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Autonomic, Behavioral and Neuroendocrine Correlates of Paternal Behavior in Male Prairie Voles

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Abstract

Socially monogamous prairie voles (*Microtus ochrogaster*) are biparental and alloparental. In the present study, we compared behavioral, cardiovascular and neuroendocrine parameters in male prairie voles with experience caring for pups (Fathers), versus reproductively inexperienced Virgin males. Father and Virgins showed generally similar responses to unrelated pups. However, in Fathers studied prior to and during pup exposure, heart rate was lower and respiratory sinus arrhythmia tended to be higher than in Virgins. Fathers also displayed comparatively lower levels of anxiety-related behaviors in an open field test. In Fathers, compared to Virgin males, we also found higher levels of oxytocin-immunoreactivity in the paraventricular hypothalamus and two brainstem regions involved in the autonomic regulation of the heart –the nucleus ambiguus and nucleus tractus solitarius. However, Fathers had less oxytocin in the bed nucleus of the stria terminalis. Vasopressin did not differ significantly in these regions. Fathers also weighed less and had less subcutaneous fat and larger testes as a percentage of bodyweight. In conjunction with earlier findings in this species, the present study supports the hypothesis that oxytocin may be involved in the adaptation to fatherhood. These findings also support the hypothesis that males, with or without prior pup experience, may show simultaneous patterns of behavioral nurturance and autonomic states compatible with mobilization and vigilance.

Keywords

Paternal care; Pup; Father; Oxytocin; Prairie vole; Autonomic nervous system; Heart rate

1. Introduction

The biological basis of mammalian maternal behavior has been traditionally linked to the hormones of birth, including the hypothalamic neuropeptide, oxytocin (OT) [1-3]. In contrast, in the absence of birth-related events, the neurobiology of paternal behavior has

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been more difficult to identify. Studies in several species have implicated OT, as well as the related neuropeptide, arginine vasopressin (AVP), in male caretaking [4-7]. For example, in human males plasma OT levels increase during the transition to fatherhood [8, 9] and fathers' behavioral and physiological readiness to engage with their infant is enhanced by the actions of OT [4, 5].

Studies in socially monogamous, biparental voles have been especially useful in identifying the possible role of OT and AVP in male caregiving behavior in males [10, 11]. In prairie voles, sexually naïve males exposed to unfamiliar pups also show an increase in peripheral OT, as well as evidence of increased central OT and AVP neuronal activity [12]. In the biparental mandarin vole, paternal behavior in fathers is accompanied by an increase in OT expression in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus [13].

Based on these findings, we hypothesized that experience as a biological father, involving, among other things, chronic pup exposure, would be associated with an up-regulation of the central OT and AVP systems. These hormonal changes might also accompany changes in the behavior and physiology of the male including anxiety-like behaviors and the reactivity of the autonomic nervous system. We have previously observed that in reproductively naïve male prairie voles, the cardiovascular response to a pup includes a sustained increase in heart rate [14]. Both OT and AVP project to brainstem autonomic regions and regulate cardiovascular activity [15, 16]. Our lab has also previously described a role for chronic OT in the capacity of parasympathetic tone to slow heart rate in voles [17]. However, autonomic responses in males with experience as fathers have not previously been studied, and we could not exclude the possibility that in naïve males the increase in heart rate was simply a response to the novelty of a pup. Furthermore, the finding of increased heart rate in males was in contrast to a general pattern of reductions in autonomic and emotional reactivity reported in maternal females of a number of species, including humans [18] and rats, which has been attributed in part to hormones of birth and lactation including OT [19, 20]. Therefore, we sought here to describe the cardiovascular response to a pup in male voles with fathering experience.

Fathering behavior in prairie voles also has been associated with reductions in body weight [21], suggesting that adaptations to prolonged exposure to pups, or other aspects of paternal behavior, might affect metabolism. Data from other species indicate that OT plays a role in the regulation of appetite, and other metabolic and autonomic functions [15, 16, 22].

In the present experiments, conducted in male prairie voles, we examine some of the behavioral, neuroendocrine, physiological and autonomic adaptations to paternal behavior. This was done by comparing behavioral and autonomic responses to an unfamiliar pup in males with experience as Fathers versus Virgin males. Radiotelemetry was used to record heart rate, including a measure of respiratory sinus arrhythmia (RSA) and a more general index of heart rate variability (SDNN), in freely behaving animals. RSA indexes parasympathetic activity, while SDNN indexes the combined outputs of sympathetic and parasympathetic innervation of the heart (see below).

