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From here to paternity: Neural correlates of the onset of paternal behavior in California mice (*Peromyscus californicus*)

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ABSTRACT

In a minority of mammalian species, including humans, fathers play a significant role in infant care. Compared to maternal behavior, the neural and hormonal bases of paternal care are poorly understood. We analyzed behavioral, neuronal and neuropeptide responses towards unfamiliar pups in biparental California mice, comparing males housed with another male ("virgin males") or with a female before ("paired males") or after ("new fathers") the birth of their first litter. New fathers approached pups more rapidly and spent more time engaging in paternal behavior than virgin males. In each cage housing two virgin males, one was spontaneously paternal and one was not. New fathers and paired males spent more time sniffing and touching a wire mesh ball containing a newborn pup than virgin males. Only new fathers showed significantly increased Fos-like immunoreactivity in the medial preoptic nucleus (MPO) following exposure to a pup-containing ball, as compared to an empty ball. Moreover, Fos-LIR in the bed nucleus of the stria terminalis (STMV and STMPM) and caudal dorsal raphe nucleus (DRC) was increased in new fathers, independent of test condition. No differences were found among the groups in Fos-LIR in oxytocinergic or vasopressinergic neurons. These results suggest that sexual and paternal experiences facilitate paternal behavior, but other cues play a role as well. Paternal experience increases Fos-LIR induced by distal pup cues in the MPO, but not in oxytocin and vasopressin neurons. Fatherhood also appears to alter neurotransmission in the BNST and DRC, regions implicated in emotionality and stress-responsiveness.

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Introduction

Parental care – providing food, warmth, shelter and protection to offspring – is a crucial component of mammalian fitness. In mammals, mothers usually play the largest or only role in rearing offspring; however, in approximately 5–6% of mammalian species (including humans), males are heavily involved in parental care as well (Kleiman and Malcolm, 1981). Several biparental rodent species, including California mice (*Peromyscus californicus*), prairie voles (*Microtus ochrogaster*), and Djungarian hamsters (*Phodopus campbelli*) have been used to study the proximate factors underlying paternal care (see for reviews: Brown, 1993; Lonstein and De Vries, 2000; Wynne-Edwards and Timonin, 2007). However, little is known about the neurobiology of paternal behavior.

The factors controlling the onset and maintenance of maternal care are well described, with most data stemming from research in female rats. Upon parturition and the onset of lactation, primiparous females shift their response to pups from avoidance (of a novel, possibly threatening stimulus) to approach (towards a non-threatening, rewarding stimulus), ultimately engaging in maternal behavior (Numan, 2007; Numan and Stolzenberg, 2008). Apparently, the distal sensory (mostly olfactory and auditory) cues from the pup are processed differently in the central nervous system of virgin or pregnant versus maternal females. This transition is heavily dependent on the specific peripartum pattern of estrogen and progesterone release (Numan and Insel, 2003) as well as central and peripheral release of the neuropeptides oxytocin, vasopressin and prolactin (Bosch and Neumann, 2008; Brunton et al., 2008; Grattan, 2001; Mann and Bridges, 2001; Numan and Insel, 2003; Numan, 2006; Russell et al., 2001).

Male rodents have been found to change their behavior towards pups upon sexual and/or paternal experience, exhibiting inhibition of infanticidal behavior in non-paternal species or enhancement of paternal behavior in biparental species (Brown, 1993). In the absence of the major hormonal and neuropeptidergic changes associated with parturition and lactation, this transition in males is thought to be influenced mainly by copulation and/or cohabitation with a (pregnant) female mate (Brown, 1986; Elwood, 1977; Elwood, 1980; Soroker and Terkel, 1988). In the California mouse, a highly monogamous and biparental species both in the wild (Ribble, 1991;

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Ribble and Salvioni, 1990) and in the lab (Dudley, 1974; Gubernick and Alberts, 1987), singly housed virgin males exhibit significantly less paternal and more infanticidal behavior towards unfamiliar, newborn pups than males housed with a pregnant female or with their female mate and pups (Gubernick and Nelson, 1989). Moreover, male California mice that attack or ignore pups as virgins become parental either after copulating with a female or after the birth of their own litter (Gubernick et al., 1994). The neuronal and hormonal mechanisms underlying this transition are not yet known.

Maternal responses to pups are thought to be controlled by a neural network that includes the medial preoptic area, bed nucleus of the stria terminalis, medial amygdala, anterior hypothalamus, periaqueductal gray and nucleus accumbens (Numan, 2007; Numan and Stolzenberg, 2008). Since fathers in biparental species often interact with neonates and juveniles using similar behaviors as mothers, including grooming, huddling and the kyphotic nursing posture, the recruitment of analogous or homologous neuronal pathways seems likely (Numan and Insel, 2003). Indeed, lesion and Fos expression studies in biparental rodents have implicated the medial preoptic area, basolateral amygdala, lateral septum, medial bed nucleus of the stria terminalis and posterior medial amygdala in the execution of paternal care (Kirkpatrick et al., 1994; Lee and Brown, 2002a, 2007).

Similar to maternal behavior, the neuropeptides oxytocin and vasopressin have been implicated in the expression of paternal behavior. Parentally naïve male California mice cohabitating with a pregnant female had higher plasma oxytocin levels than virgin males or new fathers, although no correlation with parental versus non-parental behavior was found (Gubernick et al., 1995). Vasopressin-immunoreactivity in the bed nucleus of the stria terminalis correlated positively with paternal behavior towards their own newborn pups in a group of animals consisting of both California mice and their congeners, white-footed mice (*P. leucopus*) (Bester-Meredith and Marler, 2003). Furthermore, intracerebroventricular administration of vasopressin stimulated, and vasopressin and oxytocin receptor antagonists inhibited, paternal behavior in paternally naïve meadow voles and prairie voles (Bales et al., 2004; Parker and Lee, 2001; Wang et al., 1994). These findings suggest that sexual and/or paternal experience changes vasopressinergic and/or oxytocinergic neurotransmission; however, it is unknown whether acute responses in these neuropeptidergic neurons to distal cues from pups play a role.

The goals of the present study were threefold. First, we wanted to confirm that male California mice undergo a transition in their behavioral responses to an unfamiliar pup, from non-paternal (as virgins) to paternal (either as paired males, housed with a female prior to the birth of their first litter, or as new fathers, housed with a female and their first litter). Second, we aimed to identify brain areas involved in this transition by assessing Fos responses to distal cues from pups in virgin males, paired males and new fathers. Third, we aimed to further elucidate the roles of oxytocin and vasopressin in the behavioral transition to fatherhood by comparing activation of oxytocinergic and vasopressinergic neurons in response to cues from pups among the three groups of males.

Materials and methods

Animals

Male ($n=35$) and female ($n=23$) California mice, descendants of mice purchased from the *Peromyscus* Genetic Stock Center (University of South Carolina, Colombia, USA) were born in our laboratory colony, weaned at 27–33 days of age (prior to the birth of the next litter) and housed in same-sex groups of two or three animals (littermates or unrelated, age-matched mice) until the start of the experiment.

A total of 23 breeding pairs consisting of an unrelated male and female were formed over a 10-week period. The 12 remaining virgin males were housed in male–male dyads. Three dyads consisted of littermates, and three comprised unrelated virgin males of the same age. Mice were housed in transparent polycarbonate cages ($L\times W\times H$: $44\times24\times20$ cm) containing woodchip bedding and cotton for nest building. The colony was maintained on a 14:10 h day/night cycle (lights on at 0500 h), with ambient temperature maintained at approximately 23 °C and humidity maintained at approximately 65%, and had ad libitum access to chow (Purina 5001 Rodent Diet) and water. All mice were weighed twice weekly to monitor health and pregnancies.

All procedures were approved by the University of California, Riverside Institutional Animal Care and Use Committee, and were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals.

Experimental design

The housing paradigm resulted in three experimental groups: virgin males ($n=12$, 114.3 ± 4.3 (mean ± s.e.m.) days old and weighing 43.6 ± 2.1 g on the day of perfusion), paired males ($n=12$, 120.6 ± 5.9 days and 34.7 ± 1.2 g) and new fathers ($n=11$, 133.8 ± 5.2 days and 37.7 ± 2.5 g).

One or two days after a breeding pair produced its first litter, the male mouse from that pair (a "new father") underwent a paternal behavior test to determine its behavior in response to an unfamiliar pup. Two days after the paternal behavior test, the male underwent another behavioral test (the "ball test") to determine its behavior and neuronal activation in response to a stainless steel wire mesh ball (Ø: 8 cm) that either contained an unfamiliar pup or was empty. One hour after the ball test, the male was perfused and its brain harvested for immunohistochemistry.

