

ORIGINAL ARTICLE

Neuroendocrine and Behavioural Responses to Exposure to an Infant in Male Prairie Voles

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Paternal behaviour and pair-bond formation are defining characteristics of social monogamy. However, in comparison to pair-bonding, the endocrine factors associated with the male care of young are not well studied. In the present study, plasma concentrations of oxytocin, vasopressin and corticosterone (CORT) were measured in reproductively naïve male prairie voles as a function of exposure to an infant or control manipulations (i.e. handling or exposure to a wooden dowel). Plasma oxytocin concentrations were transiently elevated within 10 min of pup exposure. Although plasma CORT concentration typically increases after handling, after 10 min of pup exposure, the concentration of plasma CORT was not increased, suggesting an attenuation of CORT release by pup exposure. Group differences in the concentrations of plasma hormones were no longer detected at 20 or 60 min after treatment. These patterns of rapid change in the concentrations of plasma oxytocin and CORT were observed in both juvenile and adult males but not detected after control procedures. Plasma vasopressin, assessed only in adult males, did not vary as a function of pup exposure or other manipulations. In the paraventricular nucleus of the hypothalamus, pup exposure also increased activation (as assessed by the measurement of c-Fos) of neurones that stained for either oxytocin or vasopressin, whereas it decreased c-Fos expression in neurones stained for corticotrophin-releasing hormone. In addition, brief pup exposure (20 min) facilitated subsequent partner preference formation when alloparental males and pup attackers were considered as a group. In the context of other studies, these data support the hypothesis that neuroendocrine changes associated with male alloparental behaviour are related to those implicated in pair-bonding.

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Studies of the neurobiology of parental behaviour have tended to focus on the hormones of gestation, birth and lactation (1,2). Males do not experience gestation, birth or lactation. However, males do participate in the direct care of their offspring in approximately 3–5% of mammalian species (3) and male parental behaviour and alloparenting by sexually naïve animals are both defining characteristics of social monogamy (4). In socially monogamous species, other family members, including the father and reproductively naïve older siblings, may exhibit positive pup-directed behaviours at levels that equal or exceed those of the mother (3,4).

The prairie vole (*Microtus ochrogaster*) is a socially monogamous, small rodent (5) that has proven particularly useful for investigating the neuroendocrine underpinnings of social bond formation, as well as parental and alloparental behaviours (5–8). Juvenile prairie voles,

living in nature, often remain with their natal family until approximately 6–7 weeks of age (4) and may be exposed to infants from subsequent litters born to their parents, providing juveniles with opportunities to engage in alloparental behaviours. During laboratory tests, a high percentage of sexually-naïve prairie voles, especially males, engage in alloparental behaviours such as pup retrieval, grooming and arched-back nursing posture (9,10); these behavioural patterns typically are not qualitatively different from behaviours seen in biological parents (10,11). Subsequent parental responsiveness towards offspring is also facilitated by previous exposure to pups (12). However, less is known about the possible neuroendocrine or behavioural consequences of exposure to a pup.

The neuropeptide oxytocin is synthesised primarily in the hypothalamus. Oxytocin is most often associated with the induction of

parturition and lactation through effects on peripheral tissues. However, oxytocin also facilitates maternal behaviour in various species, including rats (2), sheep (13), prairie voles (1) and humans (14). Studies also suggest that oxytocin may influence paternal behaviour in humans (15) and nonhuman primates (16). In general, a failure to demonstrate a single or consistent mechanism for paternal behaviour has led to controversy regarding the hormonal regulation of male parental behaviour (17–19).

Oxytocin also may reduce responses to fear-eliciting stimuli and inhibit hormones released by the hypothalamic-pituitary-adrenal (HPA) axis, including corticotrophin-releasing hormone (CRH) (20) and the adrenal steroid, corticosterone (CORT) (21). CORT release has effects on social behaviours (22), including potentially increasing the capacity of male prairie voles to respond positively towards infants. In prairie voles, the exogenous administration of CORT and the stress of swimming facilitate pair-bonding (22) and exposure to a swim stressor facilitates parental care in male but not females (23), suggesting a sex difference in the role of HPA axis hormones in parental behaviour. Meanwhile, i.c.v. CRH dose-dependently facilitates pair-bond formation in male prairie voles (24), whereas i.c.v. infusion of CRH has recently been shown to inhibit maternal behaviour in the marmoset (25).

Earlier work on stress and alloparental care suggested that the pup exposure might reduce anxiety or emotional reactivity (23). Thus, the present study aimed to examine the effects of pup exposure on the HPA axis, including the cells of the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BNST) and central amygdala (CeA) in which CRH synthesis is abundant (26). The neuropeptide CRH influences a variety of behaviours, as well as the HPA axis, with effects on multiple brain regions, including the PVN (27), CeA (28) and BNST (29). These brain areas also are involved in parental behaviours, suggesting a possible connection between stress and parenting. For example, the BNST is activated after alloparental behaviour in voles (30), whereas the CeA has been implicated in the response to infant stimuli in humans (31).

Vasopressin is structurally similar to oxytocin and shares some overlapping functions in pair-bond formation and a variety of male social behaviours (32–34). Vasopressin is associated with active coping strategies (35) and increased vigilance and arousal, as well as adaptive and defensive functions, including territoriality (36), which are observed in male mammals. Because testosterone promotes the synthesis of vasopressin, vasopressin has been the focus of studies examining male parental and alloparental care in several biparental species, including the prairie vole (33,37) and the California mouse (38,39). However, changes in central vasopressin alone are probably not sufficient to explain the appearance of male alloparental behaviour (17) because it is necessary to block both vasopressin and oxytocin receptors to produce a significant reduction in male alloparental behaviour (40).

The prairie vole, with a strong tendency to show spontaneous care for infants, provides an animal model that allows the examination of the neurobiology of male alloparental behaviour. In the present study, we examined the hypothesis that exposing a sexually naïve male prairie vole to an unfamiliar pup would have neuroen-

docrine consequences; these changes in turn would increase the tendency of males to show positive behavioural responses toward a female conspecific. Juvenile (Experiment 1) or adult (Experiment 2) male prairie voles were exposed to an unfamiliar pup or a control manipulation and blood samples were taken at multiple time intervals after the onset of treatment, allowing the measurement of plasma concentrations of oxytocin, vasopressin and CORT as a function of time after the onset of pup exposure.

An additional goal of these studies (Experiment 3) was to identify possible neural substrates for the effects of pup exposure by using c-Fos expression as an indicator of neural activation in brain regions containing high levels of CRH. The regions selected included the supraoptic nucleus (SON) and PVN, using double-labelling to target cells that synthesise oxytocin, vasopressin or CRH. In addition, c-Fos expression was measured in CRH-rich regions of the brain. The neuropeptide CRH regulates behaviour and the HPA axis, from multiple brain regions: the PVN (27), the CeA (28), which has been implicated in the response to infant stimuli in humans (31), and the BNST (29), a region activated after alloparental behaviour in voles (30). In the BNST, oxytocin, vasopressin and CRH plus c-Fos were measured. In the CeA, measurements of CRH were used to define a field of high CRH density, and c-Fos was measured in this area of the CeA.

Considerable evidence suggests that the neural processes necessary for social bonding are also affected by oxytocin and vasopressin in male and female prairie voles (34,41–43). Based on the shared pathways associated with parental behaviour and pair-bonding, we hypothesised that rapid neuroendocrine responses resulting from pup exposure would influence pair-bond formation in male prairie voles. In Experiment 4, we tested this hypothesis by comparing the time male prairie voles spent in side-by-side contact with a familiar and an unfamiliar female in a partner preference test, administered after brief exposure to an unfamiliar pup (20 min) or other control manipulations.

Materials and methods

Animals and general procedures

Male F₂ or F₃ descendants of wild prairie voles caught near Champaign, Illinois, were used in the present studies. Subjects were maintained under a 14 : 10 h light/dark cycle (lights on 06.30 h) in a temperature- and humidity-controlled vivarium. Food (Purina rabbit chow; Purina Mills, St Louis, MO, USA) and water were available *ad lib*. 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 before 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 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 09.00 h and 10.00 h.