We hypothesized that, in comparison to Virgins, experienced Fathers would show lower levels of heart rate and higher RSA and possibly SDNN. As an independent index of anxiety-related behavior, we also examined the hypothesis that Fathers would be more exploratory in an Open Field Test (OFT) [23]. Finally, we also examined the hypothesis that Fathers, in comparison to Virgin males, would show higher levels of OT- and AVP-immunoreactivity in brain regions previously implicated in brain regions involved in the regulation of social behavior, stress reactivity and the autonomic nervous system. Brain regions that contain OT and AVP-ergic cell bodies that we examined consisted of: the PVN, SON, bed nucleus of the stria terminalis (BNST) and medial pre-optic area (MPOA). OT and AVP projections were quantified by the intensity of fiber density in regions that regulate the autonomic nervous system, including: the dorsal motor nucleus of the vagus (DMX), nucleus ambiguus (NA) and nucleus tractus solitarius (NTS).

2. Methods

2.1 Subjects

Male F2 or F3 descendants of wild prairie voles caught near Champaign, Illinois were used in these experiments at 60-90 days of age. Subjects were maintained on a 14/10 hour light/dark cycle on at 06:30 AM in a temperature and humidity controlled vivarium. Food (Purina rabbit chow) and water were available *ad libitum*. Prairie vole offspring remained in their natal group with their parents in large polycarbonate cages (24 × 46 × 15 cm) containing cotton nesting material. Offspring were weaned at 21 days of age, prior to the arrival of the next litter to prevent premature exposure to pups, and then pair-housed with a same-sex sibling in smaller cages (17.5 × 28 × 12 cm) in a single-sex colony room until testing. Thus, all test subjects were sexually naïve and prior to this experiment had never been exposed to pups. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee. Experiments began during the lights-on period between 10:00 and 11:00 AM.

2.2 Experimental Design

In Experiment 1, male vole subjects were randomly assigned to one of two conditions for 6 weeks: raising pups with an age-matched female (Fathers, $n = 10$), or left in a standard cage with their same-sex sibling (Virgin, $n = 10$). In the Father condition, males were previously paired with an age-matched female 3 weeks prior to the study period to ensure that pups were present for the 6 weeks of comparison. This time frame was selected so as to permit the subjects to raise 2 litters. Males were weighed once every week and at the conclusion of 6 weeks were tested in an OFT and Allopaparental Test before being sacrificed for immunohistochemical tissue processing. Animals were returned to their home cage for 24 to 48 hours between the OFT and Allopaparental Test and between the Allopaparental Test and sacrifice. Fathers and Virgins were age-matched at the time of testing.

Upon observing robust differences between Fathers and Virgin males at 6 weeks (Experiment 1), a second cohort of males (Experiment 2, $n = 8$) was established to examine autonomic changes resulting from fatherhood. Originally, males from Experiment 2 were to

be sampled throughout a similar 6 weeks of paternal experience. However, we observed differences at the first time point (10 days after the birth of first litter) and the experiment was discontinued as signal quality in the radiotelemetric transmitters had begun to decay in some males.

In Experiment 2, males were implanted with radiotelemetric transmitters that permit the recording of heart rate, heart rate variability, locomotor activity and temperature. Following recovery from surgery, heart rate was recorded at baseline in the home cage, with their siblings. After obtaining a 10-15 min baseline, males were tested for alloparental behavior. The day after Alloparental Testing, males randomly assigned to the Father conditions were paired with an adult female and allowed to mate and raise a litter of pups. Fathers remained with their mates and pups until the pups were 10 days old, at which point the females and pups were removed. At this time, Fathers and Virgin males underwent an Alloparental Test. In each alloparental test stimulus pups were 1-3 days old and unrelated to the subjects.

