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[a] Department of Biology, University of California, Riverside, CA, USA

[b] Neuroscience Graduate Program, University of California, Riverside, CA, USA

[c] Evolution, Ecology and Organismal Biology Graduate Program, University of California, Riverside, CA, USA

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BRIEF PUP EXPOSURE INDUCES Fos EXPRESSION IN THE LATERAL HABENULA AND SEROTONERGIC CAUDAL DORSAL RAPHE NUCLEUS OF PATERNALLY EXPERIENCED MALE CALIFORNIA MICE (*PEROMYSCUS CALIFORNICUS*)

T. R. DE JONG,[a*] K. R. MEASOR,[a,b] M. CHAUKE,[a,b] B. N. HARRIS[a,c] AND W. SALTZMAN[a,b,c]

AQ:1 [a]*Department of Biology, University of California, Riverside, CA, USA*

[b]*Neuroscience Graduate Program, University of California, Riverside, CA, USA*

[c]*Evolution, Ecology and Organismal Biology Graduate Program, University of California, Riverside, CA, USA*

Abstract—Fathers play a substantial role in infant care in a small but significant number of mammalian species, including humans. However, the neural circuitry controlling paternal behavior is much less understood than its female counterpart. In order to characterize brain areas activated by paternal care, male California mice were separated from their female mate and litter for 3 h and then exposed to a pup or a control object (a glass pebble with the approximate size and oblong shape of a newborn pup) for 10 min. All males receiving a pup showed a strong paternal response towards it, whereas males receiving a pebble interacted with it only occasionally. Despite the clear behavioral differences, exposure to a pup did not increase Fos-like immunoreactiv(e)(ity) (Fos-LIR) compared to a pebble in brain areas previously found to be associated with parental care, including the medial preoptic nucleus and medial bed nucleus of the stria terminalis. Pup exposure did, however, significantly increase Fos-LIR in the lateral habenula (LHb) and in predominantly serotonergic neurons in the caudal dorsal raphe nucleus (DRC), as compared to pebble exposure. Both the LHb and DRC are known to be involved in the behavioral responses to strong emotional stimuli; therefore, these areas might play a role in controlling parental behavior in male California mice.

Key words: paternal care, Fos, lateral habenula, caudal dorsal raphe, 5-HT.

In a majority of mammalian species, mothers play the largest or only role in providing food, warmth and protection to offspring. However, fathers are significantly involved in parental care in approximately 5–6% of mammalian species, including humans (Kleiman and Malcolm, 1981). Despite the strong impact of both adequate and inadequate human paternal care on child development, mammalian paternal care is much less studied and understood than its maternal counterpart (Sarkadi et al., 2008; Kentner et al., 2009).

The California mouse (*Peromyscus californicus*), a rodent species that is highly monogamous and biparental, both in the wild (Ribble and Salvioni, 1990; Ribble, 1991; Gubernick and Teferi, 2000) and in the laboratory (Dudley, 1974; Gubernick and Alberts, 1987), is often used to study causes and consequences of paternal care. Similar to most female mammals, sexually and parentally experienced male California mice are more likely to respond paternally to a stimulus pup (grooming, huddling and even performing the kyphotic nursing posture) than virgin males (Gubernick and Nelson, 1989; Gubernick et al., 1994; de Jong et al., 2009). In females, this phenomenon is thought to be caused by a shift in perception of distal sensory cues (mostly olfactory and auditory) from pups, from threatening and aversive to non-threatening and rewarding (Numan, 2007; Numan and Stolzenberg, 2008). It is likely that a similar shift occurs in males. In females, however, peripartum release of estrogen, progesterone, oxytocin, and prolactin plays an important and well-described role in the onset of maternal behavior (Grattan, 2001; Mann and Bridges, 2001; Russell et al., 2001; Numan and Insel, 2003; Numan, 2006; Bosch and Neumann, 2008; Brunton et al., 2008), whereas these hormones seem to play a smaller and less understood role in the onset of paternal care (Wynne-Edwards and Timonin, 2007; Kentner et al., 2009).

