

Effect of Intraseptal Vasotocin and Vasoactive Intestinal Polypeptide Infusions on Courtship Song and Aggression in the Male Zebra Finch (*Taeniopygia guttata*)

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Abstract

The present experiments were conducted to test the hypothesis that septal arginine vasotocin (AVT) and vasoactive intestinal polypeptide (VIP) modulate directed song (a courtship behaviour) and aggression in male zebra finches (*Taeniopygia guttata*). Subjects were surgically fitted with a guide cannula directed at the septum. Following recovery they were tested for aggression and directed song following infusions of AVT, its antagonist (anti-vasopressin, AVP), and saline volume control. Infusion of the AVT antagonist significantly reduced all three aggressive behaviours measured (pecks, beak fences and chases); and AVT infusion significantly facilitated beak fencing. Vasoactive intestinal polypeptide treatment significantly reduced pecking. No treatment produced a change in directed song. Comparison with findings in mammals suggests that modulation of aggression by septal AVT (or AVP) is evolutionarily conserved in vertebrates, but modulation of aggression by VIP has not previously been reported for any vertebrate.

Key words: septum, vasotocin, vasoactive intestinal polypeptide, vasopressin, aggression, song, zebra finch.

The septum of vertebrates is a morphologically and histochemically conserved forebrain region which has been implicated in the control of both aggressive and courtship behaviours (1–6) and research on septal neuropeptides in rodents suggests that the behavioural functions of septum differ between species which exhibit divergent patterns of social organization (7–10). Consistent with this are recent findings demonstrating that lesions of the septum affect male aggression and courtship differently in a territorial songbird, the field sparrow (*Spizella pusilla*), than in a colonial songbird, the zebra finch (*Taeniopygia guttata*). Septal lesions facilitate aggression in male field sparrows and do not reduce courtship, but reduce both courtship song and aggression in male zebra finches (1). Neurochemical processes within the septum which underlie this species difference are unknown. Thus, the present experiments were conducted to examine the effects of intraseptal administrations of arginine vasotocin (AVT), its antagonist (anti-vasopressin (AVP), the mammalian homologue of AVT), and vasoactive intestinal polypeptide (VIP) on courtship and aggression in male zebra finches. The goal of these experiments was to identify physiological characteristics which may underlie interspecific divergence in septal

behavioural function which may be associated with social organization.

A variety of findings indicate that AVT modulates reproductive behaviours in vertebrates. These include advertisement calling in anuran amphibians (11, 12), amplexic claspings in the rough-skinned newt (*Taricha granulosa*) (13), and the production of a variety of vocalizations by female white-crowned sparrows (*Zonotrichia leucophrys*) (14). Courtship in male zebra finches is reduced by peripheral administration of AVT, and this effect is reversed by testosterone administration (15). A role for central AVT in the modulation of male aggression and courtship in birds has not been reported previously, although a recent experiment has shown that intraventricular administration of AVT inhibits copulatory behaviour in male Japanese quail (*Coturnix japonica*) (16). In contrast, the facilitation of aggression and agonistic scent marking in rodents by septal AVP has been extensively studied (17, 18).

The modulation of aggression by septal AVP in mammals suggests that septal AVT may also modulate aggression in birds, an hypothesis which is further supported by the observation that AVT/AVP exhibit conserved forebrain

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distributions across vertebrate classes (19–28). This distribution includes cell bodies and fibres in the preoptic area and nucleus of the stria terminalis, and a fibre plexus in the septum. AVT/AVP distributions are often seasonally variable, steroid dependent, and sexually dimorphic in vertebrates, including some birds, with males showing greater cell and fibre densities (see references above and citations within). In the zebra finch, AVT-immunoreactivity (–ir) in the lateral septum is reduced by gonadectomy in males (27), but sexual dimorphisms in AVT-ir in the zebra finch have not been identified (29).

A recent study in Japanese quail has demonstrated that both AVT-ir and VIP-ir fibre innervations of the caudal septum are steroid sensitive (30). Interestingly, castration reduces AVT-ir replacement but increases VIP-ir; these effects are reversed by testosterone. The implications of this differential sensitivity for behaviour remain unspecified. In fact, although VIP in vertebrates is distributed in regions known to control aggressive and reproductive behaviours [e.g. septum, preoptic area and nucleus of the stria terminalis (21, 31–34)], the potential role of VIP in aggressive behaviour has not been addressed and reproductive functions of VIP have been little investigated [but see (35)].