2.3 Open Field Test

The open field consisted of a clear plexiglas testing arena 42×42 cm square, with walls 30 cm high. Animals were transferred into the open field and placed into a standardized corner of the arena. Lighting in the room was maintained at typical colony levels for the light cycle. Testing in the arena lasted for 10 min, during which time behavior was video recorded. Behavior was later analyzed by two trained, experimentally-blind observers using Noldus Observer (Noldus Information Technology, The Netherlands). Observers recorded the amount of time spent in the center quarter of the arena, time spent autogrooming and the amount of total locomotor activity indexed by the number of lines crossed. Time spent in the center of an arena has been described as a measure of both state and trait anxiety in a variety of rodent species [23]. Inter-rater reliability was approximately 98% across the measured behaviors.

2.4 Alloparental Test

Testing of alloparental responses toward an unfamiliar 1-3 day old pup was conducted based on previously published procedures [12]. Testing occurred in a novel cage in which a male was immediately presented with a pup and behavior recorded for 20 min during video recording. In the rare instances of an adult male expressing aggression towards the pup, the test was aborted and the pup either returned to its home cage or euthanized. Behavior was later analyzed by two trained, experimentally-blind observers using Noldus Observer (Noldus Information Technology, The Netherlands). Observers recorded the latency to approach the pup and durations of: carrying the pup, licking/grooming the pup, huddling over the pup and total time in contact with the pup. OT and AVP have been previously implicated in these behaviors [6, 12]. Inter-rater reliability was approximately 95% across the measured behaviors.

2.5 Tissue Collection

Following behavioral testing, and consistent with IACUC policy, subjects immediately were anesthetized with a mixture of ketamine (67.7 mg/kg; NLS Animal Health, Owings Mills, MD) and xylazine (13.3 mg/kg; University of Illinois Hospital Pharmacy, Chicago, IL)

which was administered intraperitoneally, and then quickly euthanized via cervical dislocation. Final body weights were recorded. In addition, testes and posterior subcutaneous fat deposits on the ventral hindquarters were removed and immediately weighed separately. Wet-weights are presented for the paired organs. Brains were removed and fixed according to previously published procedures [12].

2.6 Immunohistochemistry

Following fixation with 4% buffered paraformaldehyde and immersion in 25% sucrose, tissue was cut in 40 μ m coronal plane sections using a freezing sliding microtome and stored in cryoprotectant at -20° C until processed. Brains were stained for OT and AVP using standard avidin-biotinylated enzyme complex (ABC) immunocytochemistry (Vector Labs; Burlingame, CA). Serial sets (every 3rd section) of free-floating tissue sections were rinsed in 0.05 M potassium phosphate buffered saline (KPBS) to remove excess cryoprotectant. Sections next were incubated in 1% sodium borohydride for 20 min at room temperature to reduce free aldehydes to alcohol followed by a rinse in KPBS. Sections then were incubated for 15 min in 0.014% phenylhydrazine at room temperature (RT) to block endogenous peroxidase activity and rinsed again in KPBS. Next, sections were incubated in rabbit OT (generously provided by Dr. Mariana Morris) or AVP (#64717, ICN Biomedicals Inc., Costa Mesa, CA) antisera at 1:100,000 concentration in 0.05 M KPBS + 0.4% Triton X-100 for 1 hour at RT and for an additional 48 hours at 4° C. Sections were rinsed in KPBS before being incubated for 1 hour at RT in biotinylated goat, anti-rabbit IgG (Vector Labs; 1:600 dilution in KPBS + 0.4% Triton X-100). Sections were rinsed again in KPBS and then incubated in an avidin-biotin peroxidase complex (45 μ l A, 45 μ l B per 10 ml KPBS + 0.4% Triton X-100; Vectastain ABC kit-elite pk-6100 standard; Vector Labs) for 1 hour at RT. Sections were rinsed in KPBS and then with tris buffered saline. Immunoreactivity was visualized by incubation in a solution containing 50 ml of tris buffered saline, 1.25 g nickel sulfate, 41.5 μ l of 3% H_2O_2 and 10 mg of diaminobenzidine (DAB) for 15 min at room temperature. Sections were rinsed in sodium acetate following by a series of rinses in KPBS. This protocol called for a higher concentration of primary antibody than we have used previously [12], along with nickel-DAB visualization in order to intensify the staining of OT-ir and AVP-ir fibers in brain regions outside the hypothalamus. Labeled sections were mounted on gelatin-coated slides and air-dried overnight. Sections then were dehydrated in ascending ethanol dilutions and cleared with Histoclear (National Diagnostics). Slides were subsequently cover-slipped with Histomount (National Diagnostics).

