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Role of septal vasopressin innervation in paternal behavior in prairie voles (*Microtus ochrogaster*)

(parental care/maternal behavior/bed nucleus of the stria terminalis/medial amygdala/oxytocin)

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ABSTRACT **After being paired with females, male prairie voles show major changes in their social behaviors among which is an increase in paternal responsiveness. These changes are accompanied by fluctuations in the density of the [Arg^8]vasopressin-immunoreactive (AVP-ir) fibers in the lateral septum, suggesting that septal AVP might be involved in these changes. To explore a possible involvement of septal AVP in paternal responsiveness, we tested whether injections of saline, AVP, or the V1$_a$ receptor antagonist [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyltyrosine]AVP [d(CH$_2$)$_5$Tyr(Me)AVP] into the lateral septum influenced the four most prominent paternal activities displayed by male prairie voles; grooming, crouching over, contacting, and retrieving pups. In a first experiment, sexually inexperienced males received a single injection of AVP, saline, or d(CH$_2$)$_5$Tyr(Me)AVP in the lateral septum, after which their paternal responsiveness was recorded during a 10-min period. AVP-injected animals spent more time contacting and crouching over pups, while d(CH$_2$)$_5$Tyr(Me)AVP-injected animals spent less time grooming pups than saline-injected animals. In a follow-up study, one group of animals received an injection of AVP preceded by an injection of saline or d(CH$_2$)$_5$Tyr(Me)-AVP into the lateral septum. A second group of animals received an injection of saline preceded by an injection of saline or d(CH$_2$)$_5$Tyr(Me)AVP into the lateral septum. In both groups, animals spent less time grooming, crouching over, and contacting pups if they had first been injected with d(CH$_2$)$_5$Tyr(Me)AVP. Control experiments suggested that the effects of AVP on paternal responsiveness were dose- and site-specific. These data suggest that septal AVP enhances paternal responsiveness by a V1$_a$ receptor-mediated mechanism.**

In monogamous rodent species, such as prairie voles (*Microtus ochrogaster*), males as well as females provide parental care (1). Although there is virtually no knowledge on the neural basis of parental behavior in prairie voles, there is much knowledge on the neural mechanisms underlying maternal behavior in other rodents, particularly in rats (2, 3). However, the neural mechanisms underlying paternal behavior are unknown.

One study compared, but did not find differences in, central oxytocin binding sites between sexually naive and maternal prairie voles (4), although it did find an induction of oxytocin binding sites in maternal montane voles (*Microtus montanus*), a promiscuous species in which males do not provide paternal behavior (5). A recent comparison of prairie and meadow voles (*Microtus pennsylvanicus*) suggested that the [Arg^8]vasopressin-immunoreactive (AVP-ir) innervation of the lateral septum may be involved in paternal behavior. In meadow voles, another promiscuous species in which males do not provide paternal care (1, 6), the plexus of AVP-ir fibers in the lateral septum did not differ between fathers and sexually inexperienced males, while in prairie voles, a monogamous species in which males do provide paternal care, this plexus was less dense in fathers than in sexually inexperienced males (7). A follow-up study showed that, compared with sexually inexperienced voles, the density of this plexus is dramatically lower in males that have been paired with females for 3 days (8), in which time they typically mate (9). After that, the density of the plexus returns to the levels of sexually inexperienced males, to drop again once the pups are born (8). Preliminary evidence suggests that this reduction in AVP-ir fiber density after mating is accompanied by an increase in AVP mRNA in the bed nucleus of the stria terminalis (10), the most likely source of these fibers, and therefore may reflect an increase in septal AVP release that is not immediately followed by a restoration of the AVP-ir content of the fibers (8).

These fluctuations in AVP-ir fiber density might be related to changes in social behaviors. After mating, prairie voles develop a strong partner preference and increase their aggressiveness towards other conspecifics (11). A recent study showed that intraventricular injections of AVP enhanced these behaviors in males, whereas intraventricular injections of an AVP antagonist blocked mating-induced increases in these behaviors, suggesting that endogenous AVP release contributes to mating-induced behavioral changes (12). After mating, male prairie voles also show an increase in paternal responsiveness (8). Although there are no data on the effects of septal AVP injections on parental behavior, intracerebroventricular injections of AVP increase maternal responsiveness in rats (13). Therefore, the changes in the density of the AVP-ir fiber plexus after mating may also be related to the changes in paternal responsiveness. Here we test the hypothesis that septal AVP influences paternal responsiveness in male prairie voles.