Maternal responses to pups are controlled by an extensive neural network, of which many components have been identified using immunohistochemical staining of the peptide Fos, a widely used marker for neuronal activation (Hoffman and Lyo, 2002). In maternal females, full or partial access to a pup consistently activates the medial preoptic area and the ventromedial bed nucleus of the stria terminalis, and (less consistently) the nucleus accumbens, lateral septum, central and medial amygdala, lateral habenula and ventrolateral periaqueductal grey (Calamandrei and Keverne, 1994; Fleming et al., 1994; Fleming and Walsh, 1994; Numan and Numan, 1994; Walsh et al., 1996; Lonstein et al., 1998, 2000; Kalinichev et al., 2000;

*Corresponding author. Tel: +1-951-827-5929.
E-mail address: Trynked@ucr.edu (T. R. de Jong).

Abbreviations: AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; BLA, basolateral amygdaloid nucleus; CeL, lateral division of the central amygdaloid nucleus; DRC, caudal dorsal raphe nucleus; DRI, interfascicular dorsal raphe nucleus; Fos-LIR, Fos-like immunoreactiv(e)(ity); LC, locus coeruleus; LHb, lateral habenula; LSV, ventral lateral septal nucleus; MePD, posterodorsal medial amygdaloid nucleus; MPN, medial preoptic nucleus; MPO, medial preoptic nucleus; PaL, lateral paraventricular hypothalamic nucleus; STLD, dorsal lateral division of the bed nucleus of the stria terminalis; STMPM, medial posteromedial division of the bed nucleus of the stria terminalis; STMV, ventromedial division of the bed nucleus of the stria terminalis; VLPAG, ventrolateral periaqueductal gray; VLPO, ventrolateral preoptic area.

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Lonstein and De Vries, 2000; Sheehan et al., 2000; Stack and Numan, 2000).

To date, only a few researchers have attempted to study pup-induced Fos expression in male rodents. In virgin male prairie voles (*Microtus ochrogaster*), Fos expression was increased following 3 h of paternal interaction with pups (compared with exposure to a piece of chocolate) in the lateral septum, medial preoptic area, medial bed nucleus of the stria terminalis, posterodorsal medial amygdala, nucleus reuniens, and paraventricular nucleus of the thalamus (Kirkpatrick et al., 1994). Previous work in our laboratory showed that distal pup cues increased Fos expression in the medial preoptic nucleus (MPN) of paternally experienced, but not virgin male California mice, and paternally experienced California mice showed increased Fos expression in the medial posteromedial and medioventral bed nucleus of the stria terminalis (STMV and STMPM) compared to virgin males, even after 3 h of isolation (de Jong et al., 2009). Interestingly, paternally experienced California mice also showed increased Fos expression in the caudal dorsal raphe nucleus (DRC) compared to virgin males. The dorsal raphe nucleus is a crucial component of the serotonin system and consists mainly of forebrain-projecting serotonergin neurons (Lowry, 2002; Michelsen et al., 2007). Because an intact serotonin system has recently been shown to be required for normal expression of maternal care in mice (Lerch-Haner et al., 2008), we hypothesized that paternal experience and/or pup exposure might activate the serotonin system as reflected by increased Fos expression within serotonergic neurons.

The present experiment was performed to characterize patterns of neural activation in paternally experienced adult male California mice in response to exposure to their own pup versus a control stimulus (a glass pebble with the approximate size and oblong shape of a newborn pup) or no stimulus at all. We were particularly interested in the brain areas involved with the initial responses to a pup compared to a control stimulus, rather than the brain areas responsible for ongoing paternal behavior. Therefore, we chose to expose the males to the stimulus for a brief (10-min) period. Males were isolated from their family 3 h prior to the test, to reduce Fos-like immunoreactivity (Fos-LIR) elicited by ongoing social behaviors. Control groups were isolated and tested with either a pebble or no stimulus. To assess the potential stressful effects of social isolation itself, additional control groups remained with their family until perfusion and were tested with either a pebble stimulus or no stimulus at all. Fos expression in response to the experimental manipulations was quantified in brain areas known to be involved in parental behavior, stress, reward, and behavioral arousal. In addition, Fos expression was quantified in serotonergic neurons in the dorsal raphe nucleus.