On the same test days, a "paired male" (a male mouse from a breeding pair of which the female was thought to be pregnant based on steady weight gain) underwent the same procedures. Virgin males were included on alternate sets of tests: since these males were housed two per cage, the two cage mates were tested on the same days to prevent any virgin male from being housed singly during the experiment. In each experimental group, four mice were tested with an empty ball in the ball test; the remainder was tested with a ball containing a pup.

The litter of one new father was found dead on the morning of the ball test (postnatal day 3). This male's behavior in the paternal behavior test was excluded from final data analysis, but its behavior towards an empty ball in the ball test was included. One virgin male's brain was not correctly perfused and could not be processed for immunohistochemistry.

The 12 females that were assumed to be pregnant when their mate was tested and perfused were weighed twice weekly and their cages were checked every morning for the presence of newborn pups, until their litter was born or until 46.0 ± 5.8 days after removal of the male.

Paternal behavior test

All paternal behavior tests took place between 1000 h and 1400 h, since California mice show higher levels of paternal behavior in the light phase than in the dark phase (Wright and Brown, 2002). Each male mouse was removed from its home cage and placed in a clean cage with woodchip bedding. The cage was carried into a soundproof chamber and placed on a table. The mouse was allowed to habituate to the novel environment for 10 min, during which a 0–3 day-old stimulus pup was removed from its home cage and brought to the test chamber. Stimulus pups were never from the litter of the experimental fathers, and although some stimulus pups were used for multiple tests, none of the males were tested twice with the same pup, and pups were never used more than once per day.

The pup was placed in the front right-hand corner of the test cage, and the male's behavior in response to the pup was videotaped for

10 min. Behavioral data were later analyzed using the JWatcher event-recorder program (Blumstein and Daniel, 2007), with the following parameters scored: latency to sniff the pup, latency to initiate paternal behavior (huddling or licking the pup or kyphosis), total time spent sniffing the pup, total time spent in paternal behavior, total time spent in general activity without pup contact, and total time spent resting without pup contact.

Ball test

All ball tests took place between 1300 h and 1500 h. Two hours prior to the test, the male mouse was removed from its home cage and placed in a clean cage containing bedding, food and water. This cage was carried into the soundproof test chamber and placed on a table for 2 h, to allow the mouse to habituate to the novel environment and to wash out residual neuronal activation from home cage experiences. The mesh balls had been cleaned with water and soap followed by ethanol at least one day prior to the test.

The wire mesh ball, which was either empty (for $n=4$ mice per group) or contained a pup (for $n=7$–8 mice per group), was placed in the front right corner of the test cage, and the male's behavior was videotaped for 5 min. Behavioral data were later analyzed using JWatcher, with the following parameters scored: latency to sniff or touch the ball, total time spent sniffing or touching the ball, and total time spent in general activity unrelated to the ball, and resting unrelated to the ball. The ball with or without a pup was removed from the test cage immediately following the 5-min test.

Each mouse remained in the test chamber for 1 h after the end of the test, and was then carried into a separate room, immediately anesthetized deeply with pentobarbital (Fatal-Plus solution, Vortech Pharmaceuticals, Dearborn, Michigan, USA) and perfused transcardially with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS.

Immunohistochemistry

Following perfusion, brains were post-fixed in tubes containing 40 ml 4% paraformaldehyde. After 1 h, paraformaldehyde was replaced by 0.1 M PBS and the brains were stored at 4 °C for up to one month.

Prior to cutting, brains were submerged in 30% phosphate buffered sucrose for up to 1 week until saturated. The cryoprotected brains were snap frozen on dry ice and cut into 30 μm thick sections on a cryostat set at −19 °C. Consecutive sections were collected in five vials containing 5 ml 0.1 M PBS, to enable up to five different immunohistochemical stainings. Brain tissue was protected against mold with 50 μl 0.01% sodium azide per vial. Two series were used for two different double-stainings: Fos + oxytocin and Fos + vasopressin.

Staining started by washing one vial per brain for 3 × 10 min with fresh 0.1 M PBS, followed by 30 min of 0.1% H_2O_2 and another round of 3 × 10 min 0.1 M PBS. Tissue was then preincubated for 30 min with 0.1 M PBS containing 0.1% bovine serum albumin and 0.3% Triton-X-100 (PBS-BT) and incubated overnight with PBS-BT containing rabbit-anti-c-Fos antibody (sc-253, 1:10,000, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA).

The next day, tissue was washed 3 × 20 min with 0.1 M PBS. Brains were then incubated for 90 min with PBS-BT containing donkey-anti-rabbit antibody (1:1500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), followed by 3 × 20 min washing with 0.1 M PBS. Staining was enhanced by 90 min of incubation with PBS-BT containing ABC-vector (1:800, Vectastain Elite Kit, Vector Laboratories, Inc., Burlingame, CA, USA), followed by 3 × 20 min washing with 0.1 M PBS. Tissue was then preincubated for 10 min with 0.6% Tris-buffer containing 0.3% ammonium-nickel sulphate and 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB), followed by 10 min of incubation with the same solution, adding 30% H_2O_2 (10 μl/50 ml). This resulted in a blue-black nuclear staining. The stained brain slices were then washed 3 × 15 min in 0.1 M PBS, and the whole process was repeated starting with 30 min preincubation with PBS-BT. In this second round, one series of brain sections per mouse was incubated overnight with rabbit-anti-oxytocin (1:200,000, Peninsula Laboratories, San Carlos, CA, USA), and one series of brain sections per mouse was incubated overnight with rabbit-anti-vasopressin (1:200,000, Peninsula Laboratories). Both stainings were visualized using DAB in 0.6% Tris-buffer without the nickel sulphate. This resulted in a brown cytoplasmic staining that left the black nuclear staining visible in case of co-localization of the two peptides.

Brain sections were mounted on gelatin/chrome-alum coated glass slides, dehydrated and cleared in ethanol and xylene, embedded in Entellan New (EMS, Hatfield, PA, USA) and coverslipped.

Quantification of immunoreactivity

Sections double-stained for Fos and oxytocin were used (1) to quantify Fos-like immunoreactivity (Fos-LIR) alone throughout the brain, (2) to describe the distribution of oxytocin neurons in male California mouse brains, and (3) to quantify the co-localization of oxytocin and Fos. Sections double-stained for Fos and vasopressin were used (1) to describe the distribution of vasopressin neurons in male California mouse brains and (2) to quantify co-localization of vasopressin and Fos. Since there is no specific brain atlas for the California mouse, The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 2008) was used, which proved to be highly accurate in localizing and determining nuclei in California mouse brain sections. Nomenclature and abbreviations were also adopted from this atlas, and Bregma levels in the text refer to the equivalent levels in the atlas, rather than actual Bregma levels in *P. californicus*. The selected brain areas either showed a clear peak in Fos-LIR in one particular section, in which case the same section was used for each individual mouse, or showed a homogenous spread of Fos-LIR over multiple sections, in which case neuroanatomical landmarks (fibers, nerves, ventricles) were used to select the section approximating the same mouse Bregma level for each individual California mouse.

For the quantification of Fos-LIR alone, most brain areas were selected *a priori* based on (1) their known functions in parental behavior and/or reproduction: the medial posteromedial division of the bed nucleus of the stria terminalis (STMPM, mouse Bregma level −0.22), medial preoptic nucleus (MPO, mouse Bregma level −0.34), basolateral amygdaloid nucleus (BLA, mouse Bregma level −0.82), posterodorsal medial amygdaloid nucleus (MePD, mouse Bregma level −1.46), ventromedial hypothalamic nucleus (VMH, mouse Bregma level −1.58), ventrolateral periaqueductal gray (VLPAG, mouse Bregma level −4.84); (2) their known functions in stress and anxiety: ventral lateral septal nucleus (LSV, mouse Bregma level +0.62), dorsal lateral division of the bed nucleus of the stria terminalis (STLD, mouse Bregma level +0.14), lateral division of the central amygdaloid nucleus (CeL, mouse Bregma level −1.46), dorsomedial periaqueductal gray (DMPAG, mouse Bregma level −4.72); or (3) their known functions in reward: accumbens nucleus shell, AcbSh, and, as a control, accumbens nucleus core, AcbC, both at mouse Bregma level +1.18). In addition, some brain areas were selected *a posteriori* for quantification of Fos-LIR alone based on strong immunoreactivity: anterior hypothalamic area (AHA, mouse Bregma level −0.70) and supraparafascicular thalamic nucleus (SPF, mouse Bregma level −2.30); or based on observed differences during gross microscopic evaluation of the sections: ventral medial division of the bed nucleus of the stria terminalis (STMV, mouse Bregma level −0.10) and caudal dorsal raphe nucleus (DRC, mouse Bregma level −5.02).