Finally, reproductive and related functions are suggested for AVT and VIP based on the findings that in the Japanese quail, distributions of these neuropeptides correspond closely to the locations of septal luteinizing hormone releasing hormone (LHRH)-containing cells (30, 33, 36) and AVT elements are colocalized with aromatase-containing neurones in the medial preoptic nucleus and nucleus of the stria terminalis. Similarly, VIP-ir nerve terminals make contact on putative LHRH cells in the lateral septum of the pigeon [*Columba livia* (37)].

Based on the observations that: (1) castration reduces AVT-ir in the septum; (2) intraseptal AVP administration facilitates agonistic behaviour in rodents; and (3) a lesion experiment has implicated the zebra finch septum in the control of courtship and aggression; we hypothesized that intraseptal infusions of AVT would facilitate courtship and aggressive behaviours in the male zebra finch. Conversely, administration of an antagonist (anti-AVP) was predicted to inhibit these behaviours. Generation of predictions for VIP effects was more difficult, given the absence of relevant behavioural data. Thus, the present VIP experiment tested the hypothesis (generated based on distribution) that VIP would modulate aggression and courtship, without specification of effect direction. However, the recent finding that castration increases VIP-ir in the septum of quail (30) suggests that VIP may function in a manner polar to that of AVT, an hypothesis which was confirmed in our investigations.

Results

Histology

All cannulae were successfully placed in the right septum. Unilateral damage to the septum extended $\approx 500 \mu\text{m}$ rostrocaudally, with insertions at more caudal levels producing various amounts of damage to the septal nucleus and medial septum. Figure 1 shows the placement of cannulae for all

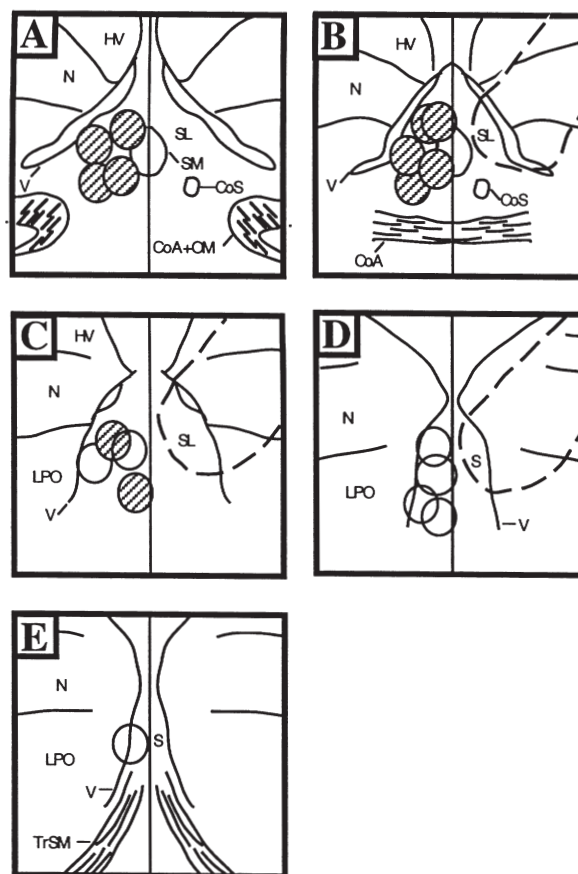


FIG. 1. Location of infusion sites in the septum from caudal (A: $200 \mu\text{m}$ caudal of the anterior commissure) to rostral (E: $600 \mu\text{m}$ rostral of the anterior commissure) levels. Shown in dashed outline on right is a reconstructed cannula path from a subject with a centre of infusion $\approx 200 \mu\text{m}$ rostral of the anterior commissure. Experiment 1 subjects are indicated by open circles. Hatch-filled circles represent subjects from experiments 2 and 3. Abbreviations: CoA, commissura anterior; CoS, nucleus septi commissuralis; HV, hyperstriatum ventrale; LPO, lobus parolfactorius; N, neostriatum; OM, tractus occipitomesencephalicus; S, nucleus septalis; SL, nucleus septalis lateralis; SM, nucleus septalis medialis; TrSM, tractus septomesencephalicus; V, ventricle.

subjects and shows the extent of a typical lesion induced by cannulation. As shown, the size of the cannula was large relative to the size of the septum, rendering meaningful comparisons between placements at a given rostrocaudal level (i.e. medial *versus* lateral; dorsal *versus* ventral, etc.) impossible. Cannulae placements in experiment 1 were rostral of the region of most dense AVT-ir and VIP-ir fibres [i.e. caudal septum (30)] and subsequent cannulations were thus directed more caudally in order to infuse directly into this region. Larger infusion volumes were used in experiment 1—however, see Materials and Methods—and likely produced diffusion to caudal levels.