MATERIALS AND METHODS

The subjects were sexually inexperienced male prairie voles from the F$_3$/F$_4$ generation of a breeding colony started with field-trapped animals. Twelve males were housed in pairs in plastic cages (44 × 24 × 20 cm) filled with peat, wood chips, and a 10-cm hay covering under a 14 hr/10 hr light/dark cycle at 21°C. When the animals were between 70 and 90 days old, 26-gauge stainless steel guide cannulas were implanted stereotaxically aimed at the lateral septum (nosebar at 0; 1.4 mm rostral, 0.4 mm unilateral, and 4 mm ventral to bregma) under ketamine anesthesia. Three days later, each subject received

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Abbreviations: AVP, [Arg^8]vasopressin; AVP-ir, AVP-immunoreactive; BST, bed nucleus of the stria terminalis; MA, medial amygdaloid nucleus; d(CH$_2$)$_5$Tyr(Me)AVP, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine]AVP.

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100-nl injections of either saline (0.9% NaCl), AVP (Sigma; 0.1 ng/100 nl of saline), or the $V1_a$ receptor antagonist [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine]AVP [Sigma; $d(CH_2)_5Tyr(Me)AVP$; see ref. 14; 1 ng/100 nl of saline] with a 33-gauge needle that extended 1 mm below the guide cannula into the lateral septum. The needle was connected to a 1-μl Hamilton syringe through PE20 tubing. The sequence of injections was counterbalanced at 2-day intervals until each subject had received each of the three injections once (Latin-square design). For saline and AVP injections, paternal responsiveness was tested according to the procedure used by Gubernick and Nelson (15). Immediately after each injection, subjects were placed into a clean aquarium (51 × 30 × 26 cm). After 5 min of adaptation, a 3- to 5-day-old prairie vole pup was placed in the center of the aquarium and the behavior was recorded for 10 min. For $d(CH_2)_5Tyr(Me)AVP$, paternal responsiveness was tested 2 hr after injection, because previous pharmacological studies had indicated that after that period $d(CH_2)_5Tyr(Me)AVP$ optimally blocks behavioral actions of AVP (16). The durations of the four most prominent paternal activities—i.e., grooming, crouching over, contacting, and retrieving the pup—were recorded with an event recorder (S&K Computer Products, Buffalo, NY). In addition, the durations of locomotion, self-grooming, and inactivity while not in contact with the pup were also recorded as nonsocial activities. Each pup was used only once. One saline-injected and one $d(CH_2)_5Tyr(Me)AVP$-injected animal attacked the pup. In these cases, the test was terminated immediately, and the time spent in paternal activities was scored as 0 sec. Data on the nonsocial activities were not acquired for these animals. The data were analyzed by orthogonal Latin-square analysis, and significant treatment effects were further examined with the post-hoc Newman–Keuls test.

In a follow-up experiment, 10 animals were housed and implanted with guide cannulas similarly as in the first experiment and injected with either $d(CH_2)_5Tyr(Me)AVP$ or with saline. Two hours later, all animals received an injection of AVP, after which their paternal responsiveness was tested. Two days later, this experiment was repeated in a counterbalanced manner. In a second similarly treated group, eight animals were injected with either $d(CH_2)_5Tyr(Me)AVP$ or saline. Two hours later all animals received an injection of saline and their paternal responsiveness was tested. Two days later, this experiment was repeated in a counterbalanced manner. Differences in paternal and nonsocial activities within these two groups were tested with a paired *t* test.

To characterize the dose–response relationship between AVP and paternal responsiveness, 10 animals that were housed and implanted with guide cannulas similarly as in the first experiment were injected in the lateral septum with 100 nl of saline containing 0, 0.01, 0.1, 1.0, or 3.0 ng of AVP, after which paternal responsiveness was tested. Each subject received a total of three injections at 2-day intervals with a different concentration each time counterbalancing the injections in the other subjects. The data were analyzed by a one-way ANOVA and significant treatment effects were further examined with the post-hoc Newman–Keuls test.