The total number of oxytocin-LIR neurons and the number of oxytocin + Fos-LIR neurons were quantified in three areas with a distinctive population of oxytocin-LIR neurons: the medioventral bed

nucleus of the stria terminalis (STMV-OT, mouse Bregma level −0.10), the anterior parvicellular paraventricular hypothalamic nucleus (PaAP-OT, mouse Bregma level −0.46) and the lateral magnocellular paraventricular hypothalamic nucleus (PaLM-OT, mouse Bregma level −0.82). The number of vasopressin-LIR neurons and the number of vasopressin + Fos-LIR neurons were quantified in two areas with a distinctive population of vasopressin-LIR neurons: the area of the STMPM surrounding the stria medullaris (STMPM-VP, mouse Bregma level −0.46) and the lateral magnocellular paraventricular hypothalamic nucleus (PaLM-VP, mouse Bregma level −0.82).

Standardized digital photographs of each brain area, as well as a millimeter scale, were taken at a magnification of 20× with a digital camera (Canon EOS-40D) mounted on a microscope (Leica Leitz DMRB). Using Photoshop CS2, a grid of lines equivalent to 0.2 × 0.2 mm (STMV-OT and STMPM-VP) or 0.1 × 0.1 mm (all other areas) was placed in each photograph so that it contained either all or the majority of immunoreactive neurons in the selected area, or, in case of larger brain areas, a portion containing a representative spread of immunoreactive neurons. The number of Fos-positive neurons within the square was counted, as well as the number of oxytocin- or vasopressin-positive neurons and the number of double-stained neurons when appropriate. The experimenter was blind to the experimental group and test condition of the mice while counting neurons.

Statistical analyses

Statistical analyses were performed using SPSS 16.0. The majority of the data was not normally distributed, and some groups were small ($n = 4$). Therefore, data were analyzed non-parametrically, and graphs depict medians with error bars delineating first and third quartiles to illustrate variability in the dataset.

For the paternal behavior test, virgin males, paired males and new fathers were compared using Kruskal–Wallis tests to detect overall effects among the experimental groups; significant overall effects were followed by nonparametric post hoc pairwise comparisons (Siegel and Castellan, 1988). The proportion of paternal males (males displaying any paternal behavior in the test) in each experimental group was compared using Fisher's exact test. In addition, spontaneously paternal virgin males were compared with fathers using the Mann–Whitney test.

For the behavioral and immunohistochemical data from the ball test, Kruskal–Wallis tests were performed on the three experimental groups for the pup and no pup test conditions separately, followed by nonparametric post hoc pairwise comparisons of virgin males, paired males and new fathers within each test condition. In addition, for each experimental group (virgin males, paired males and new fathers) separately, the responses to the pup versus the no pup condition were compared using Mann–Whitney tests.

Spearman's rho was used to correlate the number of Fos-immunoreactive neurons in each quantified brain area with the amount of time spent sniffing or touching the pup-containing ball or the empty ball, with virgin males, paired males and new fathers pooled within each test condition. Subsequently, each significant correlation was compared to the correlation in the other condition ("pup" versus "no pup") using the Fisher's Z' transformation. When a significant correlation in the "pup" condition was found to be significantly different from the corresponding correlation in the "no pup" condition, correlations were further analyzed for virgin males, paired males and new fathers separately. Conversely, significant correlations in the "no pup" condition were not further analyzed for each group separately, since group sizes in this condition ($n = 4$) were considered too small to reliably detect significant correlations.

For all analyses, effects were accepted as statistically significant when P (two-tailed) was smaller than 0.05 ($Z < \pm 1.996$).

Results

Breeding

The mates of the new fathers ($n = 11$) gave birth after 44.8 ± 3.0 (mean ± s.e.m.) days of cohabitation to an average of 1.7 ± 0.2 pups, similar to previously reported litter sizes in these species (Gubernick and Alberts, 1987). Nine of these females became pregnant again before the male was removed, 3–4 days after parturition.

Of the 12 females that had not yet given birth before their male mates (the 'paired males') were removed, six females eventually gave birth to an average of 2.3 ± 0.2 pups, 59.8 ± 6.9 days after pairing and 11.8 ± 1.3 days after the paternal behavior test. The other six females (two mates of males tested with an empty ball, four mates of males tested with a pup-containing ball) were never observed with a litter. Four of these latter females continued to gain weight after mate removal but eventually started losing weight; one stopped gaining weight after the male was removed; and one continued to gain weight but did not produce a litter within 47 days after mate removal.

Paternal behavior test

Virgin males, paired males and new fathers differed significantly in their behavior towards a 1–3 day-old unfamiliar pup (Fig. 1).

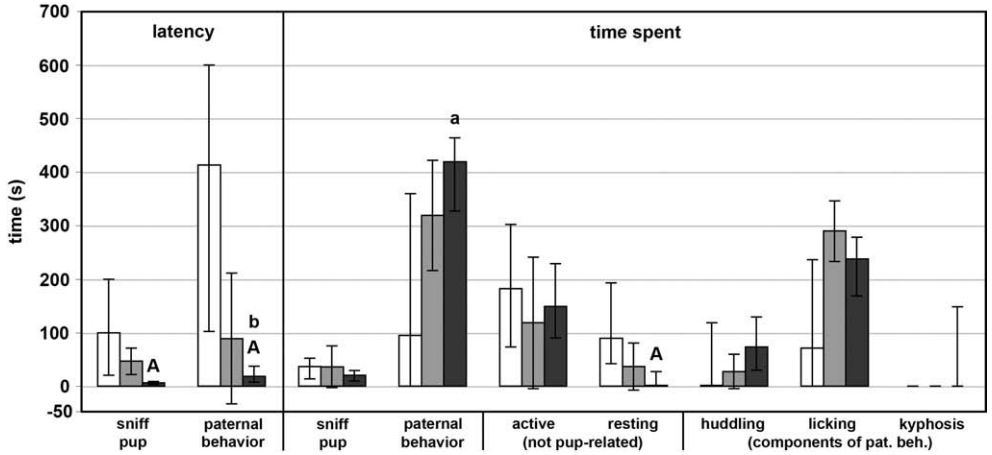

Fig. 1. Behavioral profile of California mice that were either virgin males (white bars), paired males (light gray bars) or new fathers (dark gray bars) during a 10-min paternal behavior test with a 1–3 day-old, unfamiliar, freely accessible pup. Data are medians; error bars delineate first and third quartiles. A = significantly different from virgin males, $P < 0.01$; a = significantly different from virgin males, $P < 0.05$; b = significantly different from paired males, $P < 0.05$.

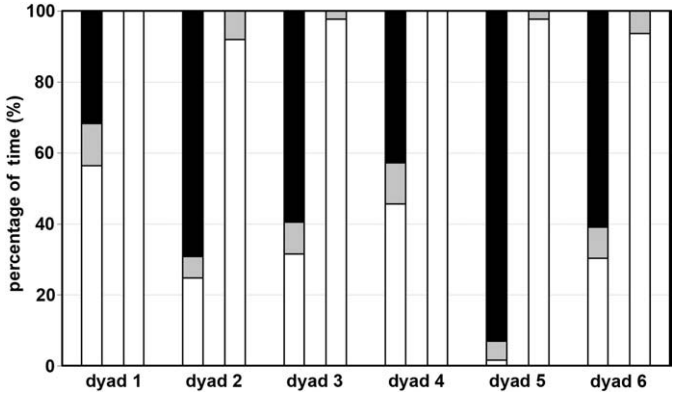

Fig. 2. Percentage of time, spent by 12 individual virgin male California mice housed in 6 dyads, performing non-paternal behavior consisting of general activity or resting (white), paternal behavior consisting of huddling, licking and kyphosis (black) and sniffing the pup (gray) in a 10-min paternal behavior test with a 1–3 day-old, unfamiliar, freely accessible pup.