2.7 Quantification of Immunoreactivity

Slides were coded and images were acquired using a Nikon Eclipse E 800 microscope, Sensi-cam camera, and IP Lab 3.7 computer software (Scanalytics Inc., Fairfax, VA). OT-ir and AVP-ir neurons were measured in three sections of the PVN where staining takes a characteristic heart shape consistent with previous studies [24, 25], and where the number of peptide stained cells was found to be highest. These sections are similar to Fig. 36 and Fig. 37 in the mouse atlas of Paxinos and Franklin (1997). Representative sections of the SON were selected at the same level as the PVN. Neuropeptide-ir was also quantified in the BNST and MPOA at a level similar to Fig. 31 in the mouse atlas. Handcounts of OT-ir and

AVP-ir neurons were carried out in the BNST, MPOA, PVN and SON by two trained, experimentally blind observers.

In the brainstem, OT-ir and AVP-ir was quantified bilaterally using standardized sampling areas in sections matched to the mouse brain atlas, Figure 93 [26]. Staining in brainstem sections was quantified when it exceeded an arbitrary threshold above background. These sections included three nuclei pertinent to autonomic regulation: DMX, NA and NTS. Three brain sections were analyzed from each subject and results were averaged bilaterally across sections. Caudal sections of each brainstem region were chosen so as to better guarantee that these neurons would represent cardiovascular control [27, 28]. The location of the NA in the vole was confirmed by staining a subset of slices with choline acetyltransferase [29], generously provided by Dr. David Wirtshafter.

2.8 Radiotelemetry Recording Equipment Implantation

Males were implanted with wireless radiotelemetry transmitters [Data Sciences International (DSI), St. Paul, MN] according to procedures described previously [30]. Briefly, telemetric transmitters were implanted intraperitoneally under aseptic conditions, following anesthesia with ketamine (67 mg/kg sc; NLS Animal Health, Owings Mills, MD) and xylazine (13.33 mg/kg sc; NLS Animal Health, Owings Mills, MD). Animals were kept under a warming lamp, and the surgical area was shaved and cleaned before any incisions were made. Rostral-to-caudal skin and muscle incisions were made on the ventral surface of the abdomen. The transmitter was inserted into the abdominal cavity, then sutured to the muscle, thereby closing the incision. The leads from the transmitter were pulled rostrally using a trochar and sleeve under the skin, and anchored in place with permanent sutures (DII placement). Skin incisions were sutured closed, and subcutaneous fluids and analgesia (Carprofen) were administered as necessary. All animals were housed for 5 days in custom-designed cages (24 × 46 × 15 cm) after surgery [31]. These cages included a divider to permit adequate healing of suture wounds in the instrumented animal without socially isolating subjects from their siblings during recovery. Animals were then returned to the standard home cages (with their sibling) for an additional 5–7 days before the onset of experiments.

2.9 Radiotelemetric Recordings

Electrocardiogram (ECG), temperature and activity signals were recorded with a radiotelemetry receiver (DSI; sampling rate 5 kHz for ECG and 256 Hz for activity, 12-bit precision digitizing). This system allows for the recording of heart rate and derived measures of heart rate variability along with temperature and locomotor activity. Radiotelemetric data were quantified according to procedures previously described [31].

Heart rate was evaluated using vendor software (Data Sciences International, St. Paul, MN), and R-wave detections were verified with a custom-designed software package (CardioEdit 1.5, Brain Body Center, University of Illinois at Chicago). The R-R intervals were analyzed for variations (heart rate variability) using a custom-designed software package, and included standard deviation of all R-R (normal-to-normal; N-N) intervals [SDNN index [32]] and amplitude of RSA [33].

