dyads wherein affiliative
555 relationships were formed. Allopreening occurred exclusively in the coloured dyads after the cohabitation (i.e., post-
556 test). Colour corresponds to the dyad in Figure 1. Data are presented as the mean with natural log-transformed.

557

558 Figure 3. Effects of V1a receptor antagonist (V1aRA) on the number of allopreening behaviour. (A) Effects of
559 V1aRA in affiliated dyads. Allopreening initiated by the dominant (DOM, left panel) and the subordinate (SUB,
560 right panel) birds was reduced drastically in the V1aRA *high-dose* conditions and slightly in the *low-dose* condition,
561 compared with the *vehicle* condition. (B) Effects of V1aRA in unaffiliated dyads. No effect of V1aRA on
562 allopreening either by the dominant (DOM, left panel) or the subordinate (SUB, right panel) was found in
563 unaffiliated dyads. Plots show the individual data at each trial. In each triad, circles represent birds affiliated with
564 another male, while triangles represent those with no affiliation. The top, middle, and bottom of the boxes depict
565 the 75, 50, and 25 percentiles, respectively, with whiskers representing the maximum and minimum values.
566 Numbers in the brackets below the boxes denote the number of samples (no. of birds × no. of trials). Asterisks
567 indicate significant differences (Wald χ^2 statistics, *** $p < 0.001$). Colours correspond to the affiliated dyads in
568 Figure 1.

569

570 Figure 4. Effects of V1aRA on the number of aggressive behaviours. (A) Effects of V1aRA in affiliated dyads.
571 Aggressive behaviour initiated by the dominant bird (DOM, left panel) drastically increased in the V1aRA *high-*
572 *dose* condition but not in the *low-dose* condition. Such increase of aggression occurred in all conditions of the
573 subordinates (SUB, right panel). (B) No effects were observed in any condition in both the dominants and the
574 subordinates of unaffiliated dyads. Plots show the individual data at each trial. In each triad, circles represent birds
575 affiliated with another male, while triangles represent those with no affiliation. The top, middle, and bottom of the

boxes depict the 75, 50, and 25 percentiles, respectively, with whiskers representing the maximum and minimum values. Numbers in the brackets below the boxes denote the number of samples (no. of birds × no. of trials). Asterisks indicate significant differences (Wald χ^2 statistics, *** $p < 0.001$, ** $p < 0.01$). Colours correspond to the affiliated dyads in Figure 1.

Figure 5. Effects of V1aRA on the number of submissive begging vocalizations. The number of submissions were compared between the vehicle control and V1aRA *low-dose* and *high-dose* groups. Dom (left panel)/Sub (right panel) refer to initiation of dominants and subordinates. (A) Effects of V1aRA in affiliated dyads. Submissive begging calls initiated by the dominant (DOM, left panel) did not change in any conditions; however, those initiated by the subordinates (SUB, right panel) increased in the V1aRA *high-dose* condition but not in the *low-dose* condition. (B) No effects were observed in any condition in both the dominants and the subordinates of unaffiliated dyads. Plots show the individual data at each trial. In each triad, circles represent birds affiliated with another male, while triangles represent those with no affiliation. The top, middle, and bottom of the boxes depict the 75, 50, and 25 percentiles, respectively, with whiskers representing the maximum and minimum values. Numbers in the brackets below the boxes denote the number of samples (no. of birds × no. of trials). Asterisks indicate significant differences (Wald χ^2 statistics, *** $p < 0.001$, ** $p < 0.01$). Colours correspond to the affiliated dyads in Figure 1.