Kruskal–Wallis tests revealed overall differences in latency to sniff the pup ($\chi^2 = 8.026$, $P = 0.018$), latency to initiate paternal behavior ($\chi^2 = 12.537$, $P = 0.002$), total time spent behaving paternally (combined huddling, licking and kyphosis, $\chi^2 = 6.727$, $P = 0.035$) and time spent resting without pup contact ($\chi^2 = 8.169$, $P = 0.017$), but not in time spent sniffing the pup ($\chi^2 = 3.623$, $P = 0.163$) or time spent being active without pup contact ($\chi^2 = 0.165$, $P = 0.921$). For the separate components of paternal behavior, the groups showed significant differences in time spent in kyphosis ($\chi^2 = 7.868$, $P = 0.020$), but not in time spent huddling with the pup ($\chi^2 = 4.019$, $P = 0.134$) or licking the pup ($\chi^2 = 5.107$, $P = 0.078$).

Post hoc pairwise comparisons showed that new fathers had a shorter latency to sniff the pup compared to virgin males ($Z = -2.805$, $P = 0.005$) but not compared to paired males ($Z = -1.848$, $P = 0.065$) and a shorter latency to initiate paternal behavior compared to both virgin males ($Z = -3.487$, $P = 0.001$) and paired males ($Z = -2.294$, $P = 0.022$). New fathers spent more time interacting paternally with the pup compared to virgin males ($Z = -2.552$, $P = 0.011$) but not paired males ($Z = -1.302$, $P = 0.193$). New fathers also spent less time resting compared to virgin males ($Z = -2.826$, $P = 0.005$) but not to paired males ($Z = -1.693$, $P = 0.091$). None of the behaviors differed significantly between virgin males and paired males.

The proportion of virgin males displaying any paternal behavior (6/12) was somewhat lower than the proportion of paired males (10/12, Fisher's exact test: $P = 0.193$) and significantly lower than the proportion of new fathers (10/10, Fisher's exact test: $P = 0.015$). An additional effect was found when the virgin male group was examined more closely: in each cage containing two virgin males, one virgin male behaved paternally whereas its cagemate did not (Fig. 2). The virgin male dyads comprised either littermates (3 out of 6 cages) or unrelated but age-matched cagemates, and paternally and non-paternally behaving virgin males did not differ in body weight, order of testing, or the sex of their littermate(s). The six spontaneously paternal virgin males did not differ from new fathers in latency to sniff the pup ($Z = -1.305$, $P = 0.192$) or time spent performing paternal behavior ($Z = -0.490$, $P = 0.624$); however, spontaneously paternal virgin males did spend more time sniffing the pup than new fathers ($Z = -2.820$, $P = 0.005$) and tended to initiate paternal behavior later in the test than new fathers ($Z = -1.952$, $P = 0.051$). There were no significant differences in any behavioral parameter between paired males with female mates that gave birth after the test and paired males with female mates that were never observed with a litter after the test ($Z \leq -1.613$, $P \geq 0.107$).

Ball test: behavior

Kruskal–Wallis tests revealed that virgin males, paired males and new fathers that were tested with an empty wire mesh ball showed no overall significant differences in behavior (Fig. 3). In contrast, virgin males, paired males and new fathers that were tested with a wire mesh ball containing an unfamiliar pup differed significantly in latency to touch the ball ($\chi^2 = 7.574$, $P = 0.023$), total time spent sniffing ($\chi^2 = 9.674$, $P = 0.008$) and touching ($\chi^2 = 6.504$, $P = 0.039$) the ball, and total time spent resting without touching the ball ($\chi^2 = 6.191$, $P = 0.045$).

Post hoc pairwise comparisons revealed that virgin males had a significantly longer latency to touch the pup-containing ball compared to both new fathers ($Z = 2.558$, $P = 0.011$) and paired males ($Z = -2.026$, $P = 0.043$), spent less time sniffing the pup-containing ball compared to both new fathers ($Z = -2.205$, $P = 0.028$) and paired males ($Z = -2.987$, $P = 0.003$), spent less time touching the pup-containing ball compared to new fathers ($Z = -2.370$, $P = 0.018$) but

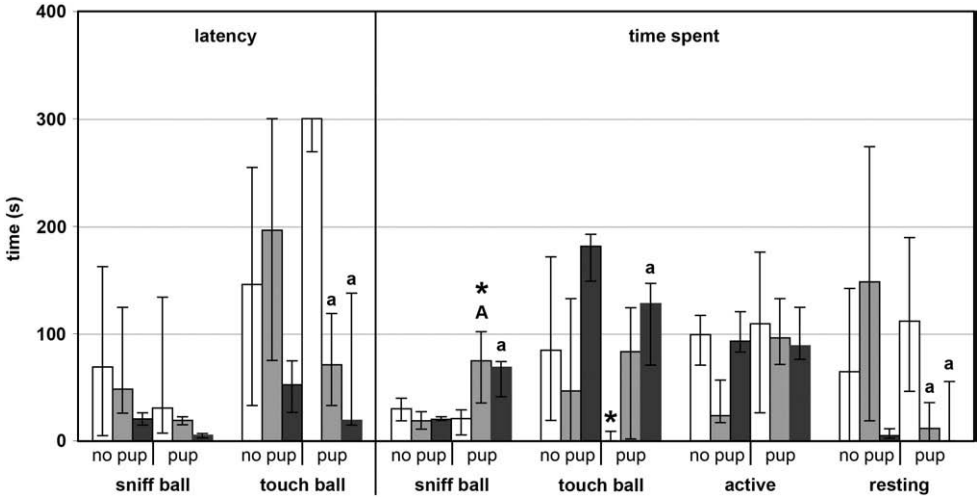

Fig. 3. Behavioral profile of California mice that were either virgin males (white bars), paired males (light gray bars) or new fathers (dark gray bars) during a 5-min test with a 1–3 day-old, unfamiliar pup contained in a wire mesh ball ("pup"), or an empty ball ("no pup"). Data are medians; error bars delineate first and third quartiles. A = significantly different from virgin males under corresponding "pup" or "no pup" condition, $P < 0.01$; a = significantly different from virgin males under corresponding "pup" or "no pup" condition, $P < 0.05$; * = significantly different from corresponding group under "no pup" condition, $P < 0.05$.

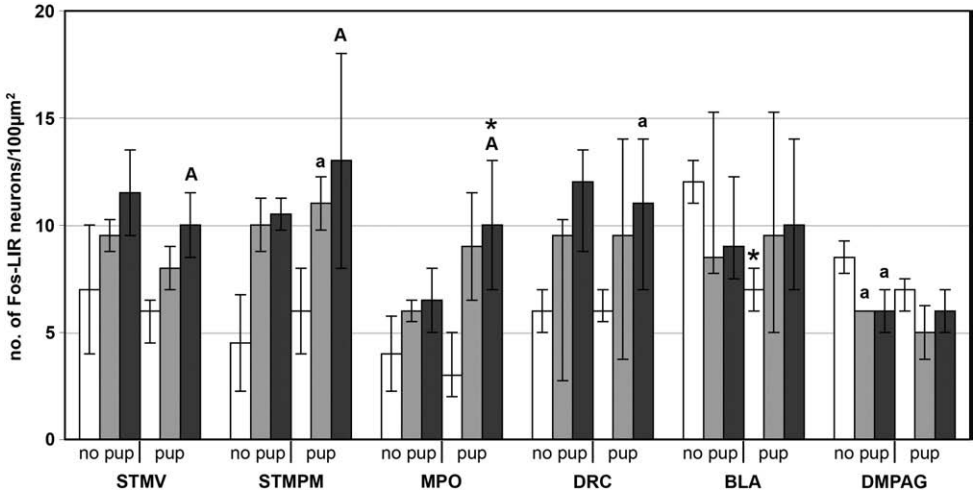

Fig. 4. Number of Fos-positive neurons per 100 μm^2 in the medioventral (STMV) and medial posteromedial (STMPM) bed nucleus of the stria terminalis, the medial preoptic nucleus (MPO), the caudal dorsal raphe nucleus (DRC), the basolateral amygdaloid nucleus (BLA) and the dorsomedial periaqueductal gray (DMPAG) of California mice that were either virgin males (white bars), paired males (light gray bars) or new fathers (dark gray bars) in response to a 5-min test with a 1–3 day-old, unfamiliar pup contained in a wire mesh ball ("pup"), or an empty ball ("no pup"). Data are medians; error bars delineate first and third quartiles. A = significantly different from virgin males under corresponding "pup" or "no pup" condition, $P<0.01$; a = significantly different from virgin males under corresponding "pup" or "no pup" condition, $P<0.05$; * = significantly different from corresponding group under "no pup" condition, $P<0.05$.

not paired males ($Z=-1.878$, $P=0.060$) and spent significantly more time resting compared to both new fathers ($Z=-2.191$, $P=0.029$) and paired males ($Z=-1.990$, $P=0.047$).