Directed song

In all experiments, subjects sang more during initial exposure of the female (stimulus male present) than during the subsequent period of free interaction with the female. No

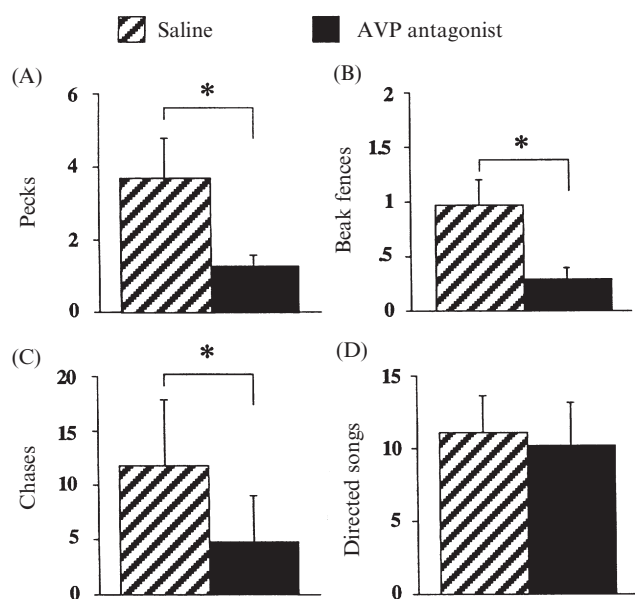


FIG. 2. Mean \pm SEM behaviour frequencies per test directed towards male (A–C) and female (D) stimuli following intraseptal infusion of 1 μ g vasopressin (AVP) antagonist or saline control (n=7): (A) pecks, (B) beak fences, (C) chases, (D) directed song. * $P < 0.05$, Wilcoxon signed-ranks.

significant treatment effects or trends were observed when analysing these data separately, and the data are thus combined for presentation in Figs 2–4.

Experiment 1 (arginine vasopressin antagonist)

Infusion of 1 μ g anti-AVP produced significant reductions in all aggressive behaviours (pecks, beak fences, and chases; Fig. 2A–C). Directed song was unaffected (Fig. 2D). Aggressive behaviour in the 2 min before exposure of the female was limited to a low frequency of pecks (with the exception of one subject which chased the stimulus male), and this data has therefore been pooled with data collected during exposure of the female. Pooling did not alter the significance of any comparison.

Experiment 2 (arginine vasotocin)

Infusion of 0.01 μ g AVT was followed by a significant increase in the number of beak fences (Fig. 3B). No significant effects on pecks, chases, or directed songs were observed (Fig. 3A,C,D).

Experiment 3 (vasoactive intestinal polypeptide)

Infusion of 0.01 μ g VIP produced a significant decrease in pecks, with no significant effects on other behaviours (Fig. 4). The lack of significance for chase and beak fence measures may be due to the small number of subjects, as five of the six subjects showed reductions in these behaviours following VIP administration.