EXPERIMENTAL PROCEDURES

Animals

Male (n=35) and female (n=35) virgin California mice were housed in breeding pairs in polycarbonate home cages (L×W×H: 44×24×20 cm^3) containing wood chip bedding and cotton for nest building. Mice were maintained on a 14:10-h day/night cycle (lights on at 0500 h.), in an ambient temperature of approximately 23 °C and approximately 65% humidity. They had *ad libitum* access to chow (Purina 5001 Rodent Diet) and water.

All mice were descendants of mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). They were born and raised in our laboratory colony, weaned at 27–33 days of age (prior to the birth of the next litter of siblings to prevent non-experimental pup exposure) and housed in same-sex groups of 2–4 animals (littermates or unrelated, age-matched mice) until the start of the experiment. The male and female in each breeding pair were never siblings or first-cousins.

All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the University of California, Riverside, Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering. The University of California, Riverside is fully accredited by AAALAC.

Experimental design

Breeding pairs were weighed twice weekly to monitor health and pregnancies. When births were due, home cages were checked every day for pups. On postnatal day 1, 2, or 3, females and pups were removed from their home cage between 08.00 h and 09.30 h and either placed in a separate cage, leaving the male alone in the home cage ("isolated"), or immediately placed back with the male ("with family"). Isolated males were divided into three groups, receiving either no stimulus (n=7), a pebble (n=7), or a pup (n=8). Family-housed males were divided into two groups, receiving either no stimulus (n=6) or a pebble (n=7). There was no experimental group of males that remained with their family and received a pup stimulus, since the presence of an actively maternal female mate would likely have changed the males' paternal behavior and neuronal activation too much to make it a valid control group. All males were semi-randomly spread over the five experimental groups, with emphasis on the equal distribution of the following parameters: number of days between pairing and birth of their first litter, litter size, age of the pups on the test day, and age and body weight of the male on the test day.

The males (with or without their families) were left undisturbed in their home cages for exactly 3 h, and were then presented for 10 min with one of their own pups, a clean glass pebble (an oblong, flattened sphere, approximately 3×1.5×1 cm^3), or no stimulus. Males were videotaped during the stimulus test for later analysis of their behavioral responses.

One h after removal of the stimulus (4 h and 10 min after (sham-) isolation), males were carried in their home cages to a separate room, immediately deeply anesthetized with an overdose of pentobarbital (ca. 200–300 mg/kg ip) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were processed for immunohistochemistry as described below. AQ: 2

Behavioral analysis

Behavioral data on the videotapes were analyzed using J-Watcher (Blumstein and Daniel, 2007), with the following parameters scored: latency to sniff the stimulus, latency to touch the stimulus (huddling or licking the pup; licking, biting or nudging the pebble), total time spent sniffing the stimulus, total time spent touching the stimulus, and total time spent resting without stimulus contact.

Immunohistochemistry of Fos and serotonin

Immunohistochemistry was performed as described previously (de Jong et al., 2009). Briefly, brains were cut into 30 μm thick

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sections and collected in five series. All brains were immunohistochemically stained for both Fos and serotonin. One series from each individual brain was incubated overnight with rabbit-anti-c-Fos antibody (sc-253, 1:5.000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and stained with 3,3′-diaminobenzidine (DAB) and ammonium nickel sulfate, resulting in a blue-black nuclear staining. Brains were then incubated overnight with rabbit-anti-serotonin (cat. no. 20080, 1:25.000, ImmunoStar Inc., Hudson, WI, USA) and stained with DAB without ammonium nickel sulfate, resulting in a brown cytoplasmic staining that left the black nuclear staining visible for quantification of Fos and serotonin double-labeling. All brain sections were mounted on gelatin/chrome-alum coated glass slides, dehydrated and cleared in ethanol and xylene, embedded in Entellan (EMS, Hatfield, PA, USA) and coverslipped.