Mann–Whitney tests comparing the response to a pup-containing ball versus an empty ball within each experimental group (virgin males, paired males and new fathers) revealed that virgin males spent less time touching the pup-containing ball than the empty ball ($Z=-1.994$, $P=0.046$). Paired males spent more time sniffing the pup-containing ball than the empty ball ($Z=-2.208$, $P=0.027$), with new fathers showing a trend in the same direction ($Z=-1.890$, $P=0.059$).

In the paternal behavior test, virgin males could be divided into individuals displaying spontaneous paternal behavior ($n=6$) and individuals displaying no paternal behavior at all ($n=6$), as described above. In the ball test, however, no significant difference could be detected between the two types of virgin males in any of the behavioral parameters.

Ball test: Fos-like immunoreactivity

Kruskal–Wallis tests revealed overall significant differences among virgin males, paired males and new fathers in Fos-LIR in response to a pup-containing ball in the STMV ($\chi^2=7.802$, $P=0.020$), STMPM ($\chi^2=10.686$, $P=0.005$), MPO ($\chi^2=9.184$, $P=0.010$) and DRC ($\chi^2=7.241$, $P=0.027$), and in Fos-LIR in response to an empty ball in the DMPAG ($\chi^2=6.741$, $P=0.034$) and DRC ($\chi^2=6.102$, $P=0.047$), as shown in Fig. 4. No overall significant differences were found among groups or between test conditions in any of the other brain areas quantified (Table 1).

Post hoc pairwise comparisons revealed that the ball containing a pup caused increased Fos-LIR in fathers compared to virgin males in the STMV ($Z=-2.737$, $P=0.006$), STMPM ($Z=-3.109$, $P=0.002$), MPO ($Z=-2.994$, $P=0.003$) and the DRC ($Z=-2.554$, $P=0.011$). In addition, the pup stimulus increased Fos-LIR in paired males compared to virgin males in the STMPM ($Z=-2.395$, $P=0.017$).

Post hoc pairwise comparisons showed that Fos-LIR in response to an empty ball was significantly increased in the DMPAG of virgin males compared to both new fathers ($Z=-2.106$, $P=0.035$) and paired males ($Z=-2.302$, $P=0.021$).

Mann–Whitney tests revealed that new fathers had increased Fos-LIR in the MPO following exposure to a pup-containing ball compared to an empty ball ($Z=-2.485$, $P=0.013$), and virgin males had decreased Fos-LIR in the BLA following exposure to a pup-containing ball compared to exposure to an empty ball ($Z=-2.384$, $P=0.017$). No other significant within-group differences in Fos-LIR were found between the two test conditions.

Double-labeling of Fos with oxytocin and vasopressin

Immunohistochemical staining of oxytocin and vasopressin resulted in a transparent but distinct brown labeling of cell bodies and fibers in distinct areas of the hypothalamus. Although the distribution of oxytocin and vasopressin overlapped in some areas, such as the supraoptic nucleus and the lateral paraventricular nucleus of the hypothalamus, distribution of the two neuropeptides differed

Table 1
Number of Fos-positive neurons per 100 μm^2 in all quantified brain areas besides the areas illustrated in Fig. 4 (see text for abbreviations) of male California mice, comparing virgin males, paired males and new fathers in response to a 5-min test with an empty wire mesh ball ("no pup", $n=4$ per group) or a ball containing a 1–3 day-old, unfamiliar pup ("pup", $n=7$–8 per group).

Fos-LIR	Virgin males		Paired males		New fathers	
	No pup (4)	Pup (7)	No pup (4)	Pup (7)	No pup (4)	Pup (7)
AcbC	28.00	12.00	28.50	26.50	13.00	21.00
	22.0–36.8	9.5–30.5	26.8–31.0	17.0–30.0	8.5–18.5	19.0–32.5
AcbSh	36.00	27.00	31.50	39.00	20.50	29.00
	27.0–46.3	17.5–34.0	23.5–41.0	26.0–43.5	17.3–26.8	21.0–35.0
LSV	25.00	27.00	27.00	32.00	23.50	24.00
	22.3–28.3	21.0–26.5	22.8–34.5	16.5–33.0	21.5–28.0	19.5–34.5
STLD	33.00	24.00	25.00	32.00	34.00	24.00
	28.8–37.0	21.0–26.5	21.5–30.0	25.5–38.3	28.3–37.8	22.0–26.5
AHC	24.00	23.00	23.00	25.00	20.50	22.50
	17.3–32.8	13.5–32.0	21.8–23.8	20.5–31.5	18.8–22.8	20.5–27.5
MePD	11.50	9.00	9.50	11.00	14.00	14.00
	9.8–12.5	7.5–12.5	7.5–10.3	8.8–13.0	10.8–17.5	11.5–15.0
CeL	23.00	18.00	18.00	19.50	23.50	20.00
	19.8–26.0	4.5–23.5	14.3–21.8	16.3–21.8	21.3–24.3	19.0–25.5
VMH	5.00	7.00	4.50	9.50	6.00	12.00
	3.8–7.3	4.0–9.5	3.8–6.0	5.8–12.0	4.8–8.3	10.3–13.0
SPF	20.50	17.00	19.50	17.00	22.50	24.00
	16.8–24.0	15.0–19.5	15.8–24.5	14.5–19.0	16.5–28.0	13.5–25.5
VLPAG	11.50	11.00	11.50	11.00	9.00	14.00
	11.0–12.3	9.5–12.0	7.8–15.0	9.3–16.0	7.8–10.8	9.5–14.5

Data are medians with first and third quartiles.

markedly in other locations, such as the STMV (oxytocin), anterior paraventricular nucleus (oxytocin) and STMPM (vasopressin). It should be noted that no clear distinction could be made between parvicellular and magnocellular neurons for either neuropeptide; therefore the PaAP and PaLM are henceforth referred to as the PaA and PaL, respectively. Blue-black Fos-LIR nuclei could readily be discerned in cell bodies positively stained for oxytocin or vasopressin (Fig. 5). Table 2 lists the number of neuropeptidergic neurons and the number of double-labeled neurons in each quantified area.

Kruskal–Wallis tests showed that there were no overall significant differences among virgin males, paired males and new fathers tested with a pup-containing ball or an empty ball in number of oxytocin neurons in the STMV (pup: $\chi^2=1.771$, $P=0.413$; no pup: $\chi^2=0.030$, $P=0.985$), PaA (pup: $\chi^2=1.685$, $P=0.431$; no pup: $\chi^2=0.346$, $P=0.841$) or PaL (pup: $\chi^2=1.220$, $P=0.543$; no pup: $\chi^2=0.618$, $P=0.734$). Similarly, there were no overall significant differences among virgin males, paired males and new fathers tested with a pup-containing ball or an empty ball in number of vasopressin neurons in the STMPM (pup: $\chi^2=2.559$, $P=0.278$; no pup: $\chi^2=0.473$, $P=0.789$) or PaL (pup: $\chi^2=1.933$, $P=0.380$; no pup: $\chi^2=0.203$, $P=0.903$). In addition, no overall significant differences were found among virgin males, paired males and new fathers tested with a pup-containing ball or an empty ball in the number of Fos + neuropeptide double-labeled neurons in the STMV-OT (pup: $\chi^2=0.772$, $P=0.680$; no pup: $\chi^2=0.688$, $P=0.709$), PaA-OT (pup: $\chi^2=3.432$, $P=0.180$; no pup: $\chi^2=1.307$, $P=0.520$), PaL-OT (pup: $\chi^2=0.391$, $P=0.822$; no pup: $\chi^2=0.070$, $P=0.966$), STMPM-VP (pup: $\chi^2=1.256$, $P=0.534$; no pup: $\chi^2=311$, $P=0.856$) or PaL-VP (pup: $\chi^2=1.933$, $P=0.380$; no pup: $\chi^2=203$, $P=0.903$).