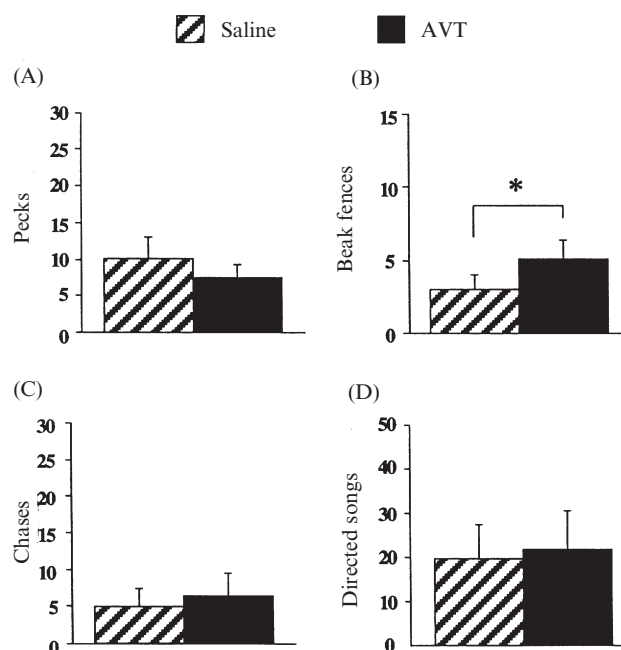


FIG. 3. Mean \pm SEM behaviour frequencies per test directed towards male (A–C) and female (D) stimuli following intraseptal infusion of 0.01 μ g vasotocin or saline control (n=10): (A) pecks, (B) beak fences, (C) chases, (D) directed song. * $P < 0.05$, Wilcoxon signed-ranks.

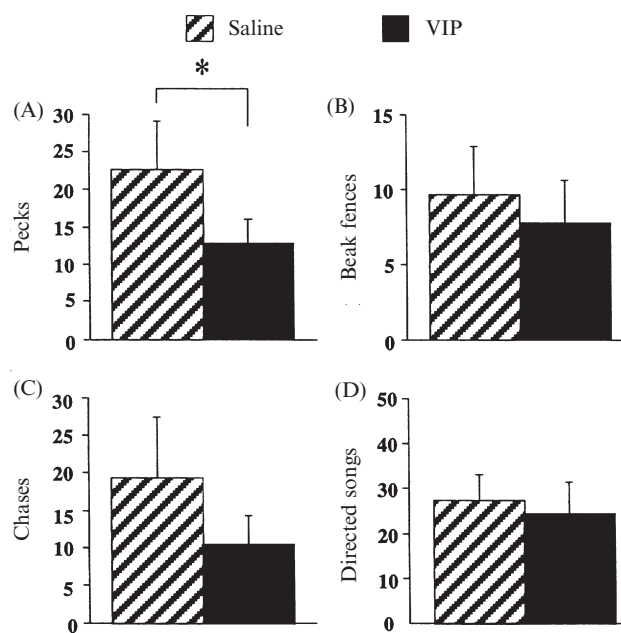


FIG. 4. Mean \pm SEM behaviour frequencies per test directed towards male (A–C) and female (D) stimuli following intraseptal infusion of 0.01 μ g vasoactive intestinal polypeptide or saline control (n=6): (A) pecks, (B) beak fences, (C) chases, (D) directed song. * $P < 0.05$, Wilcoxon signed-ranks.

Discussion

Consistent with our hypotheses, aggressive behaviour in male zebra finches was facilitated by intraseptal administration of AVT and inhibited by intraseptal administration of anti-AVP

and VIP. These findings are also consistent with the results of a pilot study which demonstrated facilitation of aggression by 0.1 µg AVT (38). However, contrary to the hypothesized role for septal AVT and VIP in the modulation of courtship, no effects on directed song were observed.

The present finding that AVT and VIP exert opposite effects on aggression is paralleled by another recent finding showing that septal AVT-ir and VIP-ir fibres in male quail are differentially sensitive to testosterone (30). Arginine vasotocin immunoreactivity in the septum decreases following castration whereas VIP-ir increases, although VIP-ir increases in the caudal septum only. Castration effects are reversed by administration of testosterone. These observations suggest that septal AVT and VIP comprise functionally antagonistic modulatory systems. However, the natural release patterns of these neuropeptides under various social circumstances have not been investigated, and further study will therefore be required to specify the roles of AVT and VIP in the modulation of aggressive behaviour.

The findings that VIP-ir is testosterone-dependent only in the caudal septum, and that both AVT-ir and VIP-ir fibres are more dense in the caudal septum (30) suggests that functional variation may exist along the septum's rostrocaudal axis. This idea was not tested in the present experiments, and although experiment 1 subjects received more rostral cannulae placements than did other subjects, the larger infusion volume in this experiment likely produced diffusion to caudal levels as well. Thus, the caudal septum was likely exposed to infusions in all experiments. Diffusion to the nucleus of the stria terminalis, a structure adjacent to the caudal septum which contains both VIP elements (32–34) and a sexually dimorphic concentration of AVT elements (39, 40), may have occurred in some subjects. However, the nucleus of the stria terminalis has not been implicated in the control of aggressive behaviour in any vertebrate taxa. Finally, infused substances likely did not reach hypothalamic levels, as infusion of AVT in or near the hypothalamus of male zebra finches inhibits directed song (unpublished observations), and no effect on directed song was observed in the present experiments.