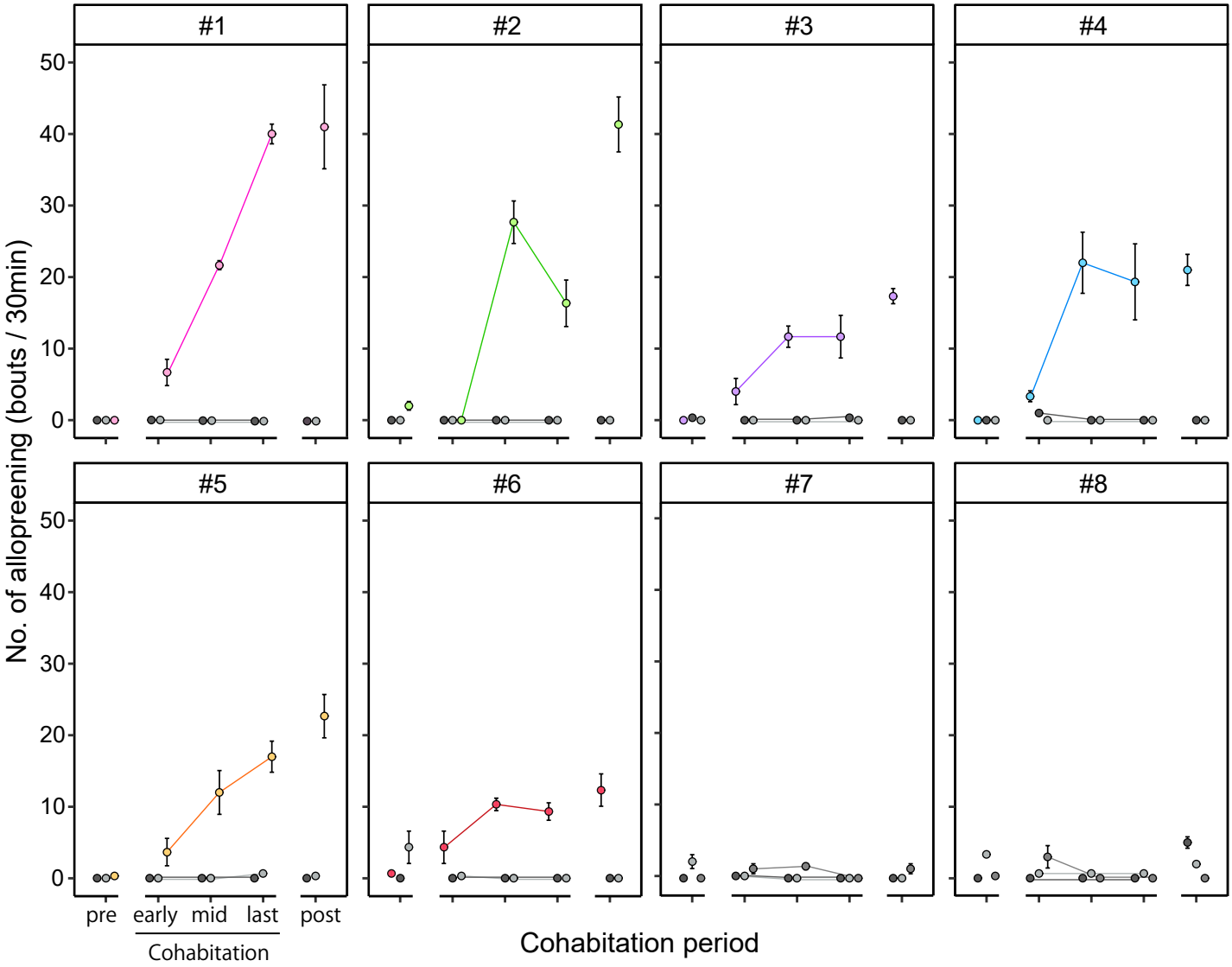
#1
#2
#3
#4
#5
#6
#7
#8
No. of allopreening (bouts / 30min)
0
10
20
30
40
50
pre
early
mid
last
post
Cohabitation
Cohabitation period

Figure 1.

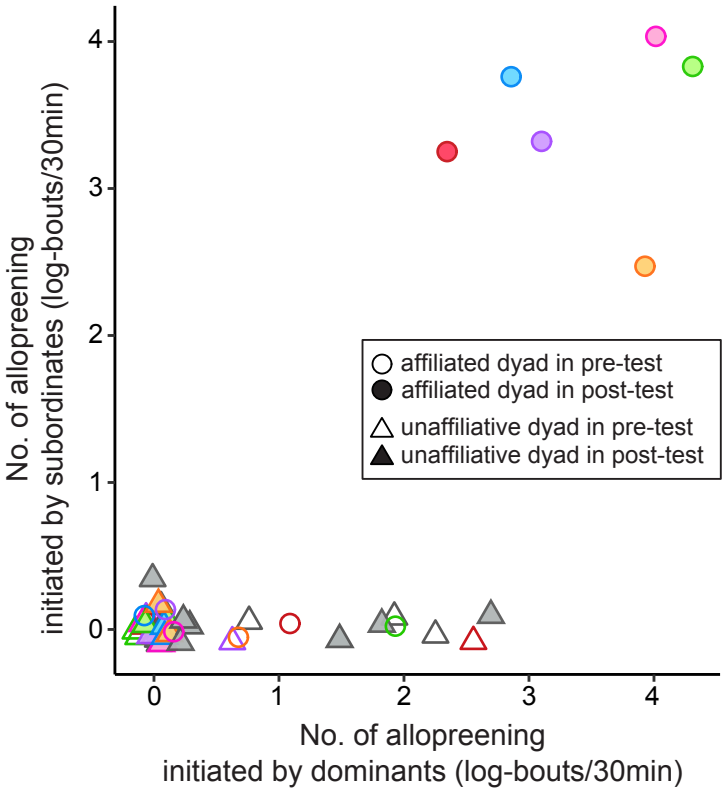
No. of allopreening
initiated by subordinates (log-bouts/30min)
No. of allopreening
initiated by dominants (log-bouts/30min)
0
1
2
3
4
affiliated dyad in pre-test
affiliated dyad in post-test
unaffiliative dyad in pre-test
unaffiliative dyad in post-test