Ball test: correlations between Fos-LIR and behavior

Table 3 lists all brain areas in which a significant correlation was found between time spent sniffing or touching the empty or pup-

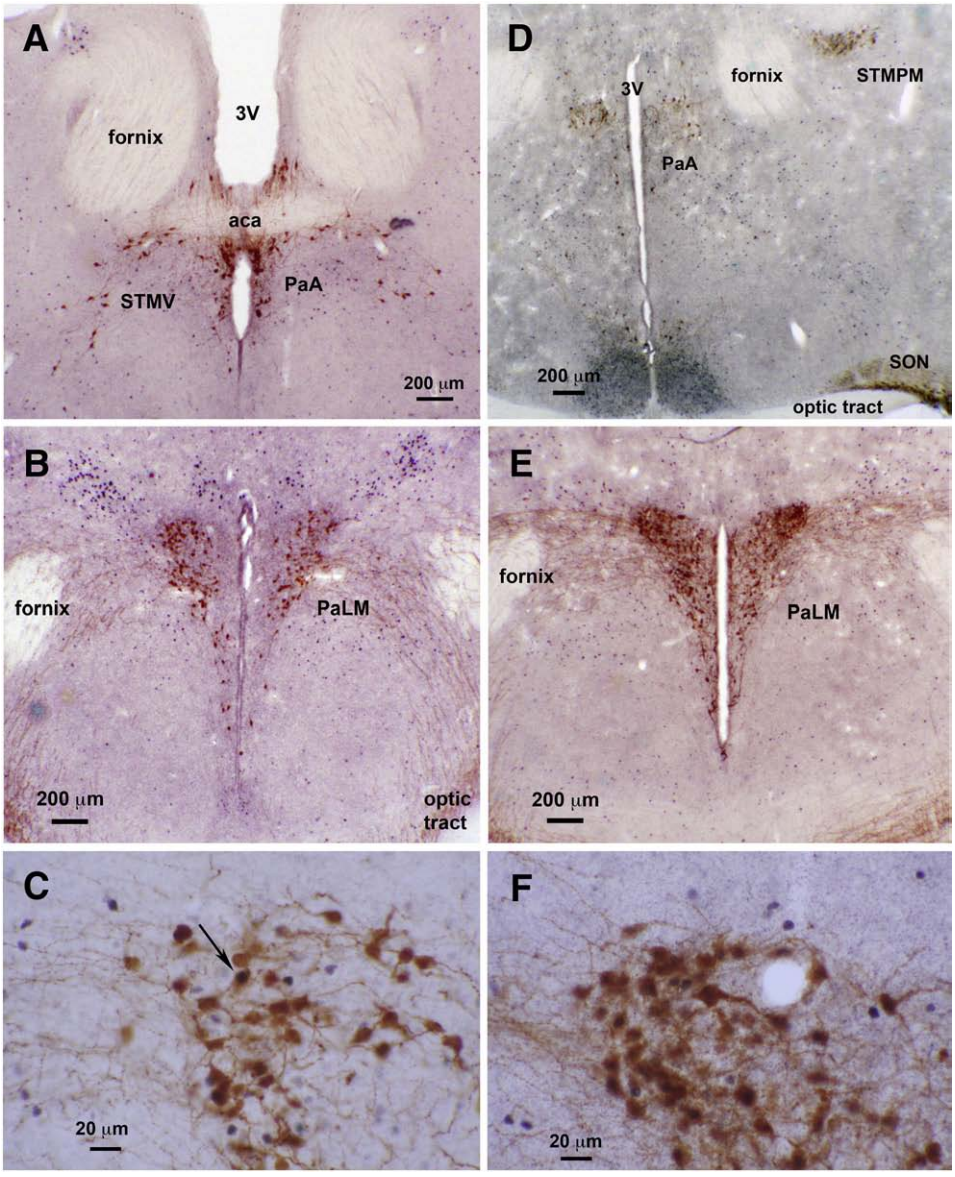

Fig. 5. Representative photomicrographs of double-labeling of Fos and oxytocin (A–C) and Fos and vasopressin (D–F) in the hypothalamus of male California mice. Fos is stained blue-black (nuclear staining); oxytocin and vasopressin are stained reddish brown (cytoplasmic staining). A: equivalent of mouse Bregma −0.10 mm; D: equivalent of mouse Bregma −0.34 mm; B, C, E and F: equivalent of mouse Bregma −0.70/−0.82 mm. The black arrow points to one of the few double-labeled cells in C; in F the majority of the neurons is double-labeled. Ace: anterior commissure; 3V: third ventricle; STMV: ventral medial division of the bed nucleus of the stria terminalis; PaA: anterior hypothalamic paraventricular nucleus; PaL: lateral hypothalamic paraventricular nucleus; STMPM: medial posteromedial division of the bed nucleus of the stria terminalis; SON: supraoptic nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Total number of oxytocinergic (OT) and vasopressinergic (VP) neurons, and number of neuropeptidergic neurons double-labeled with Fos, in five different brain areas (STMV-OT and STMPM-VP: 200 μm^2; PaA-OT, PaL-OT and PaL-VP: 100 μm^2) of male California mice, comparing virgin males, paired males and new fathers in response to a 5-min test with an empty wire mesh ball ("no pup", $n=4$ per group) or a ball containing a 1–3 day-old, unfamiliar pup ("pup", $n=7$–8 per group).

OT/VP neurons	Virgin males		Paired males		New fathers	
	No pup (4)	Pup (7)	No pup (4)	Pup (7)	No pup (4)	Pup (7)
STMV-OT	6.50	7.00	6.00	9.00	5.50	10.00
	5.5–7.5	5.0–10.0	4.0–8.5	8.5–12.5	4.0–7.0	8.0–11.5
+ Fos-LIR	0.00	0.00	0.50	0.00	0.00	1.00
	0.0–0.3	0.0–0.8	0.0–1.0	0.0–0.8	0.0–0.3	0.0–1.5
PaA-OT	24.00	13.00	27.00	20.00	19.50	22.00
	19.8–26.8	11.0–22.5	16.8–34.3	14.5–24.5	17.8–24.0	19.5–25.5
+ Fos-LIR	2.00	4.00	0.00	1.00	3.00	1.00
	1.5–2.3	2.0–5.5	0.0–1.8	0.5–1.0	2.3–3.3	0.0–4.5
PaL-OT	20.00	18.00	24.00	17.50	17.00	19.50
	17.5–22.8	13.0–25.0	21.0–26.0	10.5–20.8	14.0–21.8	16.3–26.5
+ Fos-LIR	2.50	1.00	2.00	0.50	2.50	0.00
	0.8–4.0	0.0–1.5	1.8–2.5	0.0–1.0	0.0–5.3	0.0–0.8
STMPM-VP	22.00	23.00	30.50	20.00	25.00	17.00
	16.8–29.0	15.5–29.5	23.3–39.0	17.8–23.0	20.3–29.3	17.0–20.0
+ Fos-LIR	1.50	2.00	2.50	0.00	1.50	2.00
	0.8–2.3	0.5–2.0	0.8–5.0	0.0–3.5	0.0–4.8	1.0–4.0
PaL-VP	34.00	29.00	26.50	33.00	32.50	25.00
	26.8–41.0	24.0–41.0	26.0–33.0	28.0–41.0	26.5–28.3	25.0–34.0
+ Fos-LIR	6.00	4.00	5.00	3.00	4.00	7.00
	4.0–7.5	0.5–7.5	3.8–6.3	0.0–3.5	1.5–10.3	3.0–8.0

Data are medians with first and third quartiles.

containing ball and number of Fos-LIR neurons per 100 μm^2 for all three groups of males combined. In the STMV, Fos-LIR was positively correlated with time spent touching the pup-containing ball ($\rho=0.484$, $P=0.023$, $n=22$), but not with the time spent touching the empty ball ($\rho=-0.025$, $P=0.939$, $n=12$), and these correlations differed significantly from each other ($\Delta Z=2.215$, $P=0.027$). Similarly, in the STMPM, Fos-LIR was positively correlated with time spent sniffing ($\rho=0.603$, $P=0.003$, $n=22$) and touching ($\rho=0.574$, $P=0.005$, $n=22$) the pup-containing ball, and negatively, but not significantly, correlated with time spent sniffing ($\rho=-0.313$, $P=0.322$, $n=12$) and touching ($\rho=-0.215$, $P=0.503$, $n=12$) the empty ball; these correlations differed significantly from each other (sniffing: $\Delta Z=4.003$, $P<0.001$; touching: $\Delta Z=3.450$, $P=0.001$).