To test the site specificity of the effects of AVP on paternal responsiveness, five animals were implanted with guide cannulas aimed at the lateral septum (coordinates as in the first experiment) and seven animals with guide cannulas aimed at the lateral ventricle (nosebar at 0; 1.8 mm caudal, 1.1 mm unilateral, and 2.2 mm ventral to bregma). Each subject was injected with saline or with 0.1 ng of AVP in 100 nl of saline, after which paternal responsiveness was tested. Two days later this experiment was repeated in a counterbalanced manner. Differences in paternal responsiveness were tested with a two-way ANOVA with site and type of injection as between-subject variables. Significant interactions were further examined with the post-hoc Newman–Keuls test.

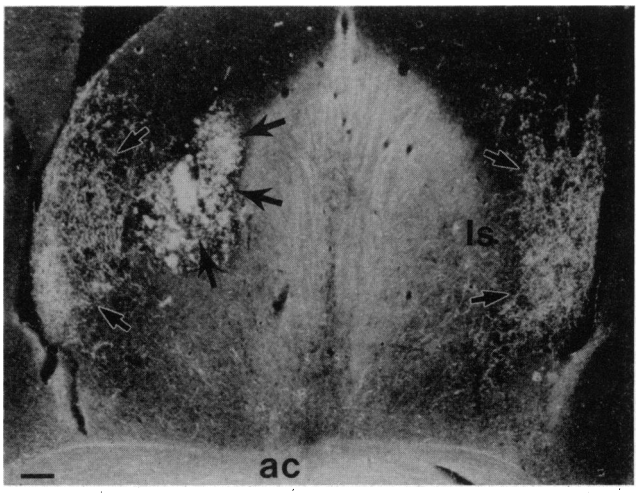

FIG. 1. Photomicrograph of a dark-field-illuminated section stained immunocytochemically for AVP. Black arrows indicate the site of the injection. The white rimmed arrows indicated the plexus of AVP-ir fibers in the lateral septum (ls). ac, Anterior commissure. (Bar = 200 μm.)

After the behavior was recorded, all subjects were sacrificed by perfusion fixation to verify injection sites histologically. Some of the sections were processed for AVP immunocytochemistry (7).

RESULTS

Histological analysis showed that the needle tracks typically ran through the medial margin of the AVP fiber plexus in the lateral septum (Fig. 1). Therefore, none of the subjects was excluded from the data analysis.

In the first experiment, the total time that voles spent displaying paternal activities (the time spent on grooming, crouching over, contacting, and retrieving combined) differed significantly per group ($F = 23.83$; df = 2, 14; $P < 0.0001$; Fig. 2). The post-hoc test indicated that voles injected with AVP spent more time displaying paternal activities than the other two groups. Significant differences were also found for specific paternal activities (Table 1). Voles injected with AVP spent more time crouching over and contacting pups

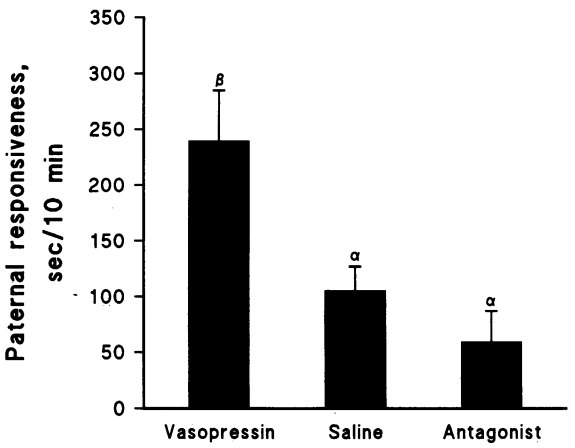

FIG. 2. Differences in paternal responsiveness among animals injected with AVP, saline, or the $V1_a$ antagonist. Animals injected with vasopressin (β) showed more paternal responsiveness than the other two groups (α; ANOVA, $P < 0.0001$). Bars indicate means + SEM.