Figure 2.

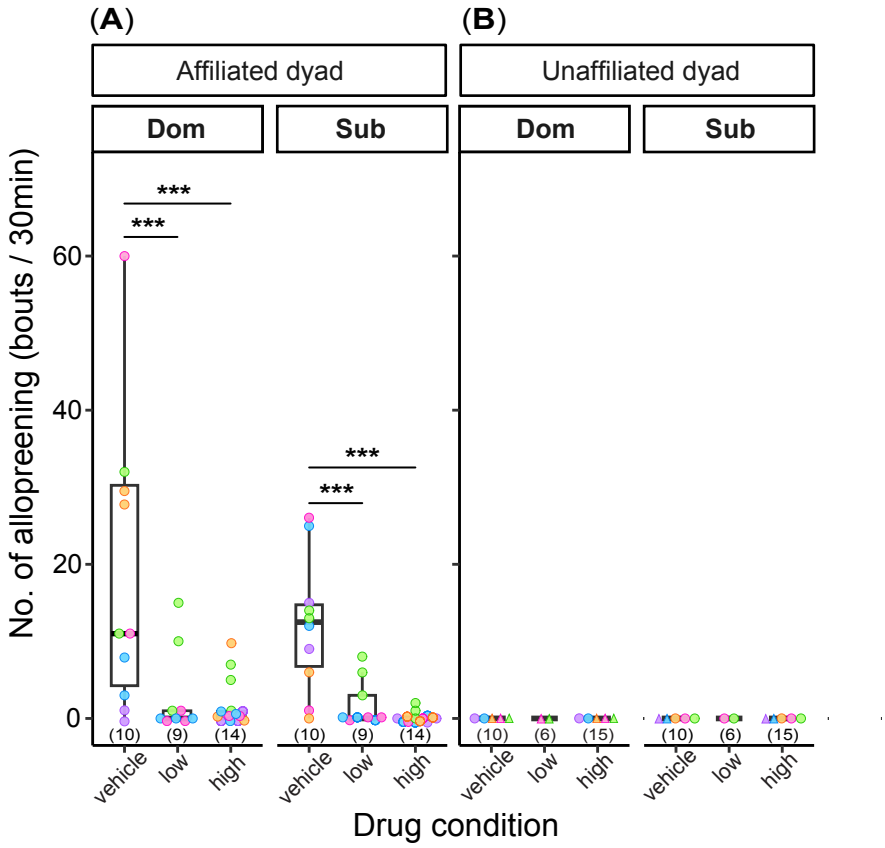
(A)
(B)
Affiliated dyad
Unaffiliated dyad
Dom
Sub
Dom
Sub
No. of allopreening (bouts / 30min)
0
20
40
60
***
***
***
***
(10)
(9)
(14)
(10)
(9)
(14)
(10)
(6)
(15)
(10)
(6)
(15)
vehicle
low
high
vehicle
low
high
vehicle
low
high
vehicle
low
high
Drug condition

Figure 3.