The present results corroborate results of lesion studies which suggest that the songbird septum participates in the regulation of aggression, but are not consistent with lesion results suggesting a modest role for the septum in the regulation of zebra finch directed song (1). This discrepancy may indicate that directed song is influenced by septal neurochemical systems other than those investigated here. Alternatively, changes in directed song observed following septal lesions may have been due to indirect effects on structures afferent and/or efferent to the septum, such as the preoptic area, which has been implicated in the control of vocalization in a range of vertebrates (6, 41–43).

The present results are also inconsistent with findings in female white-crowned sparrows, in which intraventricular administration of AVT facilitates a variety of vocalizations (14), and with findings in male canaries (*Serinus canaria*), in which peripheral administration of AVT increases or decreases song duration dependent upon the season (44). These inconsistencies may be related to species differences, sex differences, differences in the functions of the vocalizations, and differ-

ences in the site(s) of AVT action. It should be noted that only courtship song was investigated here, and it remains to be determined whether septal AVT and/or VIP modulate agonistic vocalization. The zebra finch does produce one call specific to aggressive encounters [the 'wsst' call (45)], but this call was not recorded in the present experiments due to its infrequency.

AVT/AVP has now been implicated in the control of male-typical agonistic behaviour in all classes of tetrapod vertebrates except reptiles, which to our knowledge have not been examined [present investigation (46–49)]. The septum in particular seems to be an important site for this action, as intraseptal AVP infusions facilitate flank marking (a stereotyped agonistic display) in Syrian hamsters (17), inter-male aggression in rats (18), and aggression in zebra finches (present study). These findings suggest that both AVT and septal functions may be broadly conserved across vertebrate taxa. As the present investigation provides the first evidence for modulation of vertebrate aggression by VIP, further study will be required to determine if this also represents a general vertebrate characteristic. However, VIP does exhibit a conserved distribution across classes (21, 31, 33, 34), a distribution which includes regions known to regulate agonistic behaviour [e.g. the septum and anterior hypothalamus (1, 17)].

Storage and release of both VIP and AVT/AVP in the hypothalamus and neurohypophysis is modulated by a range of environmental stressors [e.g. social defeat, restraint stress, dehydration, hypoglycemia (50–53)]. In addition, AVT is known to be colocalized with corticotropin releasing factor in the septum of snakes (54), and septal AVT/AVP has been implicated in thermoregulatory and osmoregulatory functions (22, 55, 56). Thus, the present demonstration that VIP inhibits aggression, and AVT facilitates aggression, raises the possibility that these neuropeptides may interact to modulate male-typical behaviour so as to produce adaptive responses to stress. This idea is consistent with findings in the rough-skinned newt, in which medullary AVT interacts with corticosterone in the control of sexual behaviour (57). Osmoregulatory stress in particular may be important for zebra finches, as they face substantial osmoregulatory challenges in their arid Australian habitat, and zebra finch breeding is dependent upon rainfall (58). Water deprivation reduces septal AVT-ir in male zebra finches (27), suggesting that dehydration may modulate agonistic behaviour as well.

The findings that: (1) male zebra finch aggression is modulated by intraseptal administration of AVT and VIP, neuropeptides which are known to mediate physiological responses to stress; and (2) lesions of the septum produce different effects on aggression in zebra finches and field sparrows (1); suggest that the divergence in septal function between zebra finches and field sparrows may be related to differences in environmental stressors faced by these two species. These may include differences in the abiotic environment which produce thermoregulatory and osmoregulatory challenges, as zebra finches breed in arid regions whereas field sparrows are breeders of mesic habitat. In addition, territoriality and coloniality may produce species differences in social stress (for instance, stress differences due to species divergence in the frequency or intensity of aggressive

encounters), suggesting that social organization may also be related to septal function. Ongoing investigations in field sparrows and a territorial Estrildid species (more closely related to zebra finches) will address this issue and should help clarify two important issues: (1) whether AVT and VIP functions do in fact underlie the divergence in the septal control of aggression in field sparrows and zebra finches; and (2) what environmental, social organization, or phylogenetic variables may be associated with AVT and VIP function.