Table 3
Spearman correlations in male California mice between number of Fos-positive neurons per 100 μm^2 in several brain areas and the time spent sniffing and touching an empty wire mesh ball ("no pup", $n=11$–12) or a ball containing a 1–3 day-old, unfamiliar pup ("pup", $n=20$–22) in a 5-min test.

Fos-LIR	Condition	Time spent			
		Sniffing ball		Touching ball	
STMV	No pup	−0.257		−0.025	
	Pup	0.335		0.484	*
	ΔZ	2.298	#	2.215	#
STMV (pup)	Virgin males	0.309		0.103	
	Paired males	0.361		0.349	
	New fathers	0.198		0.775	*
STMPM	No pup	−0.313		−0.215	
	Pup	0.603	**	0.574	**
	ΔZ	4.003	##	3.450	##
STMPM (Pup)	Virgin males	0.093		0.424	
	Paired males	−0.030		0.479	
	New fathers	0.855	*	−0.070	
AHC	No pup	−0.246		−0.766	**
	Pup	0.257		0.014	
	ΔZ	1.874		3.192	##
VLPAG	No pup	−0.483		−0.703	
	Pup	−0.030		0.346	
	ΔZ	1.678		4.232	##

In case of a significant correlation in the "pup" condition that was significantly different from the correlation in the "no pup" condition ($\Delta Z>\pm 1.996$), correlations were subsequently analyzed for virgin males ($n=7$), paired males ($n=8$) and new fathers ($n=7$) separately.
Significant correlations: $^{**}P<0.01$, $^{*}P<0.05$; significant differences between correlations under "no pup" versus "pup" conditions: $^{\#\#}P<0.01$, $^{\#}P<0.05$.

As described in the Materials and methods section, significant correlations in the "pup" condition that differed significantly from the corresponding correlation in the "no pup" condition were further analyzed for virgin males, paired males and new fathers separately. In the STMPM, Fos-LIR was correlated positively and significantly with time spent sniffing the pup-containing ball in new fathers ($\rho=0.855$, $P=0.014$, $n=7$), but not in paired males ($\rho=-0.030$, $P=0.954$, $n=8$) or virgin males ($\rho=0.093$, $P=0.842$, $n=7$), and there were no significant correlations with time spent touching the pup-containing ball in new fathers ($\rho=-0.070$, $P=0.877$, $n=7$), paired males ($\rho=0.479$, $P=0.230$, $n=8$) or virgin males ($\rho=0.424$, $P=0.344$, $n=7$). In the STMV, Fos-LIR was correlated positively and significantly with time spent touching the pup-containing ball in new fathers ($\rho=0.775$, $P=0.041$, $n=7$), but not in paired males ($\rho=0.349$, $P=0.396$, $n=8$) or virgin males ($\rho=0.103$, $P=0.826$, $n=7$).

For all three groups of males combined, Fos-LIR in the AHC and VLPAG showed significant negative correlations with time spent touching the empty ball (AHC: $\rho=-0.766$, $P=0.004$, $n=12$; VLPAG: $\rho=-0.703$, $P=0.011$, $n=12$), and non-significant positive correlations with time spent touching the pup-containing ball (AHC: $\rho=0.014$, $P=0.954$, $n=20$; VLPAG: $\rho=0.346$, $P=0.114$, $n=22$). These correlations differed significantly between the "pup" and "no pup" conditions (AHC: $\Delta Z=3.192$, $P=0.001$; VLPAG: $\Delta Z=4.232$, $P<0.001$).

Discussion

In the present study, we aimed (1) to confirm that male California mice undergo a transition from non-paternal behavior as a consequence of sexual and/or parental experience, (2) to identify brain regions that might play a role in this behavioral transition, and (3) to further elucidate the roles of intracerebral oxytocin and vasopressin in the onset of paternal behavior.

Transition to paternal behavior

Male California mice appeared to change their responses to unfamiliar neonatal pups following sexual and paternal experiences, with new fathers approaching stimulus pups more rapidly and spending more time in paternal interactions with the pups than virgin males, and paired males (whose mates had not yet given birth)

showing an intermediate behavioral profile. The differences between new fathers and virgin males were caused mainly by the proportion of males that behaved paternally in each group (new fathers: 10 out of 10; virgin males: 6 out of 12), rather than an overall inhibition of paternal behavior in virgin males.

Previous studies in California mice have reported similar results (Gubernick and Laskin, 1994; Gubernick and Nelson, 1989), although in those studies a smaller proportion of virgin males behaved paternally and a significant number of virgin males showed infanticidal behavior, which was not observed in the present study. These differences could be explained in part by the single housing of the virgin males in one study (Gubernick and Nelson, 1989), as compared to housing in male–male dyads in the present experiment: social isolation has been shown to increase depression-like behavior and alter oxytocin, vasopressin and CRF neurotransmission in male and female prairie voles, and to increase aggressive behavior towards both pups and intruders in female prairie voles (Grippo et al., 2007, 2008) and male house mice (Sigg et al., 1966). In the second study (Gubernick and Laskin, 1994), the virgin male California mice were either younger (35–65 days) and more often non-paternal (63 out of 81) or older (160 days) and more often infanticidal (38 out of 64) than the virgins in the present study, suggesting a possible age effect.

Interestingly, in the present study each cage housing two virgin males contained one highly paternal and one non-paternal male, suggesting that paternal behavior can be facilitated by non-reproductive cues. Dominance status seems the most likely candidate to account for the auspicious distribution of male behavior. In male house mice, subordinates exhibit significantly more paternal behavior and less infanticide than dominants (Huck et al., 1982; vom Saal and Howard, 1982). Moreover, subordination of males by their pregnant, often aggressive female mates has been suggested as a relevant factor in the onset of paternal behavior (Brown, 1993). These unexpected findings in our virgin male California mice will be further investigated in future experiments.

The paired males in the present study were used to evaluate a possible effect of copulation or cohabitation with a (pregnant) female on the onset of paternal behavior, which has been suggested previously (Brown, 1986; Soroker and Terkel, 1988; Elwood and Kennedy, 1991; Gubernick et al., 1994; Palanza and Parmigiani, 1991). Indeed, paired males showed an intermediate behavioral pattern, resembling virgin males in their long latencies to initiate paternal behavior, but otherwise not showing any significant difference from either new fathers or virgins. For half of the paired males, however, we could not confirm subsequently that their mates were pregnant at the time of testing. These females had been showing a pattern of weight gain similar to pregnant females until the time of testing, but pups were never found in the cage in the weeks after the test. It is possible that these females had indeed been pregnant, but that the highly stressful removal of a male from a pair bond (e.g., Bosch et al. (2009) in prairie voles) may have caused the females to reabsorb or abort the fetuses, or to commit infanticide immediately after parturition. Such a phenomenon has been indicated in other monogamous, biparental rodents such as prairie voles (Dewsbury, 1995; McGuire et al., 1992), Djungarian hamsters (Wynne-Edwards et al., 1987) and Mongolian gerbils (*Meriones unguiculatus*) (Norris, 1985). In California mice, absence of the father increases the interbirth interval (Cantoni and Brown, 1997) and fathers assist with the birth of pups (Lee and Brown, 2002b), further indicating that removal of the male prior to parturition may have affected reproductive success. Although the unconfirmed reproductive state of these paired males' female mates complicates the interpretation of data, there were no significant differences in the paternal behavior test between paired males whose mates were known to give birth and paired males whose mates were not.

The ball test was designed to characterize the effects of sexual and paternal experiences on Fos expression in response to visual, olfactory and auditory cues from an unfamiliar pup, while preventing differences in the performance of paternal behavior and concomitant differences in Fos-LIR. Virgin males showed a significantly longer latency to touch the pup-containing ball, and spent significantly less time sniffing and touching it, than new fathers and paired males, with the majority of virgin males (6 out of 8) not touching the pup-containing ball at all. In contrast, virgin males did not differ from paired males or new fathers in latency to sniff the pup-containing ball, suggesting decreased motivation to interact with the pup rather than increased fear of approaching the pup-containing ball.

Virgin males spent significantly less time touching a pup-containing ball than an empty ball, suggesting that the pup was an aversive stimulus for these males. In contrast, paired males spent significantly more time sniffing the pup-containing ball than the empty ball and new fathers showed a similar, but non-significant trend, suggesting that males with sexual and/or parental experience were attracted to the pup.

The three experimental groups showed no significant differences in behavioral responses towards an empty ball; however, group sizes were small ($n = 4$), and false negative results are possible.