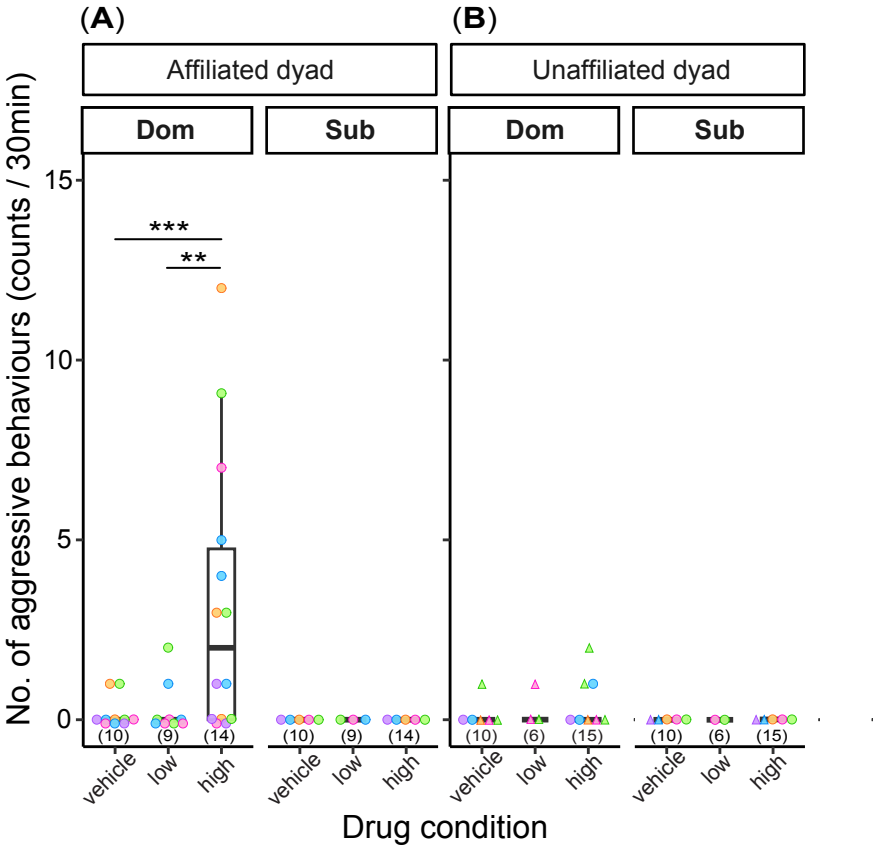
(A)
(B)
Affiliated dyad
Unaffiliated dyad
Dom
Sub
Dom
Sub
No. of aggressive behaviours (counts / 30min)
15
10
5
0
***
**
(10)
(9)
(14)
(10)
(9)
(14)
(10)
(6)
(15)
(10)
(6)
(15)
vehicle
low
high
vehicle
low
high
vehicle
low
high
vehicle
low
high
Drug condition

Figure 4.

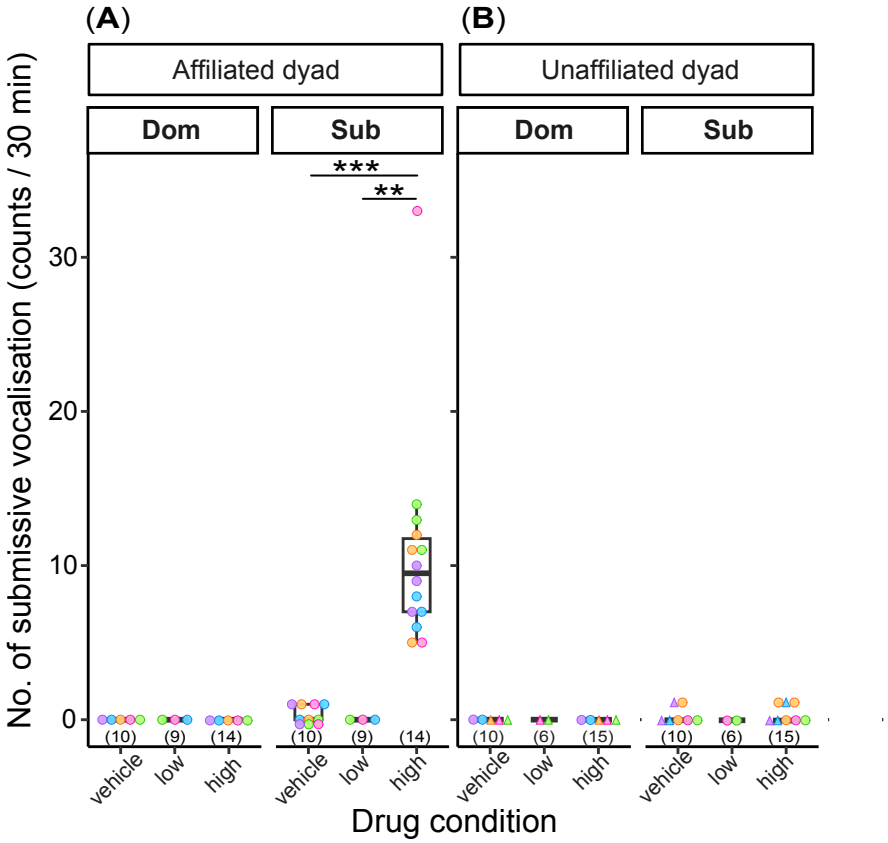
(A)
(B)
Affiliated dyad
Unaffiliated dyad
Dom
Sub
Dom
Sub
***
**
No. of submissive vocalisation (counts / 30 min)
30
20
10
0
(10)
(9)
(14)
(10)
(9)
(14)
(10)
(6)
(15)
(10)
(6)
(15)
vehicle
low
high
vehicle
low
high
vehicle
low
high
vehicle
low
high
Drug condition