RSA was assessed using time-frequency procedures [34, 35] described in detail elsewhere [30, 31, 36]. RSA was operationally defined as the natural log of the sum of the power within the respiratory bandwidth of 1.0–4.0 Hz. This procedure has been validated in prairie voles [31] and provides the greatest sensitivity to changes in vagal activity [33]. The amplitude of RSA represents the functional vagal impact on the sino-atrial node of myelinated vagal efferent pathways originating in the brainstem (NA). The ECG signal was exported into a data file and examined using CardioEdit, to ensure that all R waves were properly detected. The following procedures were implemented to minimize the possibility of violating the assumption of stationarity which can distort time series analyses of RSA: 1) the R–R intervals (heart period) were time-sampled into equal time intervals with a sampling rate of 20 Hz; 2) the time series were detrended with a moving polynomial filter that removed variance in the series below 1 Hz for RSA (i.e., 21-point cubic polynomial), 3) the spectral analysis identified the peak amplitude of RSA from the de-trended data. SDNN represents the HRV produced by the combined outputs of sympathetic and parasympathetic innervation of the heart.

2.10 Statistical Analysis

The data are presented as means $\pm$ standard errors in all text and figures. All analyses were conducted using SPSS 19 (SPSS inc., USA) with α set at 0.05. Subject body weights were compared via a repeated measures ANOVA; follow-up t-tests were conducted at individual time points upon observing a time by condition interaction. Testes weight and subcutaneous fat were both calculated as percentage of bodyweight and compared via t tests. Behaviors in the OFT and Allopaparental Test along with OT-ir and AVP-ir were compared via a two tailed t-test.

In animals instrumented for telemetry, heart period was extracted from the ECG data and were quantified (i.e., msec between successive R-waves). Heart period data have been transformed to heart rate (beats per min) in the figures to facilitate visual interpretation. Heart period, RSA, activity, and temperature data were analyzed using repeated measures analyses of variance (ANOVA) with time and stimulus/condition as within subject factors. Baseline data were averaged over 5 min, taken just before presentation of the pup and then compared via a two tailed paired t-test to examine the possible effects of fatherhood on resting autonomic parameters. Telemetric and behavioral data were compared during pup exposures, using a repeated measures ANOVA on data binned into 5 min segments.

3. Results

3.1 Body Weight

Figure 1 shows the changes in body weight across 6 weeks. The repeated measures ANOVA yielded a time by condition interaction [$F(5,14) = 3.868$, $p = 0.021$], with post-hoc analysis suggesting that the Father condition resulted in slightly smaller body weights during weeks 5 and 6 ($p < 0.05$); Fathers weighed 37.3 ± 0.7 g, while Virgins weighed 39.1 ± 1.0 g. Fathers were found to have less body fat ($0.55 \pm 0.09\%$ vs. $1.36 \pm 0.09\%$, $t(19) = 8.635$, $p < 0.001$) and larger testes ($1.80 \pm 0.16\%$ vs. $1.24 \pm 0.09\%$, $t(19) = 13.347$, $p < 0.001$) relative to body weight.

3.2 Open Field Test

Figure 2 illustrates the results of the OFT. Fathers spent significantly more time in the center of the OFT (15.6 ± 4.6 sec vs. 4.2 ± 1.7 sec, $t(19) = 3.662$, $p < 0.001$, $p = 0.002$). No group differences were seen in time spent grooming or locomotor activity.

3.3 Alloparental Test

Behavior did not differ between Fathers and Virgins in the Alloparental Test ($p > 0.05$ for all comparisons). The majority of males responded parentally towards the pup; (1 animal in the Virgin condition attacked the pup and data from that animal were not included here).

3.4 Immunohistochemistry

Figure 3 illustrates the results of immunohistochemical staining for OT-ir. No differences were found between treatments in terms of AVP-ir in the BNST, MPOA, PVN, SON or brainstem. Generally, OT-ir fiber staining was more readily visible than AVP-ir, which was very light outside the hypothalamus.

There were significantly more OT-ir neurons in the PVN in Fathers compared to Virgins (121.53 ± 8.88 vs. 99.07 ± 3.97 , $t(19) = 20.459$, $p < 0.001$). Fiber density of OT-ir also was higher in the NA and NTS in the Father condition ($3.5E+07 \pm 1.2E+07$ pixels vs. $1.4E+07 \pm 3.7E+06$ pixels, $t(19) = 3.571$, $p = 0.002$; and $1.2E+08 \pm 1.7E+07$ pixels vs. $5.4E+07 \pm 1.3E+07$ pixels, $t(19) = 6.670$, $p < 0.001$ respectively). There were significantly fewer OT neurons in the BNST of Fathers compared to Virgin males (6.7 ± 1.56 vs. 12.9 ± 1.98 , $t(19) = 6.915$, $p < 0.001$). No differences in OT-ir were detected in the DMX, MPOA or SON.