Materials and methods

Experiments were conducted in the following order: AVP antagonist, AVT, and VIP (experiments 1–3, respectively). No AVT antagonist is commercially available, but previous research in the rough-skinned newt has demonstrated that AVP antagonists may have a high affinity for AVT receptors (59).

Surgical, histological, and statistical procedures were the same in all experiments. However, implant and testing procedures were altered following experiment 1, because the extremely high levels of aggression exhibited by subjects in the control condition during experiment 1 appeared in many cases to represent the limit of the subjects' physical capabilities. The methodological changes for subsequent experiments were thus intended to elicit a more moderate level of aggression, which would allow both facilitatory and inhibitory modulations by neuropeptides to be detected. As discussed below, these changes proved largely ineffective at reducing aggression.

Animals and housing

Subject and stimulus animals were raised in our colony at Cornell University or were obtained from a commercial breeder (Canary Bird Farm, Elizabethtown, NJ, USA). A total of 18 adult male subjects were used (experiment 1, $n=7$; experiment 2, $n=10$; experiment 3, $n=6$). Five subjects used for experiment 3 were previously used for experiment 2. Nineteen adult stimulus males and 18 adult stimulus females were employed. All animals were housed in same-sex cages (hardware cloth aviaries $0.8 \times 1.9 \times 1.0$ m or hardware cloth cages $52 \times 55 \times 46$ cm). Birds were maintained on a 14 h light:10 h dark cycle and were provided with finch seed mix and water *ad libitum*.

Surgery

Surgeries were conducted stereotaxically (Brain Research Instruments, Princeton, NJ, USA) under isoflurane vapour anaesthesia which was delivered through a rubber cup placed over the beak. Isoflurane concentration was controlled using a Dräger Halothan Vapor 19.1 (Drägerwerk AG, Lübeck, Germany) and a Foregger™ anaesthesia machine (Air Products, Allentown, PA, USA), with concentrations varying from 1.3 to 3.0% of a compressed air flow. A small skull 'flap' was opened at the level of the telencephalon/cerebellum junction in order to reference the cannula; the flap was then glued back in place using skin glue. Co-ordinates were established based on pilot work (unpublished) and previous experience performing septal lesions in the zebra finch (1). Each subject received a single, 24-gauge, stainless steel cannula directed at the right septum. Insertion was made 1.7 mm lateral to the midline at 21° to avoid midline vasculature. Cannulae were affixed to the skull using dental acrylic (Hygenic Corp., Akron, OH, USA) and Crazy Glue™. Surgery duration averaged 45 min. At least 6 days recovery was allowed before testing.

Implants

Subjects received subcutaneous implants of testosterone propionate packed into 5 mm (experiment 1) or 2.5 mm (experiments 2–5) silastic tubules (1.96 O.D., 1.47 I.D.) at least 1 week prior to testing. These were placed at the lateral edge of the breast. Implants ensured that behaviour occurred at adequate levels throughout testing (particularly during the isolation of experiment 1), and ensured adequate levels of circulating steroid in the event that cannulation or infusion disrupted the regulation of gonadal steroid secretion. The implant sizes used here likely produced serum testosterone levels which equalled or exceeded serum levels in intact males, as previous research has demonstrated that in castrated male zebra finches, 7-mm implants of the type used here produced serum levels which were approximately twice those of intact males (60).

Test order

For each experiment, subjects received one experimental and one control test with the same stimulus animal(s), followed by another control and experimental test with a different stimulus animal(s). The order of treatments was reversed for presentation of the second stimulus animal(s), and the order of treatments was counterbalanced between subjects. Time of day was the same for control and experimental treatments with the same stimulus animals.

Infusions

Except for experiment 1, all neuropeptides were delivered in 0.2 μ l saline using a Hamilton syringe fitted with a 33-gauge needle which extended 0.5 mm beyond the tip of the guide cannula. Control infusions of 0.2 μ l saline were administered in the same manner; all infusions were pressure injected over a 10-s period. Experiment 1 methods were the same as above with the exception that infusion volumes of 1 μ l were employed and were pressure injected over a 30-s period. The relatively high dose and volume were used to ensure that the entire septal region was infused. Hence, this first experiment represents an attempt to determine the likelihood that septal AVT may modulate aggressive and courtship behaviour. Based on the positive findings of this experiment, experiment 2 was subsequently conducted to attempt a more localized treatment of AVT within the septum.