Table 1. Effects of AVP, saline, or the $V1_a$ antagonist on the time spent on specific paternal activities

Activity	Time, sec/10 min (mean ± SEM) AVP	Saline	$V1_a$ antagonist	ANOVA	Newman–Keuls test
Grooming	104.1 ± 18.5	86.6 ± 18.8	39.1 ± 17.1	$P < 0.001$	AVP, saline > $V1_a$
Contacting	83.1 ± 34.3	6.9 ± 3.7	11.8 ± 11.6	$P < 0.001$	AVP > saline, $V1_a$
Crouching	50.2 ± 19.3	8.5 ± 3.9	3.9 ± 3.1	$P < 0.05$	AVP > saline, $V1_a$
Retrieving	2.1 ± 1.5	3.4 ± 1.3	4.7 ± 3.2	NS	—

NS, not significant.

than voles injected with saline or $d(CH_2)_5$Tyr(Me)AVP. In addition, voles injected with $d(CH_2)_5$Tyr(Me)AVP spent less time grooming pups than voles injected with saline or AVP. No differences were found in pup retrieval. Injection order had no significant effect on any of the paternal activities. In general, time spent on nonsocial activities did not differ among the groups except for the time spent in locomotion, which was significantly less in AVP-injected animals than in saline, or $d(CH_2)_5$Tyr(Me)AVP-injected animals (257.7 ± 46.4 versus 346.5 ± 30.1 and 381.1 ± 41.6 sec, respectively; $F = 12.17$; df = 2, 12; $P < 0.001$).

In the follow-up experiment, AVP-injected voles that were pretreated with $d(CH_2)_5$Tyr(Me)AVP spent less time displaying paternal activities than AVP-injected voles pretreated with saline ($t = 2.94$; $n = 10$; $P < 0.05$; Fig. 3*A*). The same difference was found for each specific paternal activity except for pup retrieval (Table 2). In the second group, saline-injected voles that were pretreated with $d(CH_2)_5$Tyr(Me)AVP spent less time displaying paternal activities than saline-injected voles pretreated with saline ($t = 6.85$; $n = 8$; $P < 0.001$; Fig. 3*B*). The same difference was found for each specific paternal activity except for pup retrieval (Table 2). In both experiments, no significant differences were found in nonsocial activities.

AVP influenced paternal responsiveness in a dose-specific manner. Voles injected with 0.1 ng of AVP spent more time displaying paternal activities than voles injected with saline or with 0.01 or 3.0 ng of AVP ($F = 4.57$; df = 4, 25; $P < 0.01$; Fig. 4*A*). Similar differences were found for the time that the voles spent grooming ($F = 4.84$; df = 4, 25; $P < 0.01$) and contacting ($F = 2.67$; df = 4, 25; $P < 0.05$; Fig. 4*B*) the pups. Although a similar trend was found in crouching over pups, the differences were not significant. There were no differences in pup retrieval.

No interaction between site and type of injection was found for the total time that voles spent displaying paternal activities. However, when the data were analyzed with a one-way ANOVA, significant differences were found between groups ($F = 3.74$; df = 3, 18; $P < 0.05$; Fig. 5). With voles injected into the lateral septum, AVP-injected animals spent more time displaying paternal activities than saline-injected voles, whereas no differences were found in voles injected into the lateral ventricles. As for the specific paternal activities, there was a significant interaction between site and type of injection for the time spent on grooming behavior, which was increased in voles injected with AVP into the lateral septum, but not in voles injected with AVP into the lateral ventricle ($F = 12.96$; df = 1, 18; $P < 0.01$; Fig. 5). No such interaction was found in the other paternal activities. However, there were significant treatment differences in the time spent crouching and in pup retrieval, which was longer in AVP-injected animals than in saline-injected animals ($F = 4.28$; df = 1, 18; $P < 0.05$; and $F = 5.42$; df = 1, 18; $P < 0.05$, respectively).

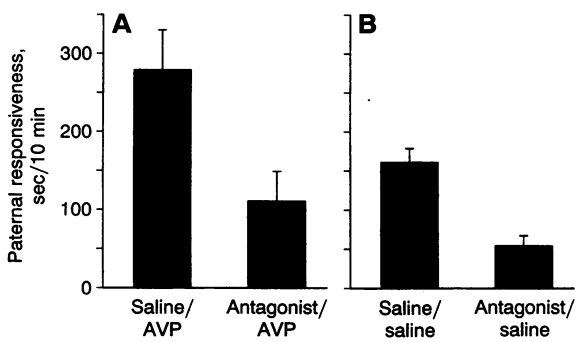

FIG. 3. (*A*) Differences in paternal responsiveness between animals that had received an injection of AVP preceded by an injection of saline (Saline/AVP) or the $V1_a$ antagonist (Antagonist/AVP) (*t* test, $P < 0.05$). (*B*) Differences in paternal responsiveness between animals that had received an injection of saline preceded by an injection of saline (Saline/saline) or the $V1_a$ antagonist (Antagonist/saline) (*t* test, $P < 0.001$). Bars indicate means + SEM.

DISCUSSION

The results of this study show that AVP injections into the lateral septum enhance paternal activities of sexually inexperienced male prairie voles in a dose-specific manner, whereas injections of the $V1_a$ antagonist $d(CH_2)_5$Tyr(Me)AVP inhibit paternal activities. The effects appear to be specific for paternal activities, since neither AVP nor $d(CH_2)_5$Tyr(Me)AVP affected nonsocial activities in a dramatic way. Only in the first experiment, the time spent in locomotion was significantly shorter in AVP-injected than in saline- or $d(CH_2)_5$Tyr(Me)AVP-injected voles, but this may be explained by the corresponding increase in the time spent crouching over and contacting pups in AVP-injected animals. Although the septal injections of AVP and its antagonists affected grooming, crouching over, and contacting pups, they did not affect pup retrieval. This may be due to the less prominent role that pup retrieval plays in parental behavior in prairie voles. As had been observed previously (1, 6), the prairie voles did not consistently show pup retrieval even when they showed high levels of the other paternal activities.

The volume and site of the septal injections suggest that AVP and $d(CH_2)_5$Tyr(Me)AVP mainly interacted with the AVP innervation of the lateral septum. In pilot experiments, similar injections of 100 nl of thionin typically showed the dye to be confined to an area with a diameter not larger than 0.5 mm and extending about 1 mm along the cannula track. Such injections would cover a substantial portion of the AVP fiber plexus in the lateral septum. In rats, this area receives its innervation mainly from the bed nucleus of the stria terminalis (BST) and medial amygdaloid nucleus (MA) (17, 18). Similarities in sex differences in this innervation suggest that in voles these fibers also come from the BST and MA: in voles as well as rats, these fibers are denser in males than in females (7, 19). Given that the needle track was at the medial margin of the AVP fiber plexus, the AVP injections probably also spread to the medial septum. AVP and $d(CH_2)_5$Tyr(Me)AVP could therefore have interfered with other structures—e.g., with the thick dispersed AVP fibers in the medial septum which appear to be derived from a source other than the BST (20). The differences in the effects of AVP and saline between animals injected into the lateral septum and lateral ventricle further support the site specificity of the effects of AVP on

Table 2. Interaction of AVP and the $V1_a$ antagonist ($V1_a$) on the time spent on specific paternal activities

Activity	Time, sec/10 min			Time, sec/10 min		
	Saline/AVP	$V1_a$/AVP	*t* test	Saline/saline	$V1_a$/saline	*t* test
Grooming	142.7 ± 26.9	62.5 ± 20.7	$P < 0.05$	87.9 ± 4.1	42.1 ± 9.7	$P < 0.01$
Contacting	93.4 ± 29.8	22.2 ± 11.4	$P < 0.05$	31.5 ± 4.4	10.2 ± 3.7	$P < 0.01$
Crouching	42.7 ± 11.8	17.6 ± 11.8	$P < 0.01$	28.6 ± 9.5	1.8 ± 1.2	$P < 0.05$
Retrieving	0.8 ± 0.7	8.9 ± 8.8	NS	13.4 ± 8.3	0.3 ± 0.3	NS

NS, not significant.

paternal behavior. There were some effects of AVP injections into the lateral ventricle, however. Although such injections did not increase the time spent grooming and contacting pups, they appeared to increase the time spent crouching over and retrieving pups, suggesting that AVP can influence specific paternal activities in sites other than the lateral septum.

The inhibiting effects of $d(CH_2)_5Tyr(Me)AVP$ on spontaneous paternal activities and on the stimulating effects of AVP on paternal responsiveness suggest that endogenous as well as exogenous AVP enhances paternal responsiveness by acting on $V1_a$ receptors. This is similar to the effects of AVP on social recognition and thermoregulation in rats, which are also functions in which the sexually dimorphic AVP projections of the BST and MA have been implicated (21, 22). However, some central effects of AVP—e.g., those on certain memory functions—can be blocked with $V1_a$ as well as V_2 and/or oxytocin antagonists (23). In addition, since the antagonist inhibited paternal activities, it is not simple to distinguish between the intrinsic effects of the antagonist and a specific blockade of the effects of injected AVP. Therefore, the pharmacological characteristics of the effects of AVP on paternal activities have to be studied in more detail, as was done for the effects of AVP on flank marking in hamsters (24, 25).

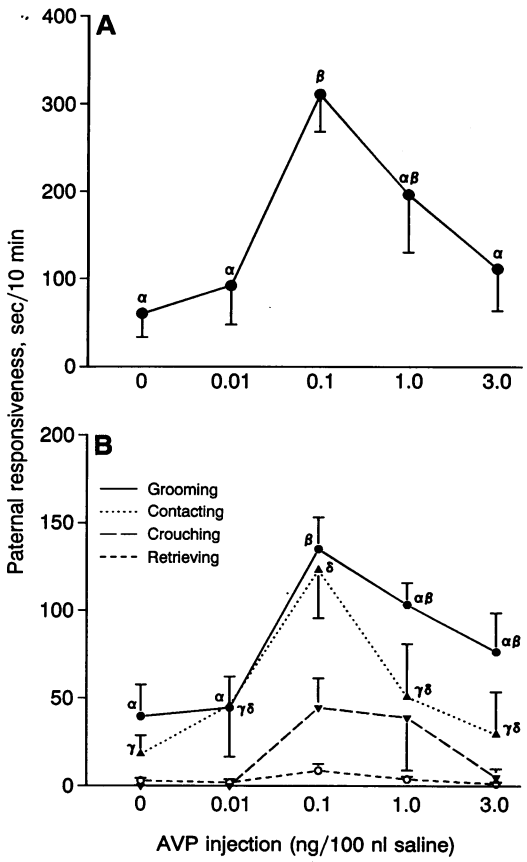

FIG. 4. Dose–response relationship for the effects of AVP on the total time that voles spent displaying paternal behavior (*A*) and on the time spent on specific paternal activities (*B*). Data points represent means and SEM. The Greek letters over the data points represent the post-hoc test results. Data points with the same letters did not differ significantly.

The effects of the injections of AVP and its antagonist suggest that endogenous as well as exogenous AVP can influence all of the prominent paternal activities except for pup retrieval. The first experiment, however, did not show differences between $d(CH_2)_5Tyr(Me)AVP$-injected animals and saline-injected animals in the total time that they spent displaying paternal activities, whereas the third experiment did show such differences. This may be related to differences in the injection procedure. In the first experiment, the behavior of $d(CH_2)_5Tyr(Me)AVP$-injected animals was recorded 2 hr after injection, while the behavior of saline-injected animals was recorded 5 min after injection. In the third experiment, voles in either group were injected at 2 hr as well as at 5 min before the behavior was recorded. Since the spontaneous paternal behavior displayed by saline-injected animals was higher in the third than in the first experiment, inhibiting effects of $d(CH_2)_5Tyr(Me)AVP$ might have been easier to detect in the third experiment.

AVP-injected animals may have spent more time displaying paternal behavior than saline-injected animals in the first experiment because injected AVP might have reached sites involved in paternal responsiveness that would normally not be exposed to endogenous AVP. Alternatively, the saline-injected animals may have spent less time displaying paternal behavior because in sexually inexperienced animals endog-

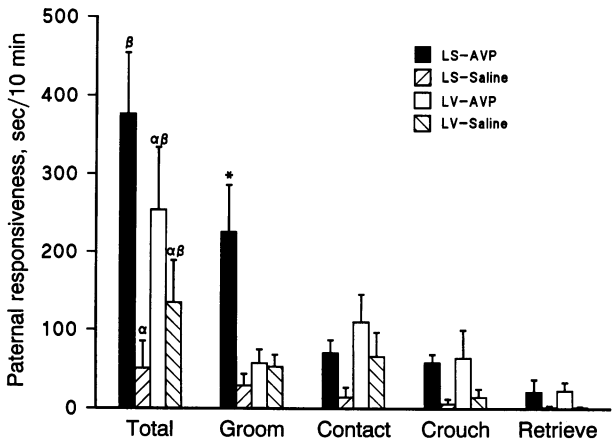

FIG. 5. Differences in the time spent on all paternal activities combined (Total) and on specific paternal activities—i.e., grooming, contacting, crouching over, and retrieving pups—between animals injected with AVP in the lateral septum (LS) (filled bars) and in the lateral ventricle (LV) (open bar) or with saline in the lateral septum (bars with hatching rising to the right) and in the lateral ventricle (bars with hatching rising to the left). *, Significant interaction between site and type of injection was only found in the time spent grooming (ANOVA, $P < 0.01$). One-way ANOVA suggested differences in the total time spent in paternal activities ($P < 0.05$). Greek letters over bars represent the post-hoc test results. Bars with the same letters did not differ significantly.

enous AVP might not be released in quantities that would optimally stimulate the circuits involved in paternal responsiveness. This would imply that if endogenous AVP release is increased, paternal responsiveness may be increased as well. One way in which AVP release may be increased is by changes in gonadal hormone levels. In other male rodents, such as rats, gerbils and mice, the density of AVP-ir innervation of the lateral septum and the AVP mRNA levels in the BST and MA—the sources of the AVP-ir innervation of the lateral septum—correlate positively with testosterone levels (18, 26–28). In prairie vole males, AVP-ir projections of the BST and MA are also testosterone-dependent (29). In addition, after mating there is an increase in testosterone levels (30) as well as in AVP mRNA labeling in the BST (10). These data suggest that mating increases the release of AVP in the septum of male prairie voles, which in turn may contribute to the increase in paternal responsiveness seen three days after mating (8). Supporting this argument is the observation that castration of male prairie voles not only inhibits AVP synthesis but also male responsiveness whereas testosterone treatment reverses these changes (29).

AVP release in the septum may also be increased by physiological challenges that may occur once the pups are born. At that time prairie vole fathers will spend a considerable amount of time licking their pups (6). In rats and other rodent species, this behavior involves ingestion of salty urine (31, 32). Since injections with hyperosmotic saline increase the release of AVP from the lateral septum in rats (33, 34), the osmotic challenge caused by ingestion of salty urine may enhance AVP release in voles as well. Another physiological challenge associated with paternal behavior may be a putative rise in body temperature, which voles may experience since they spend a considerable time huddled over pups (6). In rats, such behavior raises body temperature (35). Since a rise in body temperature also increases the release of AVP from the lateral septum of rats (36), a putative rise in body temperature in prairie vole fathers may increase AVP release as well. In either case, the data of the present study suggest that a putative increase in AVP release after pups are born would further enhance paternal responsiveness. Although data to substantiate these speculations are still lacking, the clarity of the effects of AVP on paternal behavior seen in the present study and the clear changes in the AVP projections of male and female voles in different stages of reproduction (7, 8) make prairie voles excellent subjects for the study of possible links between physiology and behavior.

In summary, the involvement of AVP-ir fibers of the lateral septum in paternal behavior in voles suggested by the results of the current study fits earlier findings of the importance of the septum and the sources of these fibers—i.e., the BST and MA—in maternal behavior in rats and mice (4, 37, 38). However, given the much denser AVP-ir projections from the BST and MA in male than in female prairie voles, and the lack of clear differences between maternal and sexually inexperienced females (7), AVP-ir projections of the BST and MA may play a lesser role in parental behavior in females than in males. The current data also fit the results of a preliminary study which showed that lesions of the MA disrupt paternal responsiveness in male prairie voles (39). These data have begun to unravel the neural mechanisms underlying paternal behavior and complement previous studies on the hormonal correlates of this behavior in rodents (15).

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