Brain activation in response to distal pup cues

Paternal experience altered Fos-LIR in response to the ball test in several brain areas. In the medial preoptic nucleus (MPO), new fathers showed increased Fos-LIR in response to a pup-containing ball compared both to virgin males tested with a pup-containing ball and to new fathers tested with an empty ball, indicating that paternal experience specifically increases MPO activity induced by distal cues from pups. A role of the MPO in the maintenance of paternal behavior has been indicated in several previous studies: lesions of the medial preoptic area, which includes the MPO, disrupted paternal care in California mouse fathers (Lee and Brown, 2002a; Lee and Brown, 2007), and full paternal interaction with a pup increased Fos expression in the medial preoptic nucleus of prairie voles (Kirkpatrick et al., 1994). The present results provide evidence for an additional role of the MPO in the onset of paternal behavior, consistent with other findings showing changes in this area following the mate's parturition in male California mice, such as increased aromatase activity (Trainor et al., 2003) and disappearance of the sex difference in cell number in the medial preoptic area (Gubernick et al., 1993). In female rats it is thought that upon parturition, estradiol, prolactin and oxytocin act in the MPO to modulate its output, leading to inhibition of avoidance responses and facilitation of approach responses towards an unfamiliar pup (Numan, 2007). A similar mechanism, involving yet unknown cues, hormones and neurotransmitters, could operate in male California mice as well.

New fathers showed significantly more Fos-LIR In the medioventral and medial posteromedial bed nucleus of the stria terminalis (STMV and STMPM) and the caudal dorsal raphe nucleus (DRC) in response to a pup-containing ball than virgin males. A similar trend was apparent following exposure to an empty ball, however, and there were no differences in Fos-LIR between the pup and no pup conditions in these three areas in any of the groups. Several explanations for these results are possible. The STMPM and STMV are known to be highly sensitive to pheromonal cues from pups, as shown in male prairie voles and female rats (Cushing et al., 2003; Kalinichev et al., 2000; Kirkpatrick et al., 1994; Numan and Numan, 1995; Sheehan et al., 2000) and the STMPM is highly sensitive to pheromonal cues from estrous females, as shown in male rats (Hosokawa and Chiba, 2005; Kippin et al., 2003; Veening and Coolen, 1998). Until 2 h prior to the test (3 h prior to perfusion) all new fathers and paired males in our study could freely interact with their female mate and/or pups in their home cage. This may have resulted in increased neuronal activation compared to virgin males in these areas at the time of perfusion 3 h later. Interestingly, the STMPM and STMV have been shown to project

specifically to the central dorsal raphe nucleus, including the caudal part analyzed in the present study (Peyron et al., 1998), and the various home cage situations may have differentially activated the STMPM and STMV, which in turn could have altered stress-induced Fos expression in the DRC. Although the role of this connection is yet unknown, all three areas are known to modulate stress and anxiety states (Abrams et al., 2005; Choi et al., 2008; Hale et al., 2008; Lowry et al., 2000; Spencer et al., 2005), and the projection has been suggested as a sex steroid-sensitive pathway to alter responses to stressful stimuli (Walker et al., 2003). In female mammals, lactation is associated with reduced anxiety and fearfulness as well as blunted neural, hormonal, and behavioral responses to stress, effects which in turn are thought to facilitate the onset and maintenance of maternal care (Brunton et al., 2008; Lightman et al., 2001). The Fos-data in the present experiment might indicate that fatherhood is similarly associated with altered emotionality and stress-responsiveness in biparental mammals (Kinsley and Lambert, 2008; M. Chauke et al., unpublished data). It should be noted, however, that Fos-LIR in the STMPM and STMV was positively correlated with time spent sniffing and/or touching the pup-containing ball but not the empty ball, when virgin males, paired males and new fathers were analyzed together, and the same correlations were present within new fathers. These results indicate that investigation of the pup in the ball was at least partly responsible for Fos-LIR in the STMPM and STMV of new fathers, but not of paired males or virgin males, suggesting that paternal experience alters responsiveness to pup cues in the bed nucleus of the stria terminalis.

The empty ball stimulus caused increased Fos-LIR in the basolateral amygdala (BLA) compared to a pup-containing ball in virgin males. The BLA is activated by unpredictable stress, novel environments and approach/avoidance conflicts (Hale et al., 2006; Reznikov et al., 2008). Since virgin males spent more time interacting with the empty ball compared to the pup-containing ball, Fos-LIR in the BLA might reflect interaction with rather than anxiety toward, the empty ball stimuli in this situation. Furthermore, the empty ball stimulus caused increased Fos-LIR in the dorsomedial periaqueductal gray (DMPAG) of virgin males compared to both paired males and new fathers. In general, Fos-LIR in the DMPAG is induced by a variety of aversive stimuli (Comoli et al., 2003; Kollack-Walker and Newman, 1995; Menard et al., 2004; Silveira et al., 2001; Zienowicz et al., 2007), suggesting that virgin males perceived the empty ball as more aversive than paired males and new fathers, despite the absence of significant differences in behavior towards the empty ball. In addition, the less time that males spent sniffing and touching the empty ball, the more Fos-LIR they exhibited in the AHC and VLPAG, indicating an avoidance-related activation of these areas, consistent with their role in defensive behavior (Canteras et al., 1997; Dielenberg et al., 2001; Keay and Bandler, 2001). Interestingly, the presence of a pup in the ball appears to “overrule” these correlations.

Vasopressin and oxytocin

The neuropeptides vasopressin and oxytocin are likely candidates to mediate the facilitation of paternal behavior in new fathers. We investigated two possible mechanisms by which these neuropeptides could influence paternal behavior: changes in the density of oxytocinergic or vasopressinergic neurons in five distinct brain areas, or altered activation of these neurons in response to 5-min exposure to visual, auditory and olfactory cues from an unfamiliar pup.

Contrary to expectations, we found no evidence for an effect of paternal experience and/or exposure to distal pup cues on activation of oxytocin- or vasopressin-containing neurons. It is possible that the short exposure time and the absence of direct physical interaction with the pup rendered the stimulus too weak to consistently recruit oxytocin and vasopressin systems. In addition, no differences were found in the density of neuropeptidergic neurons. Bester-Meredith et al. (1999) found a positive correlation between vasopressin immunoreactivity in the STMPM and the level of paternal care, although it should be noted that this correlation required combined data from in-fostered and cross-fostered California mice and white-footed mice. Alternatively, since we used a relatively low dose of neuropeptide antibody in our immunohistochemical stainings in order to optimize quantification of double-labeling with Fos, our stainings may not have been optimal for the analysis of neuropeptide levels.

A likely possibility is that the interaction between oxytocin, vasopressin and paternal experience is dependent on neuropeptide receptor expression or binding, rather than neuropeptide levels or release. Indeed, paternal experience has been associated with an increase in vasopressin 1a receptor expression in the hypothalamic paraventricular nucleus and supraoptic nucleus of paternal prairie voles, but not in non-paternal montane voles (*Microtus montanus*) (Wang et al., 2000), and paternal experience coincides with an increased level of oxytocin receptor binding in the accessory olfactory bulb, septum, bed nucleus of the stria terminalis and amygdala of facultatively paternal meadow voles (*M. pennsylvanicus*) (Parker and Lee, 2001). In addition, male meadow voles that behaved paternally towards a pup had higher levels of vasopressin receptor binding in the accessory olfactory bulb and lower binding in the septum than non-paternal males (Parker et al., 2001).

Conclusions

In conclusion, the present experiment confirms that experience with their first litter stimulates male California mice to approach an unfamiliar newborn pup, and increases the likelihood of paternal behavior towards the pup. In addition, both sexual and paternal experiences increase the time spent investigating an unreachable unfamiliar pup. These results indicate that male California mice undergo a transition in their behavioral responses to pups, as a consequence of reproductive experience. However, the relatively high proportion of spontaneously paternal virgin males indicates that non-reproductive cues, such as intrasexual dominance status, can facilitate paternal behavior as well. New fathers exhibited selective activation of the medial preoptic nucleus in response to distal cues from an unfamiliar pup, suggesting a role for the MPO in the transition to paternal behavior. Despite suggestions in the literature, we found no evidence that fatherhood altered numbers or pup-induced activation of oxytocinergic or vasopressinergic neurons. Finally, fatherhood appears to alter neurotransmission in the BNST and DRC, regions implicated in emotionality and stress-responsiveness. Together, these results implicate the MPO in the onset of paternal behavior, and point to the BNST and DRC as brain areas where paternal experience, stress and anxiety might interact.

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