3.5 Behavior and Cardiovascular Regulation

In Experiment 2, one Father lost signal integrity before the completion of the study and was removed from analysis ($n = 7$). All males responded parentally to pups both as Virgins and Fathers. Figure 4 shows the cardiovascular parameters at baseline and in response to pups in the Virgin and Father conditions. At baseline, Fathers were found to have lower resting heart rate (350 ± 10 bpm vs. 401 ± 11 bpm, $t(6) = -3.244$, $p = 0.018$). During the Alloparental Test, the binned ANOVA for activity yielded a main effect of time [$F(4,3) = 31.263$, $p = 0.009$], with activity generally following an inverted-U shaped curve. The binned ANOVA for heart rate yielded main effects of paternal behavior [$F(1,6) = 11.218$, $p = 0.015$] as well as time [$F(4,3) = 100.746$, $p = 0.002$], with the Virgin condition having higher heart rate (585.500 ± 15.527 bpm) than Fathers (502.250 ± 21.994 bpm). The binned ANOVA for RSA yielded a main effect of time [$F(4,3) = 17.160$, $p = 0.021$] with RSA generally following a U-shaped curve. There was a trend toward higher RSA in Fathers [$F(1,6) = 4.901$, $p = 0.069$]. The binned ANOVA for SDNN yielded main effects of paternal experience [$F(1,6) = 6.702$, $p = 0.041$] as well as time [$F(4,3) = 9.146$, $p = 0.050$], with the Virgin males having a lower SDNN (7.352 ± 0.642 msec) than the Fathers (9.668 ± 0.741 msec). No group differences were found for the strength of the correlation between heart rate and RSA. Temperature also was recorded but did not differ between groups.

4. Discussion

The results of the present study support the general hypothesis that a variety of physiological and endocrine adaptations accompany fatherhood in prairie voles. These include an apparent change in the regulation of the central OT system, with a reduction in heart rate prior to and in response to a pup, as well as an increase in testes weight, lower body weight and less subcutaneous fat. The causes and consequences of these adaptations are only now becoming apparent.

Behavior

As in previous studies of male prairie voles, both Fathers and Virgins showed high levels of alloparental behavior towards unfamiliar pups [12]. The absence of a behavioral difference in response to pups between males with or without reproductive experience also is consistent with earlier studies [37, 38]. However, in comparison to Virgins, Fathers in this study exhibited lower levels of anxiety-related behaviors, as indexed by increased time spent in the center of an open field. Sexual activity has been found to be acutely anxiolytic in rats [39], but we hypothesize that the results observed here are more likely due to chronic rather than acute adaptations. Lieberwirth and colleagues [40] recently reported in prairie voles that, when tested 6 days after the birth of the first litter, fathers showed increased anxiety-related behavior in the OFT and elevated plus maze. The effect was modest and did not result in significant changes in time spent in the center of the arena, but rather increased time spent in the corners. Moreover, since a number of procedural differences exist between these studies, including size of the chamber, initial placement of subjects and the difference in time since birth, comparisons are difficult. Lieberwirth et al. tested 6 days after birth, used a 56×56 cm field and began with subjects in the center as opposed to 6 weeks after birth in a 40×40 cm field with corner placement used here. Given the arousing nature of pups [14] and the weight loss their presence appears to induce in parents [21], it is reasonable to expect that paternal adjustment to the presence of pup might include time-related changes in anxiety, perhaps initially anxiogenic and eventually anxiolytic. Further efforts can now be directed at determining when these behavioral changes emerge and to what aspects of paternal behavior they pertain. A limitation to interpretation of the presented data is that Fathers also had pair-bonding and mating experience that each may have influenced the outcome of the current study. The findings from the present study do correspond to prior research in another biparental rodent, the California mouse, in which new fathers showed less anxiety-related behaviors in an elevated-plus maze compared to either virgin males or expectant fathers, but similar levels of neophobia, corticotropin releasing hormone (CRH) expression, and CRH activity [41].