Arginine vasotocin and antagonist dosages (0.01 μ g and 1 μ g, respectively) were selected to be within effective ranges established for other species (13, 61). A concentration equivalent to AVT was selected for VIP (0.01 μ g), given the absence of relevant behavioural data for this neuropeptide. Peptides were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Experiment 1

Two days prior to testing, subjects ($n=7$) were placed individually in a hardware cloth cage $52 \times 55 \times 46$ cm. They remained housed singly in these cages for the duration of testing. Two-day intervals were allowed between tests to ensure that the septum was clear of the antagonist. Tests were conducted 1 h after infusion of 1 μ g AVP antagonist ($[\beta$ -Mercapto- β , β -cyclopenta-methylenepropionyl¹,O-Me-Tyr²,Arg⁸]-vasopressin (a potent V_1 antagonist) or saline volume control, thus allowing time for competitive binding. Testing consisted of introducing a stimulus male into the subject's home cage and recording behaviour for 2 min. A female was then exposed in an immediately adjacent cage by removing an opaque barrier and behavioural observation continued for 5 min. The stimulus male was then removed and the stimulus female was placed in the subject's cage, followed by 5 min of observation. Observations were conducted from behind plastic curtains which had small windows. Behaviours recorded during the first 7 min (i.e. when the stimulus male was present) were: pecks and chases directed towards the stimulus male, beak fences (an aggressive behaviour in which two birds 'fence' with their beaks), and the number of directed songs given to the stimulus female (a key male-typical courtship behaviour of zebra finches; given while close to and directly facing a conspecific). During the last 5 min (when only the stimulus female was present) behavioural recordings were restricted to directed song, as pecks, beak fences, and chases directed towards female stimuli occur at very low levels in this context. Similarly, directed song to males is a low-frequency behaviour and was not recorded in the present experiments.

Experiments 2–5

Subjects were housed in same-sex cages $52 \times 55 \times 46$ cm with 2–5 other subjects. This arrangement was established immediately following surgery and continued until test completion. One h before each test, each subject was isolated in a small wire cage $23 \times 25 \times 46$ cm to acclimate. Following this period, subjects received an infusion and tests were begun within 5 min. For testing, a wire barrier was placed in the centre of the cage; a stimulus male was placed in the half containing the subject and a stimulus female was placed in the other half. Observations were conducted for 5 min. The stimulus male and the wire barrier were then removed, allowing the subject and stimulus female to interact. Observations continued for an additional 5 min. Behaviours recorded were as in experiment 1. Twenty-four h were allowed between tests (within and between experiments).

As previously explained, testing procedures were modified from those used in experiment 1 in an attempt to elicit a more moderate level of aggression. Thus, subjects were given testosterone propionate implants half the size of those used in experiment 1 (see 'Implants' above), and were tested in a neutral cage as opposed to a home cage in which they had been singly housed. These changes were largely ineffective (see Results). Other changes, such as the use of different cages and the deletion of the first 2-min observation period

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(before exposure of the female), were made for practical reasons and allowed the experimenter to introduce the stimulus female into the subject male's cage (following the exposure portion of the test) without additional handling of the female.

Histology

Subjects were killed by an overdose of anaesthesia and perfused through the heart with 0.9% saline followed by 10% formalin. Heads were stored for at least 1 week in 10% formalin. Brains were removed and stored overnight in a 30% sucrose/10% formalin solution, embedded in albumin gelatin and cut on a freezing microtome at 50 or 100 μm . Sections were then mounted on slides to establish cannula placement within the septum. For most subjects, this was easily determined in unstained tissue, but when necessary, sections were stained with cresyl violet to ensure accuracy.

Statistics

For each subject, the total frequency of each behaviour exhibited per condition (control *versus* experimental) was divided by the number of tests (2), and these individual subject means were then used for statistical analyses and the generation of the group means presented in the results. Wilcoxon signed-ranks tests were used to compare behaviour frequencies exhibited in experimental and control conditions. All P-values are two-tailed.

Acknowledgements

We thank Tom Smulders for technical assistance and David Winkler and Andrew Bass for comments on a draft of this manuscript. J. L. G. was supported by a predoctoral fellowship from the National Science Foundation.

Accepted 17 June 1998

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