Quantification of immunoreactivity

Since there is no specific brain atlas for *Peromyscus californicus*, The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 2008) was used, based on the overall neuroanatomical similarities between the species (de Jong et al., 2009). 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 *Peromyscus californicus*. Selected brain areas either showed a clear peak in Fos-LIR in one particular section, in which case equivalent sections were used for each individual mouse, or showed a homogenous spread of Fos-LIR over multiple sections, in which case neuroanatomical landmarks (fiber pathways, nerves, ventricles) were used to select the section approximating the same location in each individual mouse.

For the quantification of Fos-LIR alone, brain areas were selected based on (1) their known function in parental behavior and/or reproduction: the medial preoptic nucleus (MPO, mouse Bregma level −0.10), ventromedial division of the bed nucleus of the stria terminalis (STMV, mouse Bregma level −0.10), medial posteromedial division of the bed nucleus of the stria terminalis (STMPM, mouse Bregma level −0.22), basolateral amygdaloid nucleus (BLA, mouse Bregma level –0.82), posterodorsal medial amygdaloid nucleus (MePD, mouse Bregma level −1.46), lateral habenula (LHb, mouse Bregma level −1.82) ventrolateral periaqueductal gray (VLPAG, mouse Bregma level −4.84); (2) their known function in stress, anxiety and arousal: the ventral lateral septal nucleus (LSV, mouse Bregma level +0.62), ventrolateral preoptic area (VLPO, mouse Bregma level +0.02), dorsal lateral division of the bed nucleus of the stria terminalis (STLD, mouse Bregma level +0.14), lateral paraventricular hypothalamic nucleus (PaL, mouse Bregma level −0.82), lateral division of the central amygdaloid nucleus (CeL, mouse Bregma level −1.46), caudal and interfascicular parts of the dorsal raphe nucleus (DRC and DRI respectively, mouse Bregma level −5.02) and locus coeruleus (LC, mouse Bregma level −5.52); or (3) their known function in reward: accumbens nucleus shell and core (AcbSh and AcbC respectively, both at mouse Bregma level +1.18). Co-localization of Fos-LIR and serotonin-LIR was quantified in the DRC and DRI.

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^2 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 serotonin-positive neurons and the number of double-stained neurons when appropriate. The experimenter did not know the identity or test condition of the mice while counting neurons.

In an attempt to improve the interpretation of the present data, brain tissue from a previous experiment (de Jong et al., 2009) was re-analyzed using the methods described above. In that study we compared the behavioral and Fos-LIR responses of male California mice that were either paternally experienced ("fathers"), or paired with a female, but without having sired a litter yet ("paired males"), or sexually and paternally inexperienced males housed in male-male dyads ("virgin males") to a 5-min exposure to a mesh ball containing an unfamiliar pup. Control males were exposed to an empty mesh ball. For the re-analysis, Fos-LIR was measured in the LHb (which was not analyzed for the previous manuscript) and the DRC (using a larger grid size of 0.2×0.2 mm^2 to match the data from the present experiment). Data for the re-analysis are not described in the results section of the present paper, but are summarized in the discussion and in Table 3.

Statistical analyses

Statistical analyses were performed using SPSS 16.0. ANOVA was used to ascertain that the five experimental groups did not differ in the number of days between pairing and birth of their first litter, litter size, age of the pups on the test day, and age and body weight of the male on the test day. The majority of behavioral and neuroanatomical data were not normally distributed; therefore, all graphs depict medians with error bars delineating first and third quartiles to illustrate variability in the dataset, and only non-parametric tests were used to analyze the data. AQ: 3