Vasopressin

Prior studies in biparental rodents have implicated AVP in male parental behavior. For example, increases in AVP have previously been reported in male prairie voles as a function of cohabitation with a female [10, 42, 43]. However, in the present study using samples taken after 9 weeks of cohabitation and the birth of two litters, we did not observe group differences in central measures of AVP in Fathers versus Virgins. This could be due to methodological differences in measurement of AVP (i.e. mRNA levels vs.

immunoreactivity) or the duration of cohabitation (3 days in earlier studies versus 9 weeks here). In addition, the relatively light AVP staining observed in tissues outside of the hypothalamus might have precluded detection of group differences beyond the regions of AVP synthesis. Furthermore, in the present study, we did not use colchicine to enhance AVP-ir, which can be used to amplify staining intensity.

Oxytocin

Unlike AVP, measures of OT differed between the Fathers versus Virgins. Fathers showed a generally higher level of OT-ir both centrally in PVN cell bodies that synthesize OT, and in fibers, presumably reaching targets for the actions of OT. However, fewer OT-ir cells were measured in the BNST of Fathers. Paternal behavior has been associated with an increase in OT receptor density in the BNST in meadow voles tested under conditions that were associated with an increase in paternal behavior [44]. Receptor binding data comparing Fathers and Virgins are not available in prairie voles, but could be useful in the interpretation of regional difference in OT-ir.

The present findings appear to implicate OT in the Father's adaptations to paternity in male prairie voles. Previous studies revealed a transient increase in plasma OT following a brief pup exposure in Virgin male prairie voles [12]. However, the Fathers versus Virgins in the present study differed in many ways including prolonged pup exposure, extended social contact with a female partner and mating on at least two separate occasions. Each of these may release OT, possibly resulting over time in a general up-regulation of central OT synthesis. Exposure to oxytocin, of both endogenous and exogenous origins, can "feed forward" to up-regulate the OT system in voles [17], potentially accounting for apparent increases in OT synthesis in the PVN and fiber density.

Another factor which might contribute to observed peptide differences between Fathers and Virgins is testosterone. Although not measured here, earlier studies in prairie voles have suggested that introduction to a female is associated with an increase in testosterone concentrations, as well as increases in androgen-dependent AVP-ir in the BNST [11, 42]. Based on the increased testes size, testosterone levels may have been higher in Father in this study. The relationship between OT and testosterone is bi-directional, with both hormones affecting the synthesis and release of the other. For example, in other species testosterone largely does not affect OT plasma levels, though it does increase the expression of OTR in various brain regions [45, 46].

Heart rate

Heart rate was reliably lower in Fathers than in Virgin males both at baseline and during care for a pup. However, Fathers showed a sustained increase in heart rate when presented with an unrelated 1-3 day old pup, following a temporal pattern that was similar to that seen in Virgin males [14]. In both Fathers and Virgins high levels of heart rate were observed throughout the duration of exposure to a novel pup. This similarity corresponds with the absence of a behavioral difference between Fathers and Virgins and reinforces the notion that the pup is a generally arousing stimulus [14]. In previous studies of animals implanted with radiotelemetry, we have not observed a decline in resting heart rate (unpublished

observations), suggesting that the effects observed here are not merely due to the presence of the transmitter or adaptation following surgery. It should also be noted that the baseline measure of the Fathers included the presence of their female mates as well as 10 day old pups. Thus, the pup-induced cardioacceleration seen in the presence of only a single, 1-3 day old pup, could be due to either the lack of the female mate or the young age of the stimulus pup, and future efforts should examine these possibilities. While the novel environment associated with the Alloparental Test may also induce cardioacceleration, we have observed no difference in the pattern of heart rate response to a pup in animals that have been immediately transferred to a new cage compared to those allowed to habituate to the novel environment (unpublished observations).

We have previously observed that in Virgins, increases in heart rate in the presence of a pup were prolonged, while those to an adult female, pup-sized wooden dowel or a novel cage were transient [14]. We have also previously observed that male voles respond to pups with OT release as measured in plasma [47]. In exercise trained rats, OT acts in the NTS to restrain exercise induced tachycardia [48]. Furthermore, exercise training leads to increased levels of OT in the NTS [49, 50]. OT increases parasympathetic cardiac activity to slow the heart by acting on the dorsal vagal complex [51], and we observed here that vole Fathers had greater OT-ir in the NA and NTS. Thus, in the present context, OT may help to restrain heart rate in paternal voles both at rest and during pup exposure by acting on brainstem autonomic regions in an analogous fashion to the exercise trained rat. That said, more pronounced group differences were detected in SDNN; in comparison to RSA, SDNN may reflect variance contributed by both sympathetic and parasympathetic inputs to the heart [32]. Previous studies, using c-Fos as an index of neural activity, have implicated source nuclei for both the sympathetic and parasympathetic systems in the response to a pup stimulus [14]. The relationship between OT and sympathetic cardiac drive is complex, with both inhibitory [52, 53] as well as stimulatory [54, 55] effects having been described.

Humans also respond to infant cries with an acceleration of heart rate [56]. Non-parents show stronger responses than parents, as do males relative to females [57], which corresponds to some of the present findings. The response to infant cries in humans is modulated in part by OT pathways [58-60] and the cardiac response to infant crying varies as a function of polymorphisms in the gene for the OT receptor [60]. However, there are no differences between fathers and non-fathers in terms of resting heart rate in humans [61]. These and earlier studies suggest that an infant, presented as a novel stimulus, may create a novel autonomic state capable of supporting both nurturance and vigilance.

Body weight

Fathers showed a decrease in body weight in the present study, supporting results from an earlier study [21]. OT, which appears to be generally increased in Fathers, has previously been associated in other species with reductions in appetite [22], and may have contributed to the decrease in body weight and body fat seen in the present study. Furthermore, in previous work, paternal behavior in voles led to a drop in leptin, which can regulate food intake and body weight [21]. Given this lower body weight relative to virgins, the principle of allometric scaling would predict that Fathers should have displayed, if anything, greater

resting heart rate, so the observed decrease is especially noteworthy. Thus, the autonomic changes associated with paternal behavior may represent adaptation to paternal behavior and prolonged exposure to pups. Habituation to the presence of pups may explain some of this effect, as well as hormonal changes related to paternal behavior. This decreased cardioactivity may be related to the diminished anxiety-related behavior, possible due in part to increases in OT synthesis.

Summary

Consistent hormonal changes associated with paternal care across mammalian species have proven difficult to identify [62]. However, the present study supports earlier research [6, 12] implicating OT in both paternal behavior and the regulation of anxiety and the autonomic nervous system. While acutely arousing, pups also may lead to stress buffering, possibly through the release of peptides such as OT, allowing fathers to engage in caretaking toward infants, while being prepared for defensive responses if needed. The data from this study also support the broader hypothesis that OT has a role in diverse functions including social behavior, anxiety, appetite and cardiovascular performance.

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Highlights

- We measured the effects of fatherhood in prairie voles compared to virgin males.
- Fathers had lower resting heart rate and lost body weight and body fat.
- Fathers had greater oxytocin innervation in autonomic brainstem regions.
- Fathers displayed less anxiety-related behavior.

Figure 1.

(A) Average body weight over the course of 6 weeks in each treatment, weight was significantly greater in Virgins by weeks 5 and 6 (* indicates $p < 0.05$). (B) Testes and subcutaneous fat weight as a percentage of body weight. Testes were significantly larger in the Fatherhood condition and subcutaneous fat was significantly smaller (* indicates $p < 0.05$ for all comparisons).

Figure 2. Behavioral effects of the Fatherhood condition relative to Virgin animals. Males in the Fatherhood condition spent significantly less time in the center of an open field compared to Virgin males, but did not differ in grooming or locomotion (* indicates $p < 0.05$).

Figure 3.

Immunohistochemical results for OT staining in various brain regions. (A) Fathers had significantly more OT-ir neurons in the paraventricular nucleus (PVN). (B) Virgins had significantly more OT-ir neurons in the BNST than Fathers. (C) Fathers had significantly more OT-ir in the NA and NTS compared to the Virgins (* indicates $p < 0.05$).

Figure 4.

Cardiovascular parameters derived from radiotelemetry before and during an Alloparental Test. Both at baseline and throughout pup exposure, Fathers demonstrated lower heart rate (* indicates $p < 0.05$). In Fathers there was a trend towards higher RSA during pup exposure (# indicates $p = 0.069$).