Figure 5.

Table 1. Individuals used in the experiment 1 and 2

Triads used in Experiment 1				*Dyads used in experiment 2*							
Triad No.	*Dominace rank*			*Affiliated*	*Drug conditions received*			*Unaffiliated*	*Drug conditions received*		
	High	*Middle*	*Low*		*Saline*	*V1aRA low-dose*	*V1aRA high-dose*		*Saline*	*V1aRA. low-dose*	*V1aRA high-dose*
1	OW	PY	VV	PY, VV	✓	✓	✓	OW, VV	✓	✓	✓
2	RR	OW	BB	OW, BB	✓	✓	✓	RR, BB	✓	✓	✓
3	#28	YA	#27	#28, YA	✓	*n.a.*	✓	#28, #27	✓	*n.a.*	✓
4	VV	BB	#28	VV, BB	✓	✓	✓	BB, #28	✓	*n.a.*	✓
5	KR	KY	OBO	KY, OBO	✓	*n.a.*	✓	KR, KY	✓	*n.a.*	✓
6	RM	YA	VA	(RM, YA)	–	–	–	–	–	–	–
7	GK	KG	RM	–	–	–	–	–	–	–	–
8	KV	KR	LK	–	–	–	–	–	–	–	–

Birds OW, YA, VV, BB, #28, KR included in two different triads. Trio #6 (RM, YA, VA) did not receive the experiment 2 (see text for the details). *n.a.* indicates data is not available.

Table 2. Outputs of GLMMs for allopreening behaviours in affiliated dyads.

variables	estimates	s.e.	*z* -value	*p*
intercept	0.854	0.275	3.108	*<0.001*
Dominance[a]	0.325	0.257	1.266	0.206
Drug condition[b]				
Vehicle	1.639	0.217	7.554	*<0.001*
V1aRA high-dose	-0.724	0.313	-2.309	*0.021*
Dominance × Vehicle	0.068	0.281	0.241	0.811

Italicized indicates statistically significant results.

[a] *Subordinate* was set to 0.

[b] V1aRA low-dose was set to 0.

Table 3. Outputs of GLMMs for aggressive behaviours in affiliated dyads.

variables	estimates	s.e.	*z* -value	*p*
intercept	0.094	0.293	0.319	0.749
Drug condition[a]				
Vehicle	-0.193	0.315	-0.612	0.540
V1aRA high-dose	0.791	0.251	3.147	*<0.01*

Italicized indicates statistically significant results.

[a] V1aRA low-dose was set to 0.

Table 4. Outputs of GLMMs for aggressive behaviours in unaffiliated dyads.

variables	estimates	s.e.	*z* -value	*p*
intercept	0.080	0.277	0.289	0.773
Drug condition[a]				
Vehicle	-0.031	0.353	-0.089	0.929
V1aRA high-dose	0.045	0.326	0.138	0.890

Italicized indicates statistically significant results.

[a] V1aRA low-dose was set to 0.

Table 5. Outputs of GLMMs for submissive vocalizations in affiliated dyads.

variables	estimates	s.e.	*z* -value	*p*
intercept	-0.083	0.273	-0.303	0.762
Drug condition[a]				
Vehicle	0.237	0.318	0.747	0.455
V1aRA high-dose	1.929	0.253	7.617	*<0.001*

Italicized indicates statistically significant results.

[a] V1aRA low-dose was set to 0.

Table 6. Outputs of GLMMs for submissive vocalizations in unaffiliated dyads.

variables	estimates	s.e.	*z* -value	*p*
intercept	0.000	0.289	0.000	1.000
Drug condition[a]				
Vehicle	0.095	0.359	0.266	0.791
V1aRA high-dose	0.095	0.337	0.283	0.777

Italicized indicates statistically significant results.

[a] V1aRA. low-dose was set to 0.