Behavioral responses of male mice towards a pup or a pebble were compared using Kruskal–Wallis tests. Significant differences were followed by nonparametric post hoc pair-wise comparisons (Siegel and Castellan, 1988) between isolated males tested with a pup or a pebble, and between isolated and family-housed males tested with a pebble. For the neuroanatomical data, Kruskal–Wallis analyses were performed on two parameters based on experimental manipulations. The first parameter was stimulus type: we compared number of Fos-LIR neurons in various brain areas among isolated males receiving either a pup, a pebble, or no stimulus. Significant overall effects were followed by nonparametric post hoc pair-wise comparisons of the three different stimulus types. The second parameter was housing × stimulus condition: we compared number of Fos-LIR neurons in various brain areas among isolated and family-housed males receiving either a pebble or no stimulus. Significant overall differences among the four groups were followed by nonparametric post hoc pair-wise comparisons of the two housing conditions for each stimulus group, and of the two stimulus groups for each housing condition.

The neuroanatomical data were further analyzed to measure the effects of paternal experience (number of days spent with a newborn litter prior to the test and perfusion) and of litter size. For these analyses, males tested with different stimulus types and under different housing conditions were pooled and re-arranged in three groups according to their paternal experience (one, two or three days) or according to litter size (one, two or three pups), and Kruskal–Wallis tests were performed on the number of Fos-LIR neurons in various brain areas among the three groups for each parameter. Significant overall effects were followed by nonparametric post hoc pair-wise comparisons of the three degrees of paternal experience or the three different litter sizes. The same set of Kruskal–Wallis analyses and post hoc pair-wise comparisons were performed on the total number of serotonergic neurons, and on the number of Fos-positive serotonergic neurons in the caudal and interfascicular dorsal raphe nucleus.

Non-parametric Spearman's rho was used to analyze correlations among the numbers of Fos-LIR or 5-HT-IR neurons and the time spent sniffing and touching a pup or a pebble (for this last group, isolated and family-housed males were pooled). In addition, Spearman's rho was used to analyze correlations among the numbers of Fos-LIR and 5-HT-IR neurons in brain areas showing a significant isolation- or stimulus-induced effect. For all analyses, effects were accepted as statistically significant when P (two-tailed) was smaller than 0.05.

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Table 1. Background information on 35 adult male California mice, divided into five experimental groups, on time between pairing and birth of their first litter (birth latency), litter size, paternal experience (days between birth and testing), and body weight (BW) and age of the males on the test day

Housing:	With family		Isolated			ANOVA:
Stimulus:	None	Pebble	None	Pebble	Pup	
Group size	n=6	n=7	n=7	n=7	n=8	$F_{4,30}/P$
Birth latency (d)	44.00±7.38	42.43±3.40	39.71±1.03	43.57±4.10	38.13±1.72	0.647/0.615
Litter size(1, 2 or 3 pups)	2.00±0.46	1.86±0.13	2.14±0.32	1.43±0.19	1.88±0.23	1.039/0.403
Paternal experience (1, 2 or 3 d)	1.83±0.47	2.14±0.24	1.86±0.22	1.57±0.28	1.50±0.19	0.881/0.487
BW male (g)	38.85±6.16	38.19±2.32	36.44±1.02	38.35±1.68	38.45±1.97	0.268/0.896
Age male (d)	161.50±28.34	157.29±7.74	161.00±7.65	161.29±8.95	156.63±9.59	0.066/0.992

Data are means±standard error of the mean.

RESULTS

Breeding

The five experimental groups did not differ significantly in the number of days between pairing and birth of their first litter ($F_{4,30}$=0.647, P=0.615), amount of paternal experience measured as age of the pups on test days ($F_{4,30}$=0.881, P=0.487), litter size ($F_{4,30}$=1.039, P=0.403), body weight of the males on the test day ($F_{4,30}$=0.268, P=0.896) or age of the males on the test day ($F_{4,30}$=0.066, P=0.992) (Table 1).