Behavioural assays were conducted in a novel, clean cage (17.5 × 28 × 12 cm) into which sexually-naïve males were transferred.

Males were immediately presented with an unfamiliar and unrelated pup (1–3 days of age) or other nonsocial stimuli. Stimuli were placed in the test cage on the side opposite to the experimental subject. One nonsocial control condition consisted of subjects (HAN condition) that were placed in a novel, clean cage: these subjects had a hand placed briefly in the cage without leaving a stimulus at the beginning of the test to mimic stimulus placement and to control for moving subjects from their home cage to a novel cage. Experimental conditions lasted 10 or 55 min (Experiment 1); 10, 20 or 60 min (Experiment 2); and 20 min (Experiments 3 and 4). Juvenile (Experiment 1) and adult (Experiments 2) male prairie voles were sacrificed immediately after experimental treatment to obtain plasma.

During Experiment 3, subjects were exposed a given stimulus for 20 min and sacrificed 40 min afterwards for tissue collection. During Experiment 4, all subjects were allowed to cohabitate for 30 min with a novel female immediately after 20 min of stimulus exposure. Immediately after the 30 min cohabitation period, the effect of pup exposure on adult male pair-bond formation was assessed using a partner preference test (44).

Experiment 1: Juvenile males and the endocrine effects of pup exposure

Juvenile male prairie voles, approximately 22–23 days of age, were randomly divided into two groups. One group (PEX) was exposed to a pup, whereas a control group (HAN) was handled similarly but was not exposed to a pup. Blood for the assessment of plasma hormone concentrations was obtained subsequent to one of three test conditions: (i) sacrificed 10 min after the onset of stimulus exposure (PEX, $n = 8$; HAN, $n = 8$); (ii) sacrificed 45 min after completion of a 10 min stimulus exposure (PEX, $n = 8$; HAN, $n = 9$); or (iii) sacrificed after 55 min of continuous stimulus exposure (PEX, $n = 7$; HAN, $n = 4$). To control for possible changes in the pups as a result of separation from their parents, the initial pup was replaced by a fresh pup after 30 min during the third condition. A hand but no pup was placed in the cage of control animals after 30 min to maintain similarity with the experimental condition. The plasma concentrations of oxytocin and CORT were measured in all subjects (Fig. 1) in this experiment.

Behaviours from all subjects during this experiment were recorded on a camcorder and later scored by an experimentally-blind observer using BEHAVIOR TRACKER (Freeware, available at www.behaviortracker.com), version 1.0. Behavioural categories quantified included: first approach toward the stimulus (latency), stimulus retrievals (frequency), licking and/or grooming the stimulus (duration), auto-grooming (duration), crouching over the stimulus (duration), which likely serves to protect the pups and regulate their body temperature, contact with the stimulus, defined as the subject having contact with the stimulus but not crouching over it or licking and/or grooming it (duration), and 'other' behaviour, defined as all other behaviour that was not directed at the stimulus (e.g. drinking, cage exploration). Subjects that displayed a crouching posture over the pup were classified as alloparental. Because all juvenile males exposed to a pup in the present study spent time crouched over the pup, all of the juvenile males were classified as alloparental.

Experiment 2: Adult males and the endocrine effects of pup exposure

Adult male prairie voles, 60–90 days of age, were randomly divided into three groups. Similar to Experiment 1, a group (PEX) of males was exposed to a pup, whereas a control group (HAN) was handled similarly but was not exposed to a pup. Because the hormone data from Experiment 1 could have been caused by the novelty of the pup, a third group (DOW) was exposed to a novel object (a wooden dowel) similar in shape and size to the pup. Changes in the concentration of plasma hormones detected in Experiment 1 were transient; however, the duration of this change remained unclear. To

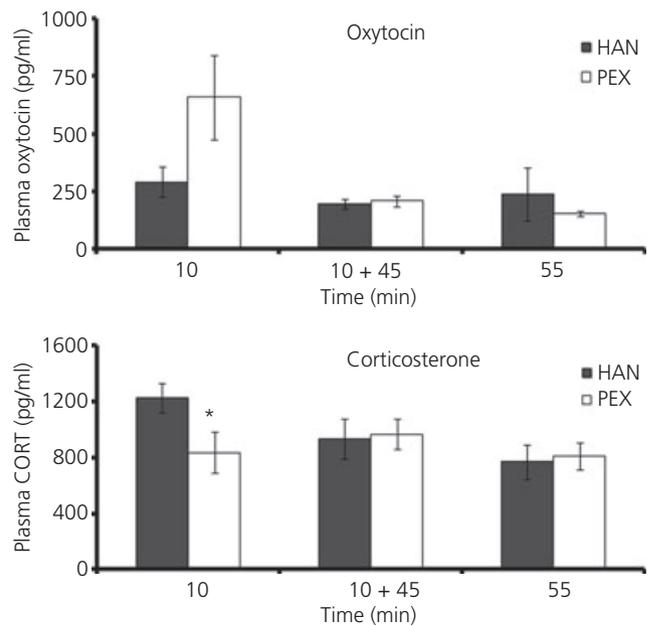


Fig. 1. Plasma concentrations of oxytocin (top) and corticosterone (CORT; bottom) in groups of juvenile male prairie voles that were exposed to a pup (PEX) or were handled without exposure to a pup (HAN). Samples were collected 10 min after the onset of stimulus exposure (PEX, $n = 8$; HAN, $n = 8$) (left), 45 min after the conclusion of 10 min of stimulus exposure (PEX, $n = 8$; HAN, $n = 9$) (middle) or immediately after 55 min of stimulus exposure (PEX, $n = 7$; HAN, $n = 4$) conditions. The original pup was replaced by a fresh pup after approximately 30 min in the 55-min stimulus exposure treatment condition. Data are expressed as the mean \pm SEM. *Significantly different from HAN ($P < 0.05$).

study the time course of changes in the concentration of plasma hormones after exposure to a pup, terminal blood samples for the assessment of plasma hormone concentrations were obtained after each of three test conditions: (i) sacrificed 10 min after the onset of stimulus exposure; (ii) sacrificed 20 min after the onset of stimulus exposure; or (iii) sacrificed 60 min after the onset of stimulus exposure. These times were chosen because 10 min of pup exposure during Experiment 1 resulted in transient changes in the concentration of plasma hormones, 20 min of pup exposure produces cell proliferation in the hippocampus (45) and our laboratory has previously used 60 min of stimulus exposure to assess c-Fos in the brain in response to social stimuli (46). An additional group (0 min), which was not exposed to any stimuli, was sacrificed immediately upon removal from the home cage to establish basal hormone concentrations. All groups consisted of nine to 12 subjects. The plasma concentrations of oxytocin, vasopressin and CORT from all subjects were measured and analysed (Fig. 2).

Behaviour from all subjects was recorded and analysed to assess alloparental behaviour similar to Experiment 1. Although adult males exposed to a dowel displayed behaviours that appear similar to alloparental behaviours (e.g. licking, and retrieval of the dowel), they spent less time engaging in these behaviours than adult males exposed to a pup. By contrast, adult males were never observed adopting a crouched posture over the wooden dowel. Thus, adult males were defined as alloparental if they adopted a crouched posture over the pup, using previously established criteria (45). Based on this definition, non-alloparental males ($n = 3$, $n = 1$ and $n = 2$ males exposed to a pup for 10, 20 and 60 min, respectively) were excluded from the analysis. In addition, the test was stopped if a pup was attacked and the pup was removed immediately from the cage, treated if necessary,

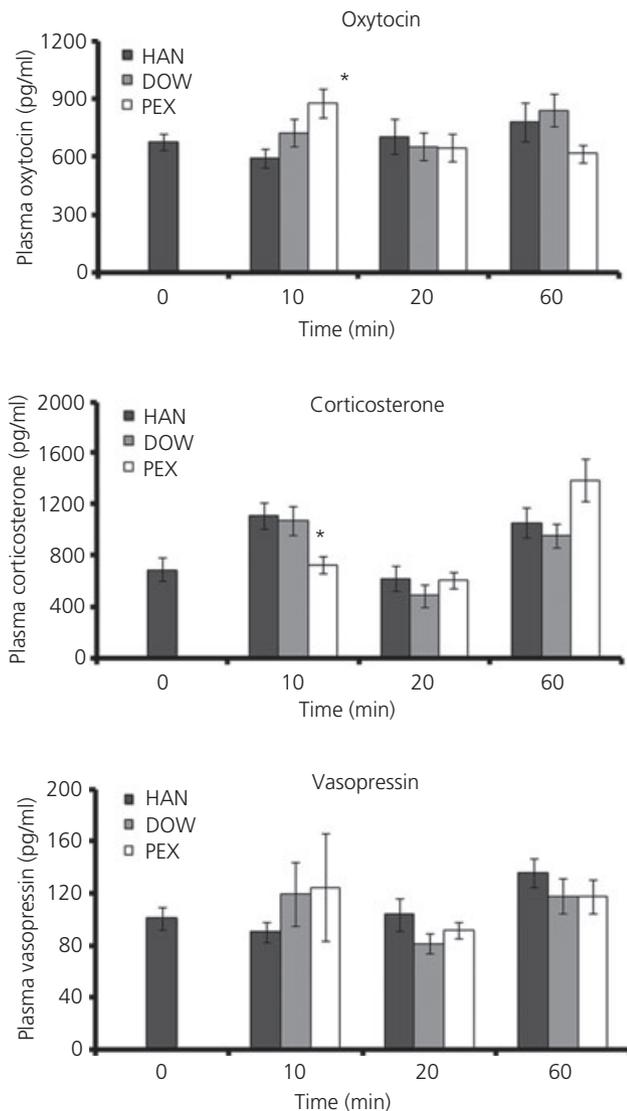


Fig. 2. Plasma concentrations of oxytocin (top), corticosterone (CORT; middle) or vasopressin (bottom) in groups of male prairie voles exposed to a pup (PEX), a dowel (DOW) or no stimulus (HAN). Plasma was collected immediately after removing the subject from its home cage (0 min) or 10, 20 or 60 min after the onset of stimulus exposure. All groups consisted of nine to 12 subjects. Data are expressed as the mean \pm SEM. *Significantly different from HAN ($P < 0.05$).

and returned to its parents. Pups were euthanised in the few instances when a severe attack occurred. Because of the infrequent occurrence of pup-attacks in adult males ($n = 1$, $n = 3$ and $n = 2$ males exposed to a pup for 10, 20 and 60 min, respectively), data from pup-attacking males also were not included in the analysis.

Experiment 3: Adult males and c-Fos staining as a function of pup exposure in brain regions containing oxytocin, vasopressin or CRH

Adult males 60–90 days of age were randomly exposed to either a pup (PEX; $n = 17$) or dowel (DOW; $n = 13$) for 20 min. After the removal of the stimulus, males remained in the testing cage for an additional 40 min, after

which time they were sacrificed as above. Twenty minutes of stimulus exposure was chosen based on pilot data and evidence showing that 20 min of pup exposure enhances cell proliferation in the hippocampus (45). The 40 min of time afterward was chosen to allow for c-Fos protein expression changes in response to the stimulus (46).

Tissue was then collected and processed for immunohistochemical staining for c-Fos, as well as either oxytocin, vasopressin or CRH. Double-labelling was possible in sections with relatively sparse staining (PVN and SON). The high intensity of staining for CRH in the CeA and BNST precluded the use of double-labelling in these areas, although it was possible to count the number of c-Fos positive nuclei within a defined field of CRH staining. It was possible to quantify oxytocin and vasopressin double-labelling within the BNST, although double-labelling was extremely rare. Because of technical difficulties titrating the concentration of primary CRH antibody, a separate cohort of animals was processed for CRH/c-Fos and treated identically to those processed for oxytocin/c-Fos and vasopressin/c-Fos (plasma hormones were not measured in these subjects). In Experiment 1, we compared the effects of a pup to those of handling and cage change. For Experiments 2 and 4, we accounted for the novelty inherent in first-time pup exposure through the addition of a second control group (i.e. a pup-sized wooden dowel). Upon seeing no difference between control groups, we consolidated the experimental design back to a single control group (the dowel) for Experiment 3, which was carried out last chronologically—i.e. after Experiment 4.

Experiment 4: Adult males and the effects of pup exposure on partner preference formation

The effect of pup exposure on social behaviour was assessed in Experiment 4 using a well-established protocol (44) designed to study pair-bond formation. Similar to Experiment 2, adult males were randomly assigned to one of three groups (PEX, DOW and HAN) and exposed to a pup or control condition for 20 min. Twenty minutes of stimulus exposure was chosen based on pilot data and evidence showing that 20 min of pup exposure enhances cell proliferation in the hippocampus (45). Subsequently, all subjects were placed in a novel cage for 30 min with a randomly selected, previously unfamiliar age- and weight-matched female. This short cohabitation was chosen because this amount of time is not sufficient for the development of a selective partner preference (47, 48). Immediately after this brief cohabitation, males spent 3 h in a testing apparatus, in which they were given a choice between spending time with the now familiar female (referred to as partner) versus another unfamiliar age- and weight-matched female (referred to as stranger) or spending time in an empty, neutral cage (44). The test apparatus consisting of three identical ($17.5 \times 28 \times 12$ cm) polycarbonate cages attached by plexiglass tubes (7.5×16.0 cm). Males were placed in the empty third chamber at the beginning of the test and were free to move about the apparatus, whereas the two stimulus females were tethered within their own separate chambers. Loose tethering restricted the movement of stimulus females to their respective chamber but did not interfere with their ability to interact with the experimental male. Although copulation between the male subject and the stimulus female was physically possible, female prairie voles do not experience a spontaneous oestrous cycle and were not sexually receptive during this test (49, 50). Nevertheless, behaviour during cohabitation and the partner preference test was monitored to verify that copulation did not occur. Similar to Experiments 1 and 2, behaviour from all subjects was recorded during the 20-min stimulus exposure and, consistent with Experiment 2, the first 10 min of the session was analysed to assess alloparental behaviour. Nineteen adult males were exposed to an unfamiliar and unrelated stimulus pup during Experiment 4 because a higher than expected number of males ($n = 7$) attacked the stimulus pup. Stimulus exposure was terminated after all pup attacks to avoid injury to pups; however, these males remained in the test cage for the remainder of the 20 min and later were tested for a partner preference. In

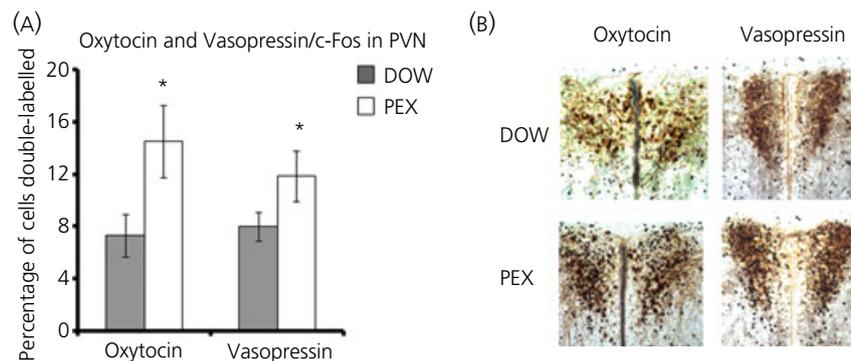


Fig. 3. The percent of c-Fos-labelled oxytocin and vasopressin neurones in the paraventricular nucleus of the hypothalamus (PVN) (A) and representative photomicrographs of the PVN in each condition (B). Subjects were exposed to either pup (PEX) or dowel (DOW) for 20 min and then sacrificed 40 min afterwards. All groups consisted of 11–13 subjects. Data are expressed as the mean \pm SEM. *Significantly different from DOW ($P < 0.05$).

addition, two males ignored the stimulus pup and did not meet the criteria (adopting a crouched posture over the pup) used to define alloparental males. Although all males were allowed to cohabitate with a female for 30 min and then tested in the partner preference paradigm, the number of non-alloparental males in this subgroup ($n = 2$) was not sufficient to include in subsequent data analysis. The exclusion of these two subjects did not affect any of the results obtained during the partner preference test. Pup-exposed, alloparental males and both control groups (DOW and HAN) consisted of ten subjects.

Behaviour during preference tests was monitored on time-lapse video with a 12 : 1 temporal reduction and scored later by an experimentally-blind observer using BEHAVIOR TRACKER, version 1.0. Social preferences were determined as a function of the difference in time spent in side-to-side contact with the partner compared to time spent in side-to-side contact with the stranger. Blood for the assessment of hormones was not collected from these subjects.

Plasma collection

In keeping with Animal Care and Use Committee policy, subjects immediately were anaesthetised deeply with a mixture of ketamine (67.7 mg/kg; NLS Animal Health, Owings Mills, MD, USA) and xylazine (13.3 mg/kg; University of Illinois Hospital Pharmacy, Chicago, IL, USA) administered i.p. and then quickly euthanised via cervical dislocation. After cervical dislocation, animals were decapitated and trunk blood was collected in ice-chilled tubes rinsed in heparin. Terminal blood samples were centrifuged at 4 °C, at 822 *g*, for 15 min to obtain plasma. Plasma was divided into aliquots and stored at -80 °C until assayed for plasma oxytocin and CORT (Experiment 1) and plasma oxytocin, vasopressin and CORT (Experiment 2).

Hormone assays

Plasma concentrations of oxytocin and vasopressin were determined using commercially available enzyme-linked immunosorbent assay kits (Assay Designs, Ann Arbor, MI, USA), previously validated for use in prairie voles (51). Plasma was diluted in assay buffer to give results falling reliably within the linear portion of the standard curve. According to the manufacturer, the minimum detection limit was 11.7 pg/ml for oxytocin and 3.39 pg/ml for vasopressin. Similarly, the inter- and intra-assay coefficients of variation were $< 8.7\%$ for oxytocin and $< 6.4\%$ for vasopressin. Cross reactivity between oxytocin and vasopressin was negligible. Plasma was diluted 1 : 6 for oxytocin measurement and 1 : 8 for vasopressin.

Plasma concentrations of CORT were assayed in Experiment 1 using a commercially available radioimmunoassay kit (MP Biomedicals Inc., Costa Mesa, CA, USA), previously validated for the prairie vole (52). Plasma was diluted as needed in assay buffer to give results falling reliably within the linear portion of the standard curve. The inter- and intra-assay coefficients of variation for CORT were $< 5\%$ and, according to the manufacturer, cross-reactivity with other steroids is $< 1\%$. Plasma was diluted 1 : 2000 for CORT measurement. In Experiment 2, plasma concentrations of CORT were assayed using a commercially available enzyme-linked immunosorbent kit (Assay Designs). Again, the inter- and intra-assay coefficients of variation for CORT were $< 5\%$ and cross-reactivity with other steroids is $< 1\%$. For the enzyme-linked immunosorbent CORT assay, plasma was diluted 1 : 500.

Tissue fixation

A spinning immersion fixation protocol (53) was used to preserve brain tissue for immunohistochemistry. Brains were carefully extracted from the skull and placed in an ice-chilled scintillation vial containing 19 ml of 4% buffered paraformaldehyde and 1 ml of 5% acrolein and spun gently for 10 min. Brains were then blocked, exposing the lateral ventricles and returned to the fixative solution for an additional 1 h and 50 min. Brains were then placed in a fresh fixative solution and spun for an additional 2 h. Subsequently, brains were immersed in a 25% sucrose solution and stored at 4 °C until sectioned. Tissue containing the hypothalamic regions of interest was prepared in 30- μ m coronal plane sections using a freezing sliding microtome. Sections were stored in cryoprotectant (54) at -20 °C until processed.

Immunohistochemistry

Brains were stained for oxytocin, vasopressin, CRH and c-Fos using standard avidin-biotinylated enzyme complex (ABC) immunocytochemistry (Vector Laboratories, Burlingame, CA, USA). Serial sets (every third 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 (RT) to reduce free aldehydes to alcohol followed by a rinse in KPBS. Sections then were incubated for 15 min in 0.014% phenylhydrazine at RT to block endogenous peroxidase activity and rinsed again in KPBS. Next, sections were incubated in rabbit c-Fos antisera (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1 : 50 000 dilution in 0.05 M KPBS + 0.4% Triton X-100 for 1 h at RT and for an additional 48 h at 4 °C. Sections were rinsed in KPBS before

being incubated for 1 h at RT in biotinylated goat, anti-rabbit immunoglobulin G (Vector Laboratories; 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 Laboratories) for 1 h at RT. Sections were rinsed in KPBS and then with Tris-buffered saline. C-Fos immunoreactivity (ir) was visualised by incubation in a solution containing 50 ml of Tris-buffered saline, 1.25 g of nickel sulfate, 41.5 μ l of 3% H₂O₂ and 10 mg of diaminobenzidine for 15 min at RT. Sections were rinsed in sodium acetate followed by a series of rinses in KPBS.

After c-Fos staining, sections were then incubated in either rabbit oxytocin antisera at a dilution of 1 : 300 000 (generously provided by Dr Mariana Morris, Wright State University, Dayton, OH, USA), rabbit vasopressin antisera at a dilution of 1 : 300 000 (#64717; ICN Biomedicals Inc., Costa Mesa, CA, USA) or goat CRH antisera at a dilution of 1 : 15 000 (T-4037; Bachem Inc., Bubendorf, Switzerland). The same procedure was carried out as above, except for the absence of nickel sulphate in the chromagen precipitation step for all three peptides, producing an orange-brown stain in contrast to the black c-Fos stain. For the CRH staining only, 2% normal rabbit serum was included during the primary incubation phase and biotinylated rabbit anti-goat secondary antibody (Vector Laboratories; dilution 1 : 600) was used to complement the anti-CRH primary antisera.

Labelled 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, Charlotte, NC, USA). Slides were then cover slipped with Histomount (National Diagnostics).

Quantification of immunoreactivity

Slides were coded and images were acquired using a Nikon Eclipse E 800 microscope (Nikon, Tokyo, Japan), a SensiCam camera (Cooke Corp., Auburn Hills, MI, USA) and IP Lab 3.7 computer software (Scanalytics Inc., Fairfax, VA, USA). A manual hand count of oxytocin-ir, vasopressin-ir and CRH-ir cell bodies observed using a \times 40 microscope objective was conducted by two trained observers who were blinded to the experimental conditions. Single- and double-labelled neurones were quantified using a standardised sampling area in accordance with procedures described previously (Ruscio *et al.*, 2007, 55,56) to ensure variability was not a function of variability in defining borders in different subjects.

Hand counts used to index cell body frequency were taken from sections matched in a rostral-caudal orientation. Oxytocin, vasopressin and CRH immunoreactive neurones were measured in a single caudal section of the PVN where staining takes a characteristic heart shape consistent with previous studies (Wang *et al.*, 1996; Ruscio *et al.*, 2007) and where the number of peptide stained cells was found to be highest. [These sections are similar to figs 36 and 37 in the mouse atlas of Paxinos and Franklin (1997)]. Representative sections of the SON were selected at the same level as the PVN.

To measure activation in brain regions also known to contain high concentrations of CRH, sections of the CeA and BNST were separately stained for CRH and chosen for measurement of c-Fos density. Sections selected corresponded to figures 31 (BNST) and 44 (CeA) in Paxinos and Franklin (1997). Vasopressin staining in the BNST was detected in a more caudal section [fig. 35 in Paxinos and Franklin (1997)] and c-Fos also was measured in this section. Regions of interest were selected according to previously published methods (55). Areas assessed in regions of interest for selected brain regions were: PVN, 1250 \times 1250 μ m; SON, 750 \times 750 μ m; BNST, 500 \times 500 μ m; and CeA, 1000 \times 1000 μ m.

Double-labelling was quantified in accordance with a previously published protocol (56). Neurones were considered to be double-labelled if a c-Fos-ir nucleus was observed entirely within the stain of a peptide-ir soma and in the same focal plane. With regard to CRH staining of soma in the BNST and CeA, the density of CRH innervation was so widespread, and somatic stain-

ing so light, that individual cells could not be readily differentiated. Therefore, CRH/c-Fos data were collected from the BNST and CeA as a count of c-Fos positive neurones within a field of CRH staining, rather than within individual cells, and therefore probably represent either CRH-producing neurones and/or neurones receiving CRH innervation.

Bilateral sections from each subject were hand counted and summed. These manual hand counts were conducted by two trained, experimentally blind raters and the results were average for all subjects. No indications of asymmetry in the staining were noted.

Statistical analysis

Data are presented as the mean \pm SEM for all analyses and figures. All statistical analyses were conducted using SPSS, version 13.0 (SPSS Inc., Chicago, IL USA) with α set at 0.05. All data were tested for assumptions of normality and equality of variance. If non-normal, data were transformed using the log or square root transformations or analysed using a nonparametric Kruskal-Wallis test when transformation was unsuccessful. In Experiment 1, independent t-tests were used to compare group (PEX versus HAN) differences in plasma hormone data from subjects 10 min after the onset of stimulus exposure, 45 min after the completion of 10 min of stimulus exposure and 55 min after the onset of stimulus exposure. In Experiment 2, ANOVA was used to compare group differences (PEX versus DOW versus HAN) in plasma hormones from subjects 10, 20 or 60 min after the onset of stimulus exposure. An ANOVA was used, with time (0, 10, 20 and 60 min) as the independent variable, to determine whether plasma hormone concentrations changed over time in response to a particular stimulus. Follow-up analyses of a significant ANOVA were conducted using Tukey's post-hoc test. Independent t-tests were used to compare group (PEX versus DOW) differences in behavioural responses during stimulus presentation and then correlations were used to assess the relationship between plasma hormones and behaviour during stimulus presentation based on *a priori* assumptions that exhibiting alloparental behaviour would increase oxytocin and decrease CORT. In Experiment 3, independent t-tests were used to compare group (PEX versus DOW) differences in the number of peptide positive neurones and the percentage of peptide positive neurones co-expressing c-Fos within the PVN and SON and in c-Fos density in the CeA and BNST. In Experiment 3, when two measures (i.e. the number of OT neurones in the PVN, and the percentage of OT neurones in the PVN co-expressing c-Fos) were found to have significantly unequal variances, log-transformed data were substituted for use in the t-test. Independent t-tests were also used to compare group (PEX versus DOW) differences in behavioural responses during stimulus presentation in Experiment 4. In addition, pair-bonding (i.e. a social preference for the partner compared to the stranger) was assessed within groups and analysed using paired t-tests. Because the assumption of homogeneity of variance could not be met for behaviours assessed during the partner preference test, the nonparametric Kruskal-Wallis test was used to assess between group differences in the amount of time males spent in each cage, the amount of time males spent interacting or in side-by-side contact with the familiar female and the amount of time spent interacting or in side-by-side contact with the unfamiliar female.

Results

Experiment 1: Juvenile males and the endocrine effects of pup exposure

Differences were noted in plasma hormone concentrations after 10 min of stimulus exposure. The concentration of plasma oxytocin observed in the PEX group tended to be higher compared to the HAN group ($t = 1.88$, $d.f. = 14$, $P = 0.08$; Fig. 1). In addition, the

concentration of plasma CORT observed in the PEX group was lower compared to the HAN group ($t = -2.20$, $d.f. = 13$, $P < 0.05$). Plasma hormones were not correlated and group differences in plasma hormone concentrations were not observed at the other time points.

Experiment 2: Adult males and the endocrine effects of pup exposure

Behaviours during the first 10 min of stimulus presentation were assessed in males in the PEX and DOW groups. Table 1 represents data pooled from all alloparental males and all males exposed to a dowel in Experiment 2. Data from males in the HAN groups are not presented in Table 1 because they did not have an opportunity to engage in many of these behaviours because a stimulus was not presented to them. The latency to approach the stimulus was significantly less in alloparental males in the PEX groups compared to the males in the DOW groups ($t = -1.994$; $d.f. = 64$; $P < 0.05$). All alloparental males in the PEX groups spent time immobile, crouched over the stimulus pup, whereas none of the males in the DOW groups spent any time immobile, crouched over the dowel ($P < 0.001$). Alloparental males in the PEX groups also spent more time licking and grooming the stimulus, as well as maintaining contact with the stimulus, compared to males in the DOW groups (all $P < 0.001$). By contrast, males in the DOW groups spent more time auto-grooming and engaged in other nonstimulus-directed behaviours compared to alloparental males in the PEX groups (all $P < 0.001$). In the DOW groups, most of this behaviour was spent investigating the wire mesh covering the test arena.

Data from subjects in Experiment 2 were obtained from terminal blood samples (Fig. 2) because non-invasive, sequential blood sampling was not possible in prairie voles. Group differences in the concentration of plasma oxytocin were detected ($F_{2,29} = 4.49$,

$P < 0.05$) in subjects exposed to experimental or control stimuli for 10 min. Post-hoc analysis revealed that the concentration of plasma oxytocin was higher in the PEX group compared to the HAN group ($P < 0.05$; Fig. 2). Group differences in the concentration of plasma oxytocin were not detected in subjects exposed to experimental or control stimuli for 20 or 60 min.

Group differences in the concentrations of plasma CORT were detected ($F_{2,29} = 3.22$, $P < 0.05$) in subjects exposed to experimental or control stimuli for 10 min. Post-hoc analysis revealed the concentration of plasma CORT was lower in the PEX group compared to the HAN group ($P < 0.05$) and tended to be lower compared to the DOW group ($P = 0.09$; Fig. 2). Statistically significant group differences in the concentrations of plasma CORT were not detected in subjects exposed to experimental or control stimuli for 20 min. There also was a trend for group differences in the concentrations of plasma CORT ($F_{2,30} = 2.64$; $P = 0.06$) in subjects exposed to experimental or control stimuli for 60 min, such that the concentration of plasma CORT after 60 min of stimulus exposure tended to be higher in PEX males compared to DOW males ($P = 0.054$).

Group differences in the concentrations of plasma vasopressin were not detected in subjects exposed to experimental or control stimuli for 10, 20 or 60 min. The relationship between plasma hormones and behaviour displayed during the stimulus presentation was assessed using correlations. The concentrations of plasma oxytocin, CORT and vasopressin were not correlated in alloparental males. However, higher concentrations of plasma oxytocin in alloparental males were associated with some pup-directed behaviour. Alloparental males with a higher concentration of plasma oxytocin tended to approach the stimulus pup more quickly ($r = -0.349$; $P = 0.06$; $n = 29$) and tended to divert their attention from the pup less by engaging in nonstimulus-directed behaviours ($r = -0.349$; $P = 0.06$; $n = 29$). The relationships between plasma vasopressin and the observed behaviours were not statistically significant, although they tended to be in the opposite directions to those seen between plasma oxytocin and behaviour. By contrast to plasma oxytocin, the concentration of plasma CORT was negatively correlated with pup-directed behaviours in alloparental males. Alloparental males with higher levels of CORT took longer to approach the pup ($r = 0.548$; $P < 0.005$; $n = 29$) and were less likely to remain immobile when crouched over the pup ($r = -0.423$; $P < 0.05$; $n = 29$). Alloparental males with higher concentrations of plasma CORT also were more likely to engage in nonpup-directed behaviours ($r = 0.369$; $P < 0.05$; $n = 29$) such as cage exploration.

Interestingly, males in the DOW groups with a higher concentration of plasma CORT took less time to approach the dowel ($r = -0.347$; $P < 0.05$; $n = 35$). There also was a negative correlation in the DOW group between nonstimulus-directed behaviours and the concentration of plasma CORT ($r = -0.392$; $P < 0.05$; $n = 35$), as well as the concentration of plasma vasopressin ($r = -0.452$; $P < 0.01$). The concentrations of plasma oxytocin and plasma vasopressin were positively correlated ($r = 0.610$; $P < 0.001$; $n = 35$) in males in the DOW groups. This correlation was not observed in pup-tested males, probably because of complex changes in oxytocin that followed pup exposure.

Table 1. Behaviour During the First 10 min of Stimulus Presentation During Experiment 2.

Behaviour	PEX groups ($n = 29$)	DOW groups ($n = 36$)
Latency to approach stimulus	16.91 ± 3.36*	32.21 ± 6.25
Time spent crouching immobile over the stimulus	49.79 ± 6.96 [‡]	0 ± 0.00
Time spent in contact with the stimulus	107.38 ± 9.31 [‡]	10.30 ± 3.41
Time spent licking and/or grooming the stimulus	251.60 ± 10.59 [‡]	44.19 ± 6.13
Time spent auto-grooming	17.90 ± 3.64 [‡]	49.35 ± 6.65
Time spent engaged in 'other' behaviours	173.33 ± 14.14 [‡]	496.16 ± 8.84

The results (mean ± SEM) represent data pooled from all alloparental males and all males exposed to a dowel in Experiment 2. Latency and time are expressed in seconds. * $P < 0.05$, [‡] $P < 0.001$: significantly different from combined DOW control groups (t-test). PEX, exposed to a pup; DOW, exposed to a dowel.

Experiment 3: Adult males and c-Fos staining as a function of pup exposure in brain regions containing oxytocin, vasopressin or CRH

Because the number of non-alloparental males was too small to allow group analysis, only males that exhibited alloparental behaviour (12 of 15) were included in the analysis of the PEX group. In addition, as a result of poor staining quality, one male from the PEX group and two males from the DOW group could not be used. The total number of c-Fos positive cells within the PVN was higher in the PEX group ($n = 11$, 204.7 ± 22.8 cells) compared to the DOW group ($n = 13$, 136.9 ± 15.7) ($t = 2.509$, $d.f. = 22$, $P = 0.02$). Table 2 represents the immunohistochemical results from Experiment 3. Specific peptide/c-Fos double-labelling is described below.

Oxytocin

Within the PVN, alloparental males in the PEX group ($n = 11$) compared to males in the DOW group ($n = 13$) showed a significantly higher percentage of oxytocin-ir neurones co-labelled for c-Fos, $14.5 \pm 2.8\%$ compared to $7.3 \pm 1.7\%$ ($t = 2.602$, $d.f. = 22$, $P = 0.016$) (Fig. 4). Group differences were not observed in either the number of oxytocin-ir cells in the PVN, SON or the BNST, or the percentage of double-labelling for c-Fos in the SON or BNST.

Vasopressin

Comparing the PEX versus DOW groups, a significantly greater percentage of vasopressin-ir neurones that were double-labelled for c-Fos was observed in the PVN (PEX = $12.2 \pm 1.9\%$, DOW = $7.9 \pm 1.0\%$) ($t = 2.171$, $d.f. = 22$, $P = 0.041$) (Fig. 4). Vasopressin-ir neurones in the PVN, SON or BNST and vasopressin neurones double-labelled with c-Fos in either the SON or the BNST did not differ between groups. There was a significant correlation between the percentages of double-labelled oxytocin and vasopressin neurones within the PVN ($r = 0.66$; $P = 0.027$; $n = 11$) in alloparental males

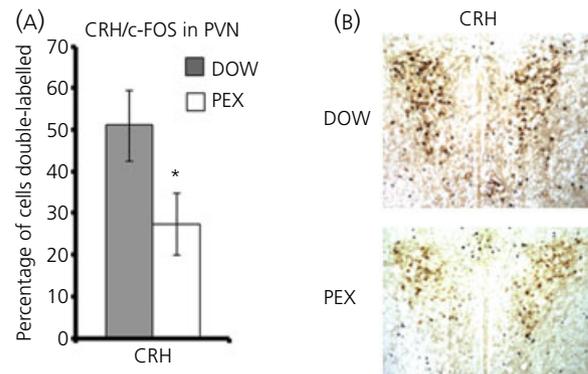


Fig. 4. The percent of recently active corticotrophin-releasing hormone (CRH) neurones in the paraventricular nucleus of the hypothalamus (PVN) (A) and representative photomicrographs of the PVN in each condition (B). Subjects were exposed to either pup (PEX) or dowel (DOW) for 20 min and then sacrificed 40 min afterwards. All groups consisted of nine to 12 subjects. Data are expressed as the mean \pm SEM. *Significantly different from DOW ($P < 0.05$).

in the PEX group, whereas there was no such correlation in males in the DOW group.

CRH

By contrast to the pattern of labelling observed for oxytocin and vasopressin, the percentage of c-Fos positive nuclei in CRH-ir neurones observed in the PVN was lower in the PEX group compared to the DOW group (PEX = $27.4 \pm 7.3\%$, DOW = $51.1 \pm 8.5\%$) ($t = 2.103$, $d.f. = 19$, $P = 0.049$) (Table 2). There was no group difference in the total number of CRH-positive cells in the PVN. We also examined c-Fos ir in two other regions, in which CRH-ir cells are common (BNST and CeA). In these regions, c-Fos counts tended to be higher in the PEX group compared to the DOW group ($P < 0.1$).

Table 2. Immunohistochemical Staining Results After Stimulus Presentation in Experiment 3.

Peptide	Brain region	#Peptide-ir Cells		c-Fos	
		PEX group ($n = 11$)	DOW group ($n = 13$)	PEX group ($n = 12$)	DOW group ($n = 9$)
Oxytocin	PVN	88.9 ± 9	67.2 ± 5	$14.5 \pm 2.8\%^*$	$7.3 \pm 1.7\%$
	SON	33.8 ± 3.9	30.9 ± 5.8	$6.4 \pm 1.4\%$	$8.9 \pm 1.9\%$
	BNST	11.6 ± 2.6	12.8 ± 2.6	$6.5 \pm 2.0\%$	$3.5 \pm 2.2\%$
Vasopressin	PVN	95.9 ± 10.3	75.6 ± 8.3	$12.2 \pm 1.9\%^*$	$7.9 \pm 1.0\%$
	SON	45.2 ± 6.4	41.2 ± 7.1	$10.7 \pm 1.7\%$	$8.1 \pm 1.1\%$
	BNST	7.7 ± 1.5	7.5 ± 0.9	$2.9 \pm 2.1\%$	$5.6 \pm 3.5\%$
CRH	PVN	40.8 ± 5.0	32.4 ± 5.0	$27.4 \pm 7.3\%^*$	$51.1 \pm 8.5\%$
	BNST	n/a	n/a	15.3 ± 3.8	7.1 ± 1.2
	CeA	n/a	n/a	20.9 ± 4.2	11.7 ± 2.3

The results are expressed as the mean \pm SEM. c-Fos data are expressed as either a percent of peptide-immunoreactive (ir) cells co-expressing c-Fos or as the number of c-Fos expressing nuclei (CRH-BNST and CRH-CeA only). *Significantly different from DOW ($P < 0.05$). PEX, exposed to a pup; DOW, exposed to a dowel. PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; BNST, bed nucleus of the stria terminalis; CeA, central amygdala.

Experiment 4: Adult males and the effects of pup exposure on partner preference formation

Similar to Experiment 2, behaviours displayed by alloparental males in the PEX group and males in the DOW group during the first 10 min of stimulus presentation were assessed in Experiment 4 and are shown in Table 3. Although latency to approach the stimulus was assessed in males that attacked the pup (PEA group) (Table 3), other behaviours were not assessed in the PEA group because the pup was removed immediately after the attack. Latency to approach the stimulus did not differ significantly between the three groups. Similar to Experiment 2, all alloparental males in the PEX group spent time immobile, crouched over the stimulus pup, whereas none of the males in the DOW group spent any time immobile, crouched over the stimulus dowel ($P < 0.005$). Alloparental males in the PEX group also spent more time licking and grooming the stimulus ($P < 0.001$), as well as maintaining contact with the stimulus ($P < 0.005$) compared to males in the DOW group. By contrast, males in the DOW groups spent more time auto-grooming and engaged in 'other' nonstimulus-directed behaviours compared to alloparental males in the PEX groups ($P < 0.05$ and $P < 0.001$, respectively).

Male prairie vole social behaviour during the partner preference test was affected by 20 min exposure to a pup (Fig. 5). Males that were exposed to a pup, regardless of whether they attacked it or responded with alloparental behaviour, spent more time in side-by-side contact with the partner compared to the stranger ($t = 3.28$, d.f. = 16, $P = 0.005$). More specifically, there was a trend for alloparental males in the PEX group to spend more time in side-by-side contact with the partner compared to the stranger ($t = 2.18$,

Table 3. Behaviour During the First 10 min of Stimulus Presentation During Experiment 4.

Behaviour	PEX group (n = 10)	DOW group (n = 10)	PEA group (n = 7)
Latency to approach stimulus	12.90 ± 4.12	26.89 ± 8.01	10.86 ± 3.12
Time spent crouching immobile over the stimulus	47.85 ± 12.58 ⁺	0 ± 0.00	
Time spent in contact with the stimulus	132.45 ± 28.25 ⁺	14.31 ± 1.44	
Time spent licking and/or grooming the stimulus	219.52 ± 31.94 [†]	29.19 ± 8.43	
Time spent auto-grooming	18.08 ± 5.55 [*]	46.85 ± 11.32	
Time spent in engaged in 'other' behaviours	182.10 ± 34.57 [†]	509.65 ± 9.92	

The results are expressed as the mean ± SEM. Latency and time are expressed in seconds. Other than latency to approach the stimulus, additional data are not shown for the group where the males attacked the pup (PEA) because the pup was removed immediately after the attack. * $P < 0.05$, ⁺ $P < 0.005$ and [†] $P < 0.001$: significantly different from the DOW control group. PEX, exposed to a pup; DOW, exposed to a dowel.

d.f. = 9, $P = 0.06$) during the partner preference test. Alloparental males spent, on average, 28.93 min with the partner and 7.86 min with the stranger. Males that attacked the pup spent significant more time in side-by-side contact with the partner compared to the stranger ($t = 2.51$, d.f. = 6, $P < 0.05$) spending, on average, 23.02 min with the partner and 1.16 min with the stranger. A small sample size ($n = 2$) prevented us from analysing how non-alloparental males spent their time during the partner preference test. By contrast, males in the two control groups (DOW or HAN) spent similar amounts of time in side-by-side contact with either the partner or the stranger (Fig. 5).

Discussion

Consistent with previous studies (10, 57), all reproductively naïve juvenile males in Experiment 1 and approximately 70% of reproductively naïve adult males in Experiments 2, 3 and 4 responded with respect to alloparental behaviour when presented with an unfamiliar 1–3-day-old pup. As little as 10 min of exposure to a pup was associated with a higher plasma concentration of oxytocin, as well as a concentration of plasma CORT that was lower in comparison to handled controls at this same time period (Figs 1 and 2). Despite age differences and slight procedural differences between Experiments 1 and 2, the patterns of effects of pup exposure on the plasma concentration of oxytocin and CORT were similar. In both experiments, the endocrine consequences of pup exposure were detected primarily in the first few minutes after stimulus exposure. In Experiment 2, in which terminal blood samples were taken closer together than Experiment 1, the effects of pup exposure on plasma hormone concentrations were no longer observed after 20 min of stimulus exposure, suggesting that the effects of pup exposure on the concentration of plasma hormones were transient, even when

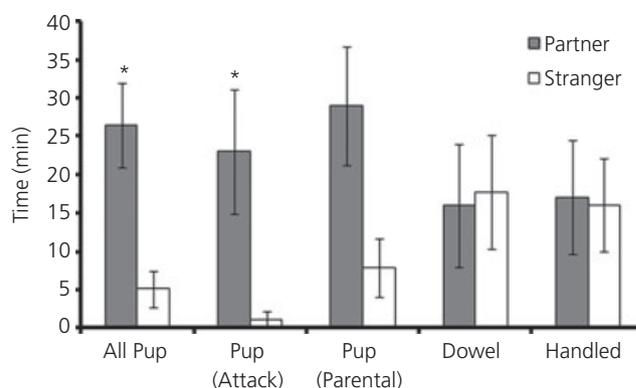


Fig. 5. Time males spent in side-by-side contact with a previously paired female (Partner) or a novel female (Stranger) during a 3-h partner preference test. Subjects were exposed to either a pup (PEX), a dowel, or merely handled for 20 min and then allowed to cohabitate with a previously unfamiliar female (Partner) for 30 min immediately before beginning the partner preference test. The experimental group (PEX) consisted of 12 subjects that exhibited alloparental behaviour and seven subjects that attacked the pup. Both control groups consisted of ten subjects. Data are expressed as the mean ± SEM. *Significantly different in time spent with Partner versus Stranger ($P < 0.05$).

males were exposed to a fresh pup half way through the treatment (Fig. 1). By contrast, the concentration of plasma oxytocin was not significantly affected in the HAN control groups in Experiments 1 and 2, suggesting that this effect was not a response to being placed in a novel environment.

Given that the changes in plasma hormone concentrations were relatively immediate and transient, future work should aim to determine whether repeated pup exposures produce the same response. The increase in the concentrations of plasma oxytocin observed after 10 min of pup exposure may influence the release of glucocorticoids by a direct action on the adrenal cortex (58–60). Although we were able to show increases in the concentration of plasma oxytocin and central oxytocinergic cell activity, the central release of oxytocin cannot be assumed. However, oxytocin may decrease HPA axis activity at more than one level. Despite the relative impermeability of the blood–brain barrier (BBB), small amounts of exogenous oxytocin administered to the circulatory system have been shown to cross the BBB (61); thus, an increase in plasma oxytocin could inhibit HPA axis activity in the PVN (21), especially if oxytocin is also being released centrally. Whether there is an involvement of oxytocin in the behavioural and hormonal effects observed in the present study remains to be determined, although a lack of correlation between plasma oxytocin and CORT does not support the notion of such an effect in the periphery.

Numerous studies have implicated vasopressin in male social behaviour, including parental behaviour (17, 37, 38, 62) and partner preference formation (7, 33, 34). The concentration of plasma vasopressin was measured only in Experiment 2 and did not differ between the PEX group and control groups. However, vasopressinergic neurones of the PVN were more active in the PEX group in Experiment 3, suggesting several interpretations. For example, the behaviourally relevant changes in vasopressin may be restricted to the central nervous system (63). Our testing schedule might have missed a peripheral surge of vasopressin, or such a peripheral release event may occur even after a comparatively small amount of stimulation, such as handling. Data from other studies in our laboratory support the latter interpretation (H. Pournajafi-Nazarloo and C. S. Carter, unpublished data).

In Experiment 3, we found that pup exposure increases double-labelling with c-Fos in oxytocin-ir and vasopressin-ir neurones and decreases c-Fos double-labelling of CRH-ir neurones in the PVN. The change in oxytocin-ir is consistent with the increase of plasma oxytocin after 10 min of pup exposure. The reductions in neuronal activity (as measured by c-Fos) in CRH-ir neurones may help to explain the diminished concentration of plasma CORT seen after 10 min of pup exposure. It is interesting that, in comparison to other treatments, pup exposure was associated with increased c-Fos expression in vasopressin-ir cells in the PVN but not in the SON and did not have a measurable effect on plasma vasopressin concentrations. Although Experiment 3 lacked a baseline measure, c-Fos is not typically induced by baseline neuronal activity (64).

In agreement with previous research, the findings of the present study suggest that changes in cells in the PVN that express either oxytocin or vasopressin may be activated in males expressing alloparental care (40). Indeed, maternal behaviour is also mediated by

the action of both neuropeptides (65). The percentages of double-labelled oxytocin-ir and vasopressin-ir cells in the PVN were significantly correlated with one another only after pup exposure, which suggests that a similar mechanism is driving activation. This relationship is unlikely to be a result of counting the same neurones twice as because the slices were 30 μm apart and it has been estimated in rats that only 1–2% of neurones express both oxytocin and vasopressin (66).

Previous research by Kirkpatrick *et al.* (30) reported that exposing a male prairie vole to a pup for 3 h induced an increase in c-Fos expression in the BNST, although the CeA was not examined. The same study showed no change in c-Fos in the PVN, which does not agree with the findings of the present study. Procedural differences, such as the duration of stimulus presentation, may account for this discrepancy. Peripheral plasma oxytocin and CORT effects occurred shortly after the initial presentation of a pup, and it is likely that changes in c-Fos, measured within approximately 1 h after the onset of pup exposure, were in response to early initial reactions to the pup.

The comparatively lower degree of c-Fos expression in CRH-ir cells in the PVN after pup exposure was in the opposite direction from that seen for oxytocin-ir and vasopressin-ir cells and may be consistent with the absence of an increase, or even a decline in the concentration of plasma CORT observed in pup-exposed males. However, there was a trend ($P < 0.1$) toward increases in c-Fos expression within the BNST and CeA; the activity in these brain regions may be associated with emotional reactions to social stimuli, rather than effects on the HPA axis. Because of the density of the CRH-ir neurones in the BNST and CeA, we were unable to determine whether these neurones were specifically CRH-synthesising cells or merely innervated by nearby CRH terminals. However, these preliminary findings suggest that the response of the CRH system to pup exposure may be region-specific, with different effects in regions regulating behavioural versus endocrine responses. Cells activated in the CeA and BNST also could be inhibitory neurones, which in turn might attenuate of CRH activity in other forebrain nuclei, possibly consistent with the apparent inhibition observed in the PVN.

Although variations in the production and/or reception of oxytocin have been shown to modulate many pro-social behaviours, including pair-bonding (41,43), parental care (2, 16, 41, 43, 67) and alloparental care (40, 68, 69), the role of social stimuli in the regulation of the release of oxytocin remains poorly understood. Somatosensory stimulation has been shown to increase the concentration of plasma oxytocin (70, 71) and may account for the increased concentration of plasma oxytocin observed after 10 min of pup exposure; however, a variety of stressors also have been shown to acutely increase the concentration of plasma oxytocin in rats (72). In adult male prairie voles, we also have observed increases in the concentration of plasma oxytocin after acute stressors, including physical restraint and lipopolysaccharide injections; however, in male prairie voles, the concentration of plasma oxytocin did not increase after either acute isolation for 1 h (present study; H. Pournajafi-Nazarloo and C. S. Carter, unpublished data) or after long-term social isolation for ≥ 4 weeks (56). It

remains to be determined whether the increase in the concentration of plasma oxytocin observed after 10 min of pup exposure is caused by positive somatosensory stimulation in male prairie voles generated by the pup or because male prairie voles perceive the pup as a stressful stimulus. However, the attenuation of HPA axis reactivity suggests a more complex response than the classic stress response.

The physiological function of the oxytocin released into the circulation during pup exposure is unclear; however, some research shows that oxytocin has an anxiolytic function and can attenuate behavioural and endocrine responses to stress (21, 73). Although the concentration of plasma oxytocin was higher in alloparental males in the PEX group compared to males in the HAN group after 10 min of stimulus exposure, the concentration of plasma CORT was lower in the PEX group compared to males in the HAN group after 10 min of stimulus exposure. Changes detected in the concentration of plasma hormones parallel the central results, where activity in CRH-ir cells in the PVN, as measured by c-Fos expression, was lower, and higher in oxytocin-ir cells. In comparison to handled controls, the initial effect of pup exposure on the concentration of plasma CORT appears to be an attenuation of measurable concentrations of CORT. Exposure to an unfamiliar, opposite-sex adult was also associated with an apparent attenuation of the plasma CORT response in adult male prairie voles (22, 74), suggesting that socially-stimulated release of oxytocin may restrain the HPA axis and other neural systems after exposure to either a pup or an unfamiliar adult.

When data from both alloparental males and those that attacked pups were combined in Experiment 4, partner preference formation was facilitated after pup exposure; this effect was not seen in males in the control groups. When pup attackers are excluded, the effect on partner preference only approaches significance ($P = 0.06$). Plasma hormones were not assessed in the males tested for partner preference formation in Experiment 4; however, data from Experiments 1, 2 and 3 showing that alloparental males respond to pup exposure with a short, transient increase in the activation of oxytocin are consistent with several studies implicating oxytocin being involved in partner preference formation in male prairie voles (41, 43, 47, 75). Data from Experiments 1 and 2 concerning the concentration of plasma CORT are not necessarily inconsistent with our previous data showing that elevations in the concentration of plasma CORT facilitate pair-bonding in male prairie voles (22, 24) because the mechanism of the pro-social effects of CORT remains unknown. Although we had a comparatively high number of pup attacks in Experiment 4 (10,17), it is not surprising that partner preference formation was facilitated in males that attacked the pups, considering that previous studies have shown that stressful events can facilitate the formation of pair-bonds in males (22, 24). It is also important to note that, although alloparental care and pair-bonding are both distinguishing features of the prairie social behaviour of vole, they are not always co-expressed in a given animal. We hypothesise that two potentially separate, but overlapping, pathways facilitate pair-bonding in males, depending on whether they respond to an unfamiliar pup with alloparental behaviour or they attack it. Additionally, female voles experience

post-partum oestrus, so the presence of a newborn pup may be a salient signal of a potentially receptive female.

There are several limitations to the present study that warrant caution when interpreting the results. Obviously, the ability to repeatedly sample plasma hormones from the same animal would be of great advantage. However, obtaining the volume of plasma necessary for hormonal analysis could trigger haemorrhage-induced changes in those same hormones. Although we speculate that the transient increase in the concentration of plasma oxytocin contributed to the attenuated plasma CORT response in males that responded with alloparental behaviour when exposed to a pup for 10 min, this series of experiments did not assess this hypothesis directly. Measurement of plasma CORT after treatment with exogenous oxytocin or oxytocin antagonists would help to address this question. The present studies also revealed negative correlations in individuals between plasma oxytocin and plasma CORT in males that were not pup-exposed, although they did not detect a correlation between plasma oxytocin and plasma CORT in any of the PEX groups. Furthermore, we were unable to correlate oxytocin/c-Fos with CRH/c-Fos because difficulties in titrating the proper concentration of primary antibody forced us to run a separate cohort for CRH/c-Fos. However, the lack of a correlation between circulating oxytocin and CORT does not confirm that these hormones are unrelated because activation of additional intervening mechanisms might account for the lack of correlation. The correlations between plasma CORT and alloparental behaviours are complex because retrievals are positively (and licking/grooming are inversely) related to the concentration of plasma CORT (23). Finally, the addition of another social stimulus beyond the pup would clarify which of the observed effects are specific to alloparenting and which can be generalised to more broadly defined affiliative social behaviours.

In summary, a short transient increase in the concentration of plasma oxytocin was observed in males that displayed alloparental behaviour during 10 min of pup exposure. The transient increase in the concentration of plasma CORT observed in control subjects was not observed in alloparental males and may possibly be attributed to the anxiolytic action of oxytocin. These peripheral effects were paralleled by corresponding changes in the central activation of oxytocin and CRH neurones. Although vasopressin neurones also were activated by pup exposure, we did not observe an increase in the concentration of plasma vasopressin. The response to pup exposure, even in males that were not overtly alloparental, also facilitated subsequent partner preference formation, possibly through similar neuroendocrine mechanisms.

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