Available online at www.sciencedirect.com
ScienceDirect
Hormones and Behavior 50 (2006) 699–707
Hormones and Behavior
www.elsevier.com/locate/yhbeh

Paternal behavior influences development of aggression and vasopressin expression in male California mouse offspring

Cristianne R.M. Frazier [a,1], Brian C. Trainor [b,*,1], Catherine J. Cravens [a,1], Tina K. Whitney [b], Catherine A. Marler [a,b,*]

[a] Department of Zoology, University of Wisconsin-Madison, USA
[b] Department of Psychology, University of Wisconsin-Madison, USA

Received 24 March 2006; revised 9 June 2006; accepted 12 June 2006
Available online 24 August 2006

Abstract

Parental care has been demonstrated to have important effects on offspring behavioral development. California mice (*Peromyscus californicus*) are biparental, and correlational evidence suggests that pup retrieving by fathers has important effects on the development of aggressive behavior and extra-hypothalamic vasopressin systems. We tested whether retrievals affected these systems by manipulating paternal retrieval behavior between day 15 and 21 postpartum. Licking and grooming behavior affect behavioral development in rats, so we also experimentally reduced huddling and grooming behavior by castrating a subset of fathers. Experimentally increasing the frequency of paternal pup retrieving behavior decreased attack latency in resident–intruder in both male and female adult offspring, whereas experimental reduction of huddling and grooming had no effect. In a separate group of male offspring, we examined vasopressin immunoreactivity (AVP-ir) in two regions of the posterior bed nucleus of the stria terminalis (BNST): the dorsal fiber tracts (dBNST) and the ventral cell body-containing region (vBNST). Experimentally increasing retrievals led to an apparent shift in AVP-ir distribution. Specifically, offspring from the high retrieval group had more AVP-ir than offspring from the sham retrieval group in the dBNST, whereas the opposite was observed in the vBNST. Experimental reduction of paternal grooming was associated with increased AVP-ir in the paraventricular nucleus and also increased corticosterone and progesterone, similar to observed effects of maternal grooming on HPA function. This study provides further evidence that paternal behavior influences the development of aggression and associated neural substrates.

Keywords: Aggression; Paternal behavior; Arginine vasopressin; Bed nucleus of the stria terminalis; Testosterone; Corticosterone; *Peromyscus*; Mice; Huddling; Grooming

Parental care is an important early environmental factor associated with offspring behavioral and neuroendocrine development. Individual differences in rat maternal licking and grooming affect the behavioral and neurological stress responses of offspring (e.g. Meaney, 2001; Gonzalez et al., 2001). For instance, rat pups that are licked and groomed more by mothers exhibit a tighter regulation of glucocorticoid negative feedback in response to acute stress as adults (Liu et al., 1997) and also increased exploration of novel environments (Caldji et al., 1998). In rats, individual differences in maternal care are propagated across generations through nongenomic mechanisms (Francis et al., 1999). These studies of variation in the maternal behavior of rats and the consequences for her offspring demonstrate the significant impact of the early social environment on offspring development.

In contrast, little is known about the influence of parental behavior of fathers on offspring development. Correlational studies suggest that paternal care has important effects on the development of behavior and the neuroendocrine system in the biparental California mouse (*Peromyscus californicus*) (Bester-Meredith et al., 1999; Bester-Meredith and Marler, 2001;

* Corresponding authors. B.C. Trainor is to be contacted at 1835 Neil Ave, Columbus, OH 43210, USA. Fax: +1 614 688 4733. C.A. Marler, 1202 West Johnson Street, Madison, WI 53706-1611, USA. Fax: +1 608 262 4029.
E-mail addresses: trainor.7@osu.edu (B.C. Trainor), camarler@wisc.edu (C.A. Marler).
[1] These authors contributed equally to this work.

0018-506X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.yhbeh.2006.06.035

Bester-Meredith and Marler, 2003a,b; Bester-Meredith et al., 2005). Male California mice exhibit high levels of paternal care (Gubernick and Alberts, 1987), and experimental removal of fathers in the field reduces pup survival (Gubernick and Teferi, 2000). A recent study suggested that paternal grooming promoted the development of novel object recognition in California mice (Bredy et al., 2004), although at present the effects of paternal behavior on social behavior are unclear. In the early stages of pup development, males primarily huddle and groom their young with little retrieving. Between day 15 and 21 postpartum (P15–21), the pups become more active outside the nest and males shift from frequent huddling behavior to a more active retrieval routine (Bester-Meredith et al., 1999; Marler et al., 2003). Pups can be retrieved back to the nest or other locations. Frequently, pups are partially retrieved such that the pup is grabbed by the parent but is then released or escapes before it is picked up. Retrieving and grabbing could be interpreted as protective behaviors (Marler et al., 2005) that remove pups from a dangerous situation (see Discussion). In addition to being biparental, male and female California mice also express high levels of aggression and territorial behavior (Ribble and Salvioni, 1990; Bester-Meredith et al., 1999; Bester-Meredith and Marler, 2001) that are plastic in response to social experience both during development (see below) and adulthood (Oyegible and Marler, 2005).

The neuropeptide arginine vasopressin (AVP) is closely associated with aggressive behavior in many mammalian species (Marler et al., 2003; De Vries and Boyle, 1998; Goodson and Bass, 2001), including humans (Coccaro et al., 1998; Thompson et al., 2004). Dense clusters of AVP immunoreactive (-ir) cells in the posterior bed nucleus of the stria terminalis (BNST) and medial amygdala project to the lateral septum and have been shown to be androgen dependent during development (De Vries and Miller, 1998). In male California mice, intracerebroventricular infusions of AVP receptor (V_{1a}) antagonists decrease aggression in resident–intruder tests (Bester-Meredith et al., 2005). Cross-fostering studies reveal that male California mouse pups raised by less aggressive and less paternal white-footed mouse (*Peromyscus leucopus*) are less aggressive in resident–intruder tests and also have less AVP-ir staining in the bed nucleus of the stria terminalis (BNST) compared to in-fostered male California mice (Bester-Meredith and Marler, 2001).

Peromyscus cross-fostering studies suggest that AVP in the BNST pathway may specifically influence paternal retrieving behavior. The amount of AVP-ir staining in the BNST is significantly higher in fathers observed retrieving pups during cross-fostering compared to fathers that did not retrieve pups (Bester-Meredith and Marler, 2003b). Furthermore, the decrease in paternal retrieving experienced by California mouse pups when raised by white-footed mice is associated with both decreased aggression (Bester-Meredith and Marler, 2003a) and the amount of paternal retrieving they perform on their biological offspring (Bester-Meredith and Marler, 2003a). Thus, paternal retrieving behavior may be a postnatal environmental cue contributing to the development of offspring aggression that is mediated by AVP in the BNST.

To test directly whether paternal behavior influences offspring aggression and AVP, we experimentally manipulated paternal behavior in two ways. First, we experimentally increased paternal retrieval and grabbing behavior. Second, we castrated fathers to investigate the effects of paternal huddling and grooming on the development of pup behavior and AVP expression. Castration reduces male huddling and grooming behavior of 1- to 3-day-old pups, a decrease which is only partially compensated for by changes in the mothers' behavior (Trainor and Marler, 2001). When pups are older and parents show low levels of huddling, castration reduces grooming but not huddling (Marler et al., 2003). Sexually inexperienced adult male and female offspring were then tested in R–I and neutral arena aggression tests. Another group of male offspring was used in the analysis of AVP-ir distribution in five brain regions: the dorsal fiber tract region of the posterior BNST (dBNST), the ventral cell body-containing region of the posterior BNST (vBNST), medial amygdala, supraoptic nucleus, and paraventricular nucleus. Adult male offspring plasma was also assayed for the presence of the steroid hormones testosterone, progesterone, and corticosterone.

Materials and methods

We used reproductively experienced, randomly selected *P. californicus* male–female pairs and their male and female offspring (Table 1). Animals were maintained in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*. During the standard housing phases, mice were group housed in 48.3×26.7×15.6 cm cages with free access to Purina 5015 mouse chow and water. Rooms were maintained at 25°C under a 13L:11D light cycle with lights on at 05:00. During manipulations and testing phases, we housed parents and pups in Plexiglas observation chambers that were partitioned into one small (22×29×30 cm) and one large compartment (30×29×30 cm) using a Plexiglas insert containing two small holes to allow for passage between the compartments. The large compartment contained a running wheel, water bottle, and food available *ad libitum*. We conducted behavioral manipulations and aggression tests under dim red lights 1 h after the initiation of the dark phase (19:00–21:00). Fifty-five California mouse mating pairs were randomly assigned to one of four treatment groups: (1) intact/sham retrieval, (2) castration/sham retrieval, (3) intact/high retrieval, (4) castration/high retrieval.

Castration surgeries

To alter huddling and grooming behavior, male parents underwent either a castration or sham procedure 3 days after the birth of a litter. The three-day

Table 1
Sample sizes for retrieval manipulations, castration manipulations, aggression tests, and immunocytochemistry

	Sham retrieval intact	Sham retrieval castrate	High retrieval intact	High retrieval castrate
Total pairs	17	13	10	15
Total pups	34	32	33	34
Average pups per litter	2	2.5	3.3	2.3
Male R–I	6	4	8	7
Male neutral	5	4	7	3
Male AVP	7	6	7	8
Female R–I	8	9	6	9
Female neutral	8	9	5	7

interval allowed males to inseminate females during the postpartum estrus and provide a consistent rearing environment for the newly conceived, experimental litter (Trainor and Marler, 2001). Surgeries were performed using a single incision at the scrotum under isoflurane anesthesia. Castrated males had both testes removed using a sterile cautery, and the incision was closed using Nexaband adhesive. Sham castrations were identical except that the testes were not removed. Each male received analgesia treatment (ketoprofen, 1 mg/kg) and recovered in isolation for 1 week and was then returned to his mate and pups. The first litter was weaned at 30 days of age and not used in the experiment. Seven days after the parturition of the experimental litter, mating pairs and their pups were transferred to the observation cages. At 14 days post-parturition (P14), the bedding of each cage was changed.

Retrieval manipulations and behavioral observations

We conducted retrieval manipulations every night on P15–21, when paternal retrieval peaks (Bester-Meredith et al., 1999). The female and pups were removed from the cage, and the female was temporarily placed in a standard cage, leaving the father in place. During a high retrieval manipulation, the pups were immediately returned to the observation cage but on the opposite side of the nest. In a sham manipulation, the pups were replaced directly into the nest. During retrieval manipulation observations, we recorded paternal retrieval and grabbing frequencies of pups. Retrievals occurred when the male grasped a pup with his mouth posterior to the shoulder and lifted it off of the ground. Grabbing occurred when the male grasped the pup on the shoulder or flanks with its mouth but did not pick the pup off of the cage floor. We scored the amount of time the father spent in the nest, grooming a pup, huddling with a pup, and running in the wheel. Paternal grooming was defined as licking a pup. Paternal huddling was scored when the pup was in contact with the ventral surface of the father. Previous work demonstrates that paternal huddling behavior is reduced during P15–21 (Bester-Meredith et al., 1999; Marler et al., 2003) when pups become endothermic. We also recorded measurements of pup activity: the amount of time pups spent in the nest, active outside of the nest, and running in the wheel. A pup that was active outside of the nest had to be both outside of the nest and actively locomoting. It was difficult to distinguish individual pups under dim red light, so we recorded the behavior of the whole litter. Thus, if one pup was in the nest, huddled by the male, and another pup was running in a wheel, we recorded all three behavior types as occurring simultaneously. Male and pup behavior were videotaped for 20 min and then the female was returned.

To characterize parental behavior outside of the retrieval manipulations, we recorded overnight behavior at eight 10 min intervals from 22:00 to 5:00 on three pairs and their pups from each treatment group. Using a computerized event recorder, an observer blind to treatment groups scored parental and pup behavior during the 20 min manipulations and overnight observations. On P22, the parents and pups were returned to a standard cage. On P31, pups were weaned into standard cages with same-sex, same-age weanlings. Between 2 and 3 male offspring and 2 and 4 female offspring were housed in each cage. Offspring matured under the housing conditions described above for 6 months, which is 90 days longer than the average age of dispersal in the field (Ribble, 1992).

Aggression tests

We tested the sexually naive, 6-month-old male and female offspring for resident–intruder and neutral arena aggression. Resident–intruder tests are used to estimate territorial aggression in rodents, including California mice (Bester-Meredith and Marler, 2001; Gray et al., 2002); neutral arena tests examine aggression outside the context of a territory (Mossman and Srivastava, 1999; Bester-Meredith and Marler, 2001). All intruders were sexually inexperienced and both age- and sex-matched with residents. We tested one male offspring from 25 of the 55 pairs described above for R–I aggression (Table 1). Nineteen of these male offspring were also tested in neutral arena tests 6 weeks after the resident–intruder tests (Table 1). At 6 months of age, we also tested two female offspring from 32 of the 55 pairs described above in resident–intruder tests and 6 weeks later for neutral arena aggression (Table 1). In all tests, intruders were the same sex as the resident (experimental animals). For female resident–intruder tests, only diestrous females were used as intruders (Davis and Marler, 2003). A vaginal lavage was conducted on each experimental female after each aggression test to determine the stage of the estrous cycle. We did not lavage experimental females prior to testing because this can affect aggressive behavior (E.S. Davis, personal observation).

For resident–intruder tests, the adult offspring was placed in an observation cage 48 h prior to testing. At the beginning of each test, the intruder was placed in the smaller chamber of the observation cage. All tests were terminated upon attack by the resident. If no attack occurred after 10 min, the intruder was removed and an attack latency of 600 s was assigned to the experimental animal. The intruder never attacked the resident first. For neutral arena aggression tests, both the experimental offspring and their opponent were placed simultaneously into separate compartments of a clean observation cage and the attack latency for the experimental offspring was measured. All tests were run for 10 min independently of an attack. Tests were videotaped and later an observer blind to treatments recorded the attack latency. Aggression was measured using attack latency: the amount of time from introduction of the non-experimental animal to an attack by the experimental animal.

AVP immunocytochemistry

We used another subset of 6-month-old male offspring for AVP-ir and hormone analysis (Table 1). The brains of these sexually inexperienced adult male offspring were collected via decapitation at which time trunk blood was collected for hormone assays (see below). Brains were immediately fixed in 5% acrolein for 3 h, transferred to fresh 5% acrolein for another 2.5 h, refrigerated overnight in a 30% sucrose buffer solution, and frozen on dry ice. Fifty-micrometer coronal sections were collected using a vibratome and refrigerated overnight in PBS.

Immunocytochemical assays contained equal representations of each treatment group. Free-floating sections were processed for AVP immunoreactivity at room temperature unless stated otherwise (Bester-Meredith et al., 1999). Briefly AVP immunoreactivity was located with rabbit anti-AVP serum (ICN Laboratories, Costa Mesa, CA) in a 1:5000 dilution for 90 min at 37°C, followed by incubation with a goat–anti-rabbit IgG (GAR) in a 1:150 dilution for 45 min at 37°C, and then a 1:300 rabbit peroxidase–anti-peroxidase (PAP) application for 45 min at 37°C. Sections were incubated twice with GAR and PAP before visualization of the antibody complex using fast-acting DAB tablets (Sigma) as the chromogen.

Microscopic images were captured under bright-field illumination. The percentage of AVP-ir staining, i.e., the number of pixels covered by AVP-ir cells and fibers within a defined area (Bamshad et al., 1993; Bester-Meredith and Marler, 2003b), in the dBNST, vBNST, medial amygdala, supraoptic nucleus, and paraventricular nucleus was analyzed by an observer blind to treatment groups using Scion Image Software. Percent AVP-ir staining was quantified for two different areas of the posterior BNST (Bregma 0.22 through −0.46 mm; Franklin and Paxinos, 1997); the dorsal fiber tracts (dBNST) and the ventral region containing the majority of cell bodies (vBNST) (Fig. 3). Both regions of the posterior BNST may contain the cells and fibers of BSTpr and BSTfi as described in rats (Dong and Swanson, 2004). The number of 50 μm sections containing AVP-ir fiber tracts in the dBNST per individual ranged from 2 to 12 with 5.7 sections being the average ($n=27$). The light intensity and camera setting were constant across the sections to standardize measurements. Percent AVP-ir was expressed as the number of pixels covered by stained cells and fibers within a defined area (Bester-Meredith and Marler, 2001, 2003b). For each brain region, a defined box size was kept constant throughout all percent staining measurements of that brain area. A box size of 160 × 400 pixels was used for the dBNST, 300 × 100 for the vBNST, 444.3 × 103.8 for the MA, 415.19 × 1139.2 for the PVN, and 500 × 74.61 pixels for the SON. The section with the maximum percent AVP-ir staining from each brain area was identified and used for analysis.

Testosterone, progesterone, and corticosterone measurements

Hormone assays were completed at the Wisconsin Regional Primate Research Center Assay Laboratory. Steroids were extracted twice with ethyl ether and separated using celite chromatography. Hormone assay procedures and

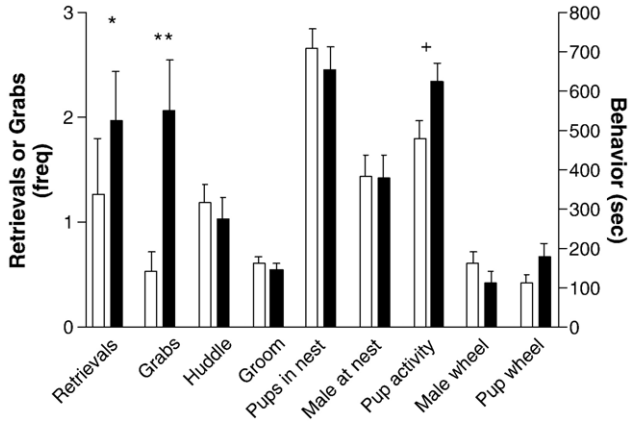

Fig. 1. Paternal and pup behavior during 20-minute retrieval manipulations averaged across intact and castrated males. Effects of retrieval manipulation: *$p<0.05$, **$p<0.01$, +$p=0.059$. Open bars=sham retrieval; black bars=high retrieval.

assay validation information has been previously described in detail (Bester-Meredith and Marler, 2001; Trainor and Marler, 2001; Davis and Marler, 2003). The intra-assay coefficients of variation were 6.7% for T, 1.7% for B, and 7.5% for P.

Statistical analysis

We averaged across all testing sessions for all measures of pup and parental behavior. During the 20 min manipulations, four male parents in the sham retrieval group (1 with an all female litter) displayed outlying levels of paternal retrievals that were more than 2 standard deviations from the mean of all 55 pairs and were thus excluded from all analyses. The average number of retrievals in the outliers (mean±SE, 10.1±1.95) was ten times higher than the average of the sham retrieval group (1.0±0.33). We used Q–Q plots to assess normality and to decide which transformations yielded the most normally distributed data. We also checked cell variances for homogeneity of variance. Mann–Whitney *U* tests were used to analyze group differences in retrieving and grabbing behavior because of heterogeneous variance. Based on analyses of Q–Q plots, we log transformed attack latencies and hormone levels (Zar, 1996). Because more than one female offspring from the same litter was tested for aggression, we used the average attack latency of those two offspring for analysis. We used two-way ANOVA to test the effects of treatments on attack latency, estrus cycle, AVP-ir staining, and hormone levels. One brain had staining undetectable from background and thus was not included in any AVP analyses. The number of male offspring in a litter was used as a covariate in AVP analysis because of the positive effect male offspring presence and position *in utero* has on development of male sibling androgen systems (vom Saal, 1983) which in turn can affect AVP (De Vries and Boyle, 1998; Lonstein et al., 2005).

Results

Retrieval and castration manipulations

During 20 min retrieval manipulations, fathers in the high retrieval group retrieved ($U=223$, $n_{sham}=27$, $n_{high}=24$, $p=0.049$) and grabbed ($U=191.5$, $n_{sham}=27$, $n_{high}=24$, $p=0.011$) pups significantly more than fathers in the sham retrieval group (Fig. 1). Retrieving and grabbing frequencies were also positively correlated ($r=0.78$, $p<0.001$). Castrated and intact fathers did not differ with respect to pup retrieving and grabbing.

There was no significant effect of the retrieval manipulation, castration, or interaction of the two manipulations on the amount of time the father or the pups spent in the nest or running in the wheel ($p>0.08$; Fig. 1). There was, however, a non-significant trend for pups from the high retrieval group to spend more time active outside the nest ($F_{1,47}=3.74$, $p=0.059$; Fig. 1). There was a non-significant positive correlation between pup activity and paternal grabbing frequency ($r=0.273$, $p=0.052$). Although there may be a connection between grabbing frequency and pup activity, pup activity explains less than 10% of the variation in grabbing behavior, which suggests that these variables are largely independent.

To test if manipulations affected the initial separation of pups from fathers, we analyzed the *latency* of the father to come in contact, groom, or huddle with their pups after replacement. There was no effect of retrieval manipulation, castration, or their interaction on these three variables (p's>0.2), suggesting that on average males from all groups initiated paternal contact with their young at similar times. We detected no effects of retrieval manipulation, castration, or their interaction on total time spent huddling or grooming during the 20 min manipulations (all p's>0.5; Fig. 1). While we castrated males to decrease huddling and grooming behavior, the lack of an observed effect of castration on huddling behaviors during P15–21 was expected because during this time males groom their offspring but do not huddle and the overall frequency of grooming was low during retrieval manipulations. A negative effect of castration on grooming was detected during overnight observations when the parents and pups were undisturbed ($F_{1,8}=10.63$, $p=0.01$; Table 2). We found no effects of castration or retrieval manipulations on any other measure of paternal, maternal, or pup behavior during overnight observations (Table 2). One important observation was that there was no evidence that the retrieval manipulation influenced maternal retrievals during the overnight observations. We did not expect to see differences in retrieval behavior here because retrieval behavior was not manipulated during these observations.

Table 2
Effects of castration and retrieval in overnight observations ($n=6$ per group)

Behavior	Castration P	Retrieval P	Interaction P
Paternal pup grooming	**0.01**	0.27	0.11
Paternal huddling	0.53	0.6	0.26
Maternal huddling	0.85	0.36	0.95
Nursing	0.38	0.47	0.84
Pup activity	0.77	0.63	0.27
Pups in nest	0.09	0.74	0.25
Paternal retrieving	0.87	0.15	
Paternal grabbing	0.53	0.75	
Maternal retrieving	0.27	0.69	
Maternal grabbing	0.87	0.11	

Mann-Whitney *U*-tests (for retrieving and grabbing) and two-way ANOVAs (for all other behaviors). Bold type indicates $p<0.05$.

Effects of paternal behavior on aggression

Resident–intruder tests

As adults, male offspring from the high retrieval group exhibited significantly shorter attack latencies compared to same-sex offspring from the sham retrieval group ($F_{1,18}=5.10$, $p=0.037$; Fig. 2). There was no difference in attack latency between males raised by castrated fathers versus intact fathers ($F_{1,18}=0.02$, $p=0.888$; Fig. 2), and there was no significant interaction ($F_{1,18}=2.24$, $p=0.16$). The same group differences in attack latency were observed in female offspring (Table 3, retrieval: $F_{1,28}=4.71$, $p=0.039$; castration: $F_{1,28}=0.17$, $p=0.68$; interaction: $F_{1,28}=0.15$, $p=0.70$). There was no effect of estrous cycle on attack latency in either the high retrieval ($F_{3,11}=0.16$, $p=0.92$) or sham retrieval females ($F_{3,13}=0.96$, $p=0.40$), although sample size per stage of estrous may have been too small to detect an effect.

Because pup activity was correlated with paternal grabbing, we used partial correlations to test the influence of these two variables on male R–I attack latency. Retrieving and grabbing frequencies were positively correlated but not normally distributed (see above). Analyses of Q–Q plots indicated that a square-root-transformed (Zar, 1996) of the combined retrieving/grabbing score for each male offspring yielded the most normally distributed data and were thus used for the following analysis. Male adult offspring attack latency was negatively correlated with pup activity ($r=-0.52$, $p=0.03$) and their fathers' paternal retrieving/grabbing frequency ($r=-0.64$, $p<0.01$). However, a partial correlation (controlling for pup activity) indicated that there was a significant negative relationship between attack latency and retrieving/grabbing ($r=-0.47$, $p=0.036$). In contrast, a partial correlation (controlling for retrieving/grabbing) indicated that there was no significant relationship between male attack latency and pup activity ($r=-0.31$, $p=0.18$). These results show that paternal retrieving and grabbing account for a significant amount of variation in offspring aggression that is not accounted for by variation in pup activity.

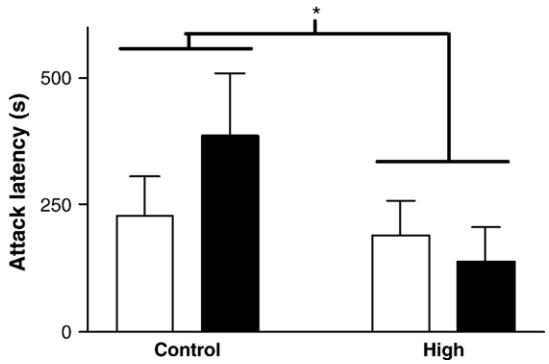

Fig. 2. Male offspring attack latency in resident–intruder aggression test. Control males experienced sham retrieval manipulations during development; high retrieval males experienced experimentally increased retrievals during development. Effect of retrieval manipulation: *$p<0.05$. Open bars=pups raised by intact fathers; black bars=pups raised by castrated fathers.

Table 3
Mean attack latencies and male baseline hormone levels of adult offspring

	Control		Retrieval	
	Intact	Castrated	Intact	Castrated
Male attack latency (s)	228±78*	386±123*	188±69	138±67
Female attack latency (s)	251±44*	261±63*	114±29	200±67
Male testosterone (ng/mL)	1.39±0.61	0.74±0.29	0.83±0.13	1.15±0.20
Male progesterone (ng/mL)	3.32±1.50**	1.41±0.53	2.71±1.02**	1.85±0.7
Male corticosterone (ng/mL)	86.2±44.6	228.0±113.7	49.7±16.3	162.4±44.2

* Effect of retrieval ($P<0.05$), ** effect of castration ($P<0.05$).

Neutral arena tests

A subset of males and females used in resident–intruder aggression tests were examined for aggression in a neutral arena. We found no effect of the retrieval manipulation in males or females or castration of the father in males or females on offspring neutral arena test attack latency. Male (Fisher Exact Test, $p=0.59$) and female (Fisher Exact Test, $p=0.46$) offspring in the high retrieval group and male (Fisher Exact Test, $p=0.37$) and female (Fisher Exact Test, $p=0.54$) offspring raised by castrated fathers were no more likely to attack than offspring in the low retrieval group.

Effects of paternal behavior on AVP-ir staining and hormone levels

AVP-ir was characterized in two regions of the posterior division of the BNST: the dorsal fiber tracts (dBNST) and the ventral cell body-containing region (vBNST) (Fig. 3). Male offspring from the high retrieval group had higher maximum percent AVP-ir staining in the fiber tracts of the dBNST than the sham manipulated group (Figs. 4, 5a, $F_{1,19}=4.64$, $p=0.04$). Although there was no difference in vBNST AVP-ir between the high retrieval and sham manipulated groups ($F_{1,19}=3.11$, $p=0.09$), the ratio of AVP-ir staining in the dBNST relative to AVP-ir staining in the vBNST (dBNST: vBNST) was significantly increased in male offspring in the high retrieval group compared to the sham retrieval group (Fig. 5b, $F_{1,19}=9.73$, $p=0.006$), possibly indicating a shift in AVP-ir staining distribution from the vBNST where the majority of the cell bodies are located to the fiber tracts of the dBNST. We detected no significant effects of retrieval manipulations on AVP-ir staining in the other brain regions examined (all p's>0.7).

We saw no effect of retrieval manipulations on steroid hormone levels in adult male offspring (Table 3, all p's>0.2). There was, however, a non-significant positive correlation between adult testosterone levels and the ratio of dBNST: vBNST AVP-ir staining ($r=0.37$, $p=0.074$). Testosterone did not correlate with AVP-ir staining in the other brain regions examined (p's>0.3). The amount of time the pups spent active

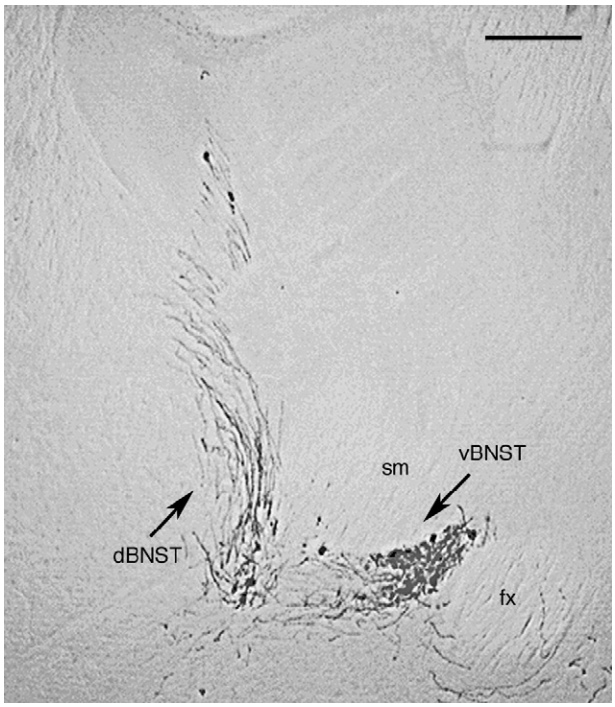

Fig. 3. Representative AVP-ir staining in the posterior dBNST fiber tracts and vBNST cell body region. sm, stria medullaris. fx, columns of the fornix (scale bar=250 μm).

outside the nest was also positively correlated with testosterone levels in adult offspring ($r=0.461$, $p=0.02$).

Male pups with castrated fathers had more AVP-ir in the paraventricular nucleus ($F_{1,19}=9.86$, $p=0.005$). These pups also had less AVP-ir staining in the dBNST compared to pups raised by intact fathers (Fig. 5, $F_{1,19}=4.51$, $p=0.047$). There was no effect of castration of the father on vBNST AVP-ir ($F_{1,19}=1.99$, $p=0.18$) or the ratio of dBNST:vBNST AVP-ir ($F_{1,19}=0.03$, $p=0.87$; Fig. 5b), suggesting that castration of fathers did not affect AVP distribution between the cell bodies and the fiber tracts in the BNST of adult male offspring. Castration of fathers had no effect on any measure of AVP-ir staining in the medial amygdala or supraoptic nucleus of adult offspring (p's>0.3).

Offspring raised by castrated fathers may have had higher adrenal activity as reflected by increased progesterone (Table 3, $F_{1,19}=5.04$, $p=0.036$) and corticosterone levels (Table 3, $F_{1,19}=3.77$, $p=0.066$) than pups raised by intact fathers. Additionally, paternal grooming of pups was significantly negatively correlated with male offspring adult corticosterone levels ($r=-0.44$, $p=0.028$).

Discussion

The hypothesis that increased retrieving and grabbing of offspring by fathers causes an increase in territorial aggressiveness when the offspring mature (Bester-Meredith and Marler, 2001, 2003a,b) was supported. Experimentally increased paternal retrieving and grabbing led to increased adult male offspring resident–intruder aggression and AVP-ir staining in the fiber tracts of the posterior BNST. This is the first experimental demonstration that a specific paternal behavior significantly affects the postnatal development of aggressive behavior and associated neural substrates. We also provide evidence that AVP in the fiber projections of the posterior BNST may be involved.

Retrievals appear to function as a behavioral component in "paternal programming" of the development of aggression. A previous cross-fostering study with California mice and white-footed mice suggested that paternal retrieving behavior can be passed between generations, possibly through behavioral mechanisms (Bester-Meredith and Marler, 2003b), and this was associated with a change in aggression between generations (Bester-Meredith and Marler, 2001, 2003a). The current study provides further evidence that paternal retrieving behavior can induce aggressive changes in the next generation. This provides an interesting comparison to the "maternal programming" of the development of stress responses and maternal behavior in rats in response to maternal licking and grooming behavior (Meaney, 2001; Gonzalez et al., 2001; Novakov and Fleming, 2005). It also raises the question of which sensory components of retrievals cause changes in offspring behavior/physiology. Effects of maternal behavior on offspring behavior can be mediated by the

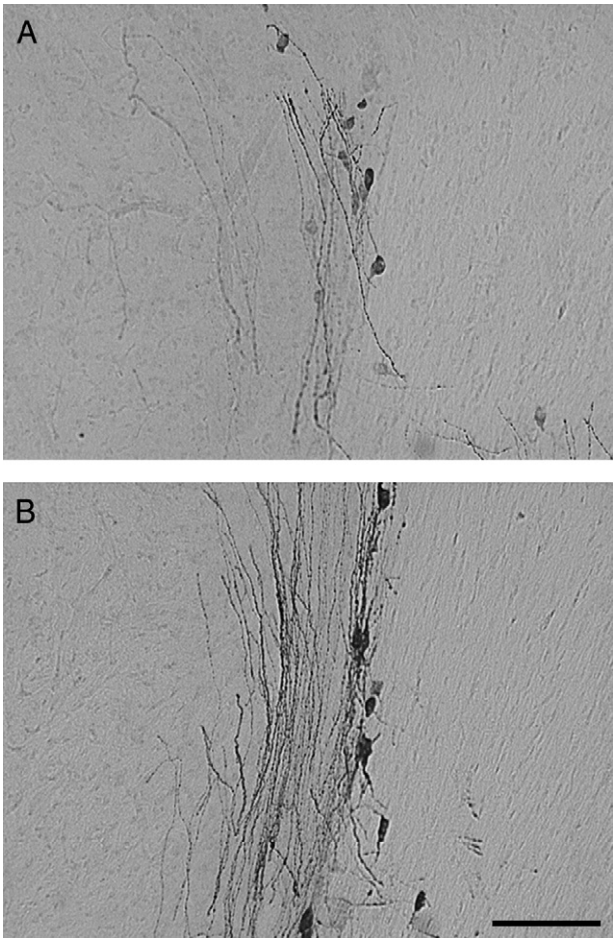

Fig. 4. Representative photomicrographs from the dBNST of male offspring from the sham retrieval group (A) and the high retrieval group (B). Scale bar=100 μm.

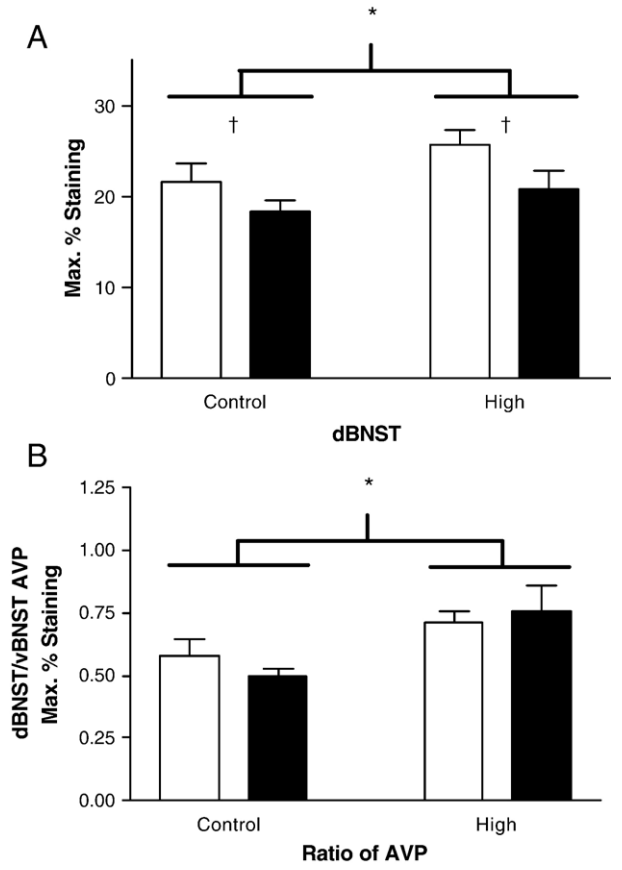

Fig. 5. Effects of retrieval manipulation and castration of father on AVP-ir in the dBNST (A), and the ratio of AVP in the dBNST to the vBNST (B). *Effect of retrieval manipulation ($p<0.05$). †Effect of castration of father ($p<0.05$). Open bars, pups raised by intact fathers; black bars, pups raised by castrated fathers.

tactile stimulation of licking or by stroking with a paintbrush (Gonzalez et al., 2001). It is likely that tactile stimulation from the father gripping the pup posterior to the shoulders could be an immediate cause for the observed long term effects. Previous studies on 10- to 24-day-old rat pups have described a stereotyped posture adopted by pups during retrieval, consisting of immobilization and the tucking in of both the front and hind legs (Brewster and Leon, 1980). This posture is stimulated by tactile stimulation and facilitates retrieving as localized lidocain treatment (which inhibits the immobilization response) increases maternal retrieval latency (Brewster and Leon, 1980). Pup retrieval in California mice occurs at a much later stage in development compared to rats, but anecdotal observations indicate that California mouse pups exhibit this immobilization response when retrieved by fathers. Future studies will examine the effect of artificial retrievals during development on adult aggression. In our study, handling by the experimenter was controlled for between the two groups. Thus, experimental handling cannot account for the observed group differences.

One mechanism that may play an important role in the "paternal programming" of offspring aggression is the neuropeptide AVP. Male offspring from the high-retrieval group had more AVP-ir staining in the dBNST fiber tracts. Furthermore, there was an apparent shift in AVP-ir distribution from the cell bodies of the vBNST to the fiber tracts of the dBNST in the high retrieval group. A variety of studies indicate that centrally acting AVP modulates components of aggressive behavior. Intracerebroventricular injections of V_{1a} antagonists decrease R–I aggression in California mice (Bester-Meredith et al., 2005). In hamsters, microinjections of AVP into the anterior hypothalamus and septum are known to increase aggressive behavior (Ferris and Delville, 1994; Ferris and Potegal, 1988). Furthermore, changes in BNST AVP and corresponding increases in resident–intruder aggression, but *not* in neutral arena aggression, were previously observed in California mice raised by white-footed mice (Bester-Meredith and Marler, 2001). Similarly, we found that experimentally increasing paternal retrieval rate resulted in decreased attack latency in offspring resident–intruder tests and increased AVP-ir staining in the dBNST, but had no effect on neutral arena aggression in male offspring. The distribution of AVP in male California mice differs from other rodents in that there is very little AVP-ir staining in the lateral septum. However, compared to male white-footed mice, male California mice have high densities of septal V_{1a} receptors, suggesting that AVP projections from the BNST may activate receptors in the septum (Bester-Meredith et al., 1999). While we did not explore the potential involvement of AVP in adult female aggression, AVP has been observed to promote aggression in female prairie voles (Stribley and Carter, 1999).

In rats, AVP localization in the terminal regions of the lateral septum is positively regulated by testosterone (De Vries et al., 1985) in a dose-dependent manner (Magnusson and Meyerson, 1996). We have observed that retrievals cause an immediate increase in testosterone in male California mouse pups (Brett Moore, Elizabeth Florek, Catherine Auger, and Catherine Marler, unpublished data). This raises the hypothesis that increased testosterone caused by retrievals mediate transportation of AVP to the dBNST and release onto the lateral septum V_{1a} receptors contributes to the observed effect of paternal retrieving on adult offspring aggression. There was some evidence in our study that males with higher testosterone may have increased AVP-ir staining in the fiber tracts of the dBNST relative to the vBNST. Future studies will examine how transient increases in testosterone during this stage of development impact the AVP neurochemical system.

Previous studies have demonstrated that stressors can alter vasopressinergic systems within the brain. In golden hamsters, repeated threats and attacks by male conspecifics shortly after weaning reduce aggression and are accompanied by a 50% decrease in AVP in the anterior hypothalamus (Delville et al., 1998). This raises the question of whether paternal retrievals are perceived as stressful by offspring. Although some pups resist retrieval by either running away or gripping the running wheel, most available evidence suggests that paternal retrieval (which increases aggression) is not analogous to social subjugation (which decreases aggression). In addition, experimentally increased retrievals did not affect measures of adrenal activity (but see below for effects of huddling and grooming). This suggests that paternal retrieving and grabbing behavior represent a different form of social interaction than social subjugation, although further investigation is needed. Retrieving and

grabbing could be interpreted as a protective behavior by removing pups from a dangerous situation (Marler et al., 2003), perhaps representing a protective style of parenting analogous to those found in primates (Fairbanks, 1996). Exactly how and why a protective paternal behavior would translate into more aggressive offspring is not yet clear. One possibility is that retrievals promote development of aggressive behaviors and prepare offspring to obtain a territory and other resources under difficult environmental conditions (Ribble and Salvioni, 1990).

Although there was no difference in aggressive behavior between pups raised by intact or castrated fathers, several observations suggest that pups raised by castrated fathers had altered adrenal function. Based on studies of maternal behavior (Caldji et al., 2000), we expected that pups that were groomed less by castrated fathers would have altered adrenal function. Our data suggest that paternal huddling and grooming may effect the development of the hypothalamic–pituitary–adrenal axis in ways similar to maternal licking and grooming. We observed a negative correlation between paternal grooming and male offspring corticosterone levels and increased progesterone levels in males raised by castrated fathers. These observations suggest that paternal grooming may decrease offspring baseline adrenal activity. We also observed that AVP-ir in the PVN was increased in offspring with castrated fathers. Vasopressin acting in the PVN can stimulate activation of the hypothalamic–pituitary–adrenal axis and have behavioral effects associated with depression (Rivier and Vale, 1983; Merali et al., 2006), so increased AVP-ir in the PVN could contribute to the observed increases in adrenal activity of pups raised by castrated fathers. California mouse dams compensate for about 50% of the decrease in huddling behavior expressed by castrated fathers (Trainor and Marler, 2001). Although the impact of changes in paternal huddling and grooming may be somewhat ameliorated, there clearly is an impact. Overall, while paternal huddling and grooming affect the development of offspring stress–response pathways with respect to progesterone and possibly corticosterone, these paternal behaviors apparently do not regulate male offspring aggression in the way that retrievals do. This indicates that, for pups, the experience of being retrieved is substantially different from being huddled or groomed by an adult male during development.

We considered the possibility that pup activity itself could influence the development of aggression; offspring attack latency was significantly correlated not only with retrieval/grabbing but also with pup activity. Using partial correlations, we found that male attack latency and retrieval/grabbing were significantly correlated when controlling for pup activity, whereas male attack latency and pup activity were not correlated when controlling for retrieval/grabbing behavior. These results suggest that the effects of the retrieval manipulation on pup activity alone cannot explain the observed differences in aggression; increases in retrieval and grabbing behavior appear to be integral to the increase in aggression. Our results also suggest that the retrieval manipulation did not cause an increase in pup separation from the father; there were no differences in latency to come in contact, groom, or huddle. We also detected no effects on total time huddling and grooming from the retrieval manipulation. Even so, similar to other species (Wuensch and Cooper, 1981), when male California mice are raised without their fathers, they display *less* aggression (Wallace, Bester-Meredith and Marler, unpublished data). We conclude that differences in paternal retrieving and grabbing, but not pup activity or separation from the father, mediate the increase in offspring aggression. It is possible that paternal retrieving or pup activity could have important effects on other behaviors such as biting or wrestling. Ongoing studies will address this question.

This line of research on mice has potential implications for primates, including humans. The pattern of AVP receptor distribution in rhesus macaques (*Macaca mulatta*) in the BNST, lateral septum, amygdala, hypothalamus, and brainstem is similar to rodents (Young et al., 1999). Correlational studies of humans with personality disorders revealed a positive relationship between aggressive life history and cerebrospinal AVP concentrations (Coccaro et al., 1998). Furthermore, intranasal AVP administration in men increases corrugator EMG responses to neutral facial expressions, bringing them to levels similar to the responses of control subjects when viewing angry faces (Thompson et al., 2004). Neglected (not abused) children have lower levels of AVP in the urine than control children in response to positive social interactions (Fries et al., 2005). Identifying the mechanisms by which social stimuli modulate animal behavior, including aggression, may contribute to our understanding of how the environment shapes human development.

In summary, we identified a novel effect of the parental behavior of fathers – the retrieving and grabbing of pups – on adult offspring aggression. Furthermore, we have begun to identify some of the underlying mechanisms for this behavioral change. In particular, there may be a role for variation in AVP-ir distribution in the BNST. Behavioral development in *Peromyscus* can serve as a useful model system for studies of both the behavioral and mechanistic changes that occur in mammals in response to the behavior of fathers towards their offspring.

Acknowledgments

We thank P. Martin, A. Seif, C. Clark, D.A. Geddes, J.A. Hutchens, J.L. Krajewski, and M. Swaab for technical support and P. Marler for reviewing the manuscript. Research support was provided by a Hilldale Fellowship to C.R.M.F., NIMH F31 MH64328 to B.C.T., NSF IBN-0110625 and IBN-9703309 to C.A.M., and by NIH P51 RR000167 (Wisconsin National Primate Research Center, University of Wisconsin-Madison) at a facility supported by Research Facilities Improvement Program grants RR15459-01 and RR020141-01.

References

Bamshad, M., Novak, M.A., Devries, G.J., 1993. Sex and species-differences in the vasopressin innervation of sexually naive and parental prairie voles, microtus ochrogaster and meadow voles, *Microtus pennsylvanicus*. J. Neuroendocrinol. 5, 247–255.

Bester-Meredith, J.K., Marler, C.A., 2001. Vasopressin and aggression in cross-fostered California mice (*Peromyscus californicus*) and white-footed mice (*Peromyscus leucopus*). Horm. Behav. 40, 51–64.

Bester-Meredith, J.K., Marler, C.A., 2003a. The association between male offspring aggression and paternal and maternal behavior of *Peromyscus* mice. Ethology 109, 797–808.

Bester-Meredith, J.K., Marler, C.A., 2003b. Vasopressin and the transmission of paternal behavior across generations in mated, cross-fostered *Peromyscus* mice. Behav. Neurosci. 117, 455–463.

Bester-Meredith, J.K., Young, L.J., Marler, C.A., 1999. Species differences in paternal behavior and aggression in *Peromyscus* and their associations with vasopressin immunoreactivity and receptors. Horm. Behav. 36, 25–38.

Bester-Meredith, J.K., Martin, P.A., Marler, C.A., 2005. Manipulations of vasopressin alter aggression differently across testing conditions in monogamous and nonmonogamous *Peromyscus* mice. Aggress. Behav. 31, 189–199.

Bredy, T.W., Lee, A.W., Meaney, M.J., Brown, R.E., 2004. Effect of neonatal handling and paternal care on offspring cognitive development in the monogamous California mouse (*Peromyscus californicus*). Horm. Behav. 46, 30–38.

Brewster, J., Leon, M., 1980. Facilitation of maternal transport by Norway rat pups. J. Comp. Physiol. Psychol. 94, 80–88.

Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P.M., Meaney, M.J., 1998. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc. Natl. Acad. Sci. U. S. A. 95, 5335–5340.

Caldji, C., Diorio, J., Meaney, M.J., 2000. Variations in maternal care in infancy regulate the development of stress reactivity. Biol. Psychiatry 48, 1164–1174.

Coccaro, E.F., Kavoussi, R.J., Hauger, R.L., Cooper, T.B., Ferris, C.F., 1998. Cerebrospinal fluid vasopressin levels—correlates with aggression and serotonin function in personality-disordered subjects. Arch. Gen. Psychiatry 55, 708–714.

Davis, E.S., Marler, C.A., 2003. The progesterone challenge: steroid hormone changes following a simulated territorial intrusion in female *Peromyscus californicus*. Horm. Behav. 44, 189–198.

De Vries, G.J., Boyle, P.A., 1998. Double duty for sex differences in the brain. Behav. Brain Res. 92, 205–213.

De Vries, G.J., Miller, M.A., 1998. Anatomy and function of extrahypothalamic vasopressin systems in the brain. Prog. Brain Res. 119, 3–20.

De Vries, G.J., Buijs, R.M., Van Leeuwen, F.W., Caffe, A.R., Swaab, D.F., 1985. The vasopressinergic innervation of the brain in normal and castrated rats. J. Comp. Neurol. 233, 236–254.

Delville, Y., Melloni, R.H., Ferris, C.F., 1998. Behavioral and neurobiological consequences of social subjugation during puberty in golden hamsters. J. Neurosci. 18, 2667–2672.

Dong, H.W., Swanson, L.W., 2004. Projections from bed nuclei of the stria terminalis, posterior division: implications for cerebral hemisphere regulation of defensive and reproductive behaviors. J. Comp. Neurol. 471, 396–433.

Fairbanks, L.A., 1996. Individual differences in maternal style: causes and consequences for mothers and offspring. Adv. Stud. Behav. 25, 579–611.

Ferris, C.F., Delville, Y., 1994. Vasopressin and serotonin interactions in the control of agonistic behavior. Psychoneuroendocrinology 19, 593–601.

Ferris, C.F., Potegal, M., 1988. Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters. Physiol. Behav. 44, 235–239.

Francis, D., Diorio, J., Liu, D., Meaney, M.J., 1999. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science 286, 1155–1158.

Franklin, K.B.J., and Paxinos, G., 1997. The Mouse Brain in Stereotaxic Coordinates, 2nd ed. Academic Press, New York.

Fries, A.B.W., Ziegler, T.E., Kurian, J.R., Jacoris, S., Pollak, S.D., 2005. Early experience in humans is associated with changes in neuropeptides critical for regulating social behavior. Proc. Natl. Acad. Sci. U. S. A. 102, 17237–17240.

Gonzalez, A., Lovic, V., Ward, G.R., Wainwright, P.E., Fleming, A.S., 2001. Intergenerational effects of complete maternal deprivation and replacement stimulation on maternal behavior and emotionality in female rats. Dev. Psychobiol. 38, 11–32.

Goodson, J.L., Bass, A.H., 2001. Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Res. Rev. 35, 246–265.

Gray, S.J., Jensen, S.P., Hurst, J.L., 2002. Effects of resource distribution on activity and territory defence in house mice, *Mus domesticus*. Anim. Behav. 63, 531–539.

Gubernick, D.J., Alberts, J.R., 1987. The biparental care system of the California mouse, *Peromyscus californicus*. J. Comp. Psychol. 101, 169–177.

Gubernick, D.J., Teferi, T., 2000. Adaptive significance of male parental care in a monogamous mammal. Proc. Roy. Soc. B 267, 147–150.

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P.M., Meaney, M.J., 1997. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic–pituitary–adrenal responses to stress. Science 277, 1659–1662.

Lonstein, J.S., Rood, B.D., De Vries, G.J., 2005. Unexpected effects of perinatal gonadal hormone manipulations on sexual differentiation of the extrahypothalamic arginine–vasopressin system in prairie voles. Endocrinology 146, 1559–1567.

Magnusson, K., Meyerson, B.J., 1996. Evidence for regulatory mechanisms maintaining testosterone-dependent AVP concentrations in terminal regions. Peptides 17, 263–268.

Marler, C.A., Bester-Meredith, J.K., Trainor, B.C., 2003. Paternal behavior and aggression: endocrine mechanisms and nongenomic transmission of behavior. In: Slater, P.J.B., Rosenblatt, J.S., Snowdon, C.T., Roper, T.J. (Eds.), Adv. Stud. Behav., Vol. 32. Academic Press, San Diego, pp. 263–323.

Marler, C.A., Trainor, B.C., Davis, E.S., 2005. Aggression and paternal behavior. Curr. Dir. Psychol. Sci. 14, 163–166.

Meaney, M.J., 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu. Rev. Neurosci. 24, 1161–1192.

Merali, Z., Kent, P., Du, L., Hrdina, P., Palkovits, M., Faludi, G., Poulter, M., Bedard, T., Anisman, H., 2006. Corticotropin-releasing hormone, arginine vasopressin, gastrin-releasing peptide, and neuromedin B alterations in stress-relevant brain regions of suicides and control subjects. Biol. Psychiatry 59, 594–602.

Mossman, C.A., Srivastava, N.P., 1999. Does aggressive behavior of *Peromyscus leucopus* influence isolation of habitat islands? Am. Mid. Nat. 141, 366–372.

Novakov, M., Fleming, A.S., 2005. The effects of early rearing environment on the hormonal induction of maternal behavior in virgin rats. Horm. Behav. 48, 528–536.

Oyegible, T.O., Marler, C.A., 2005. Winning fights elevates testosterone levels in California mice and enhance future ability to win fights. Horm. Behav. 48, 259–267.

Ribble, D.O., 1992. Dispersal in a monogamous rodent, *Peromyscus californicus*. Ecology 73, 859–866.

Ribble, D.O., Salvioni, M., 1990. Social organization and nest cooccupancy in *Peromyscus californicus*, a monogamous rodent. Behav. Ecol. Sociobiol. 26, 9–15.

Rivier, C., Vale, W.W., 1983. Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion *in vivo*. Endocrinology 113, 939–942.

Stribley, J.M., Carter, C.S., 1999. Developmental exposure to vasopressin increases aggression in adult prairie voles. Proc. Natl. Acad. Sci. U. S. A. 96, 12601–12604.

Thompson, R., Gupta, S., Miller, K., Mills, S., Orr, S., 2004. The effects of vasopressin on human facial responses related to social communication. Psychoneuroendocrinology 29, 35–48.

Trainor, B.C., Marler, C.A., 2001. Testosterone, paternal behavior, and aggression in the monogamous California mouse (*Peromyscus californicus*). Horm. Behav. 40, 32–42.

vom Saal, F.S., 1983. Variation in infanticide and parental behavior in male mice due to prior intrauterine proximity to female fetuses: elimination of prenatal stress. Physiol. Behav. 30, 675–681.

Wuensch, K.L., Cooper, A.J., 1981. Preweaning paternal presence and later aggressiveness in male *Mus musculus*. Behav. Neural Biol. 32, 510–515.

Young, L.J., Toloczko, D., Insel, T.R., 1999. Localization of vasopressin (V-1a) receptor binding and mRNA in the rhesus monkey brain. J. Neuroendocrinol. 11, 291–297.

Zar, J.H., 1996. Biostatistical Analysis. Prentice Hall, Upper Saddle River NJ.