Behavioral response to different types of stimuli

Behavioral responses to either a pebble or a pup were measured for 10 min in three groups of adult male California mice: two groups of males that were isolated from their family for 3 h prior to the test and received either a pebble or a pup, and one group of males that remained with their family and received a pebble (Fig. 1). A Kruskal–Wallis test showed an overall significant difference among the three groups in the time spent touching the stimulus (χ^2=11.806, df=2, P=0.003). Post hoc pair-wise comparisons showed that isolated male mice spent significantly more time touching a pup compared to a pebble ($Z_{1,13}$=2.428, P=0.015). Among mice that were tested with a pebble, time spent touching the pebble did not differ between isolated and family-housed males ($Z_{1,12}$=0.832, P=0.405).

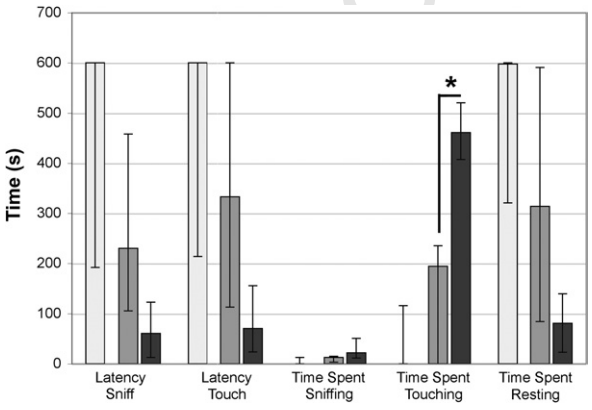

Fig. 1. Behavioral response of male California mice exposed for 10 min to a glass pebble while either housed with their female mate and pups (light gray bars, n=7) or isolated (medium gray bars, n=7), or exposed to a pup while isolated (dark gray bars, n=8). Data are medians; error bars delineate first and third quartiles. * P<0.05.

Fos-like immunoreactivity in response to different types of stimuli

The effect of a brief interaction with a pup compared to a pebble or no stimulus on Fos-LIR in the brains of isolated adult male California mice was analyzed. A Kruskal–Wallis test revealed overall significant differences in Fos-LIR among the three experimental groups in the LHb (χ^2=11.592, df=2P=0.003, Figs. 2 and 3) and DRC (χ^2=8.159, df=2, P=0.017, Fig. 2). No overall significant differences were found in any of the other brain areas quantified (Table 2). Post hoc pair-wise comparisons revealed that pup stimuli caused increased Fos-LIR compared to both pebble stimuli and no stimuli in the LHb (pup vs. pebble: $Z_{1,13}$=2.970, P=0.003; pup vs. no stimulus: $Z_{1,13}$=2.709, P=0.007) and DRC (pup vs. pebble: $Z_{1,13}$=2.173, P=0.030; pup vs. no stimulus: $Z_{1,13}$=2.428, P=0.025) in isolated males. No significant differences in Fos-LIR were found between isolated mice receiving a pebble stimulus and those receiving no stimulus.

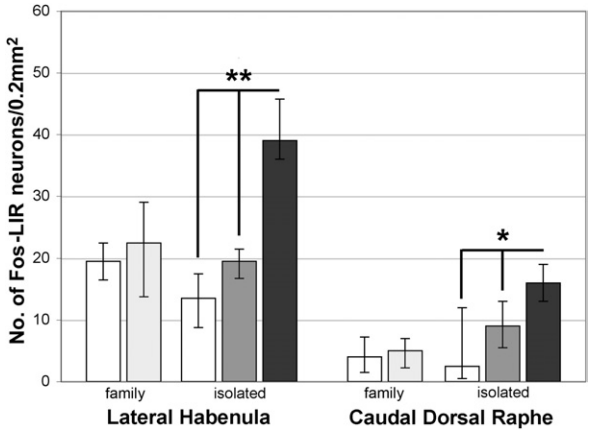

Fig. 2. Number of Fos-LIR nuclei measured in a square of 0.2×0.2 mm² in the lateral habenula and caudal dorsal raphe nucleus of male California mice that were housed with their female mate and pups (family) or isolated, and were exposed for 10 min to either no stimulus (white bars, n=6 and 7), a pebble (light and medium gray bars, n=7 each) or a pup (dark gray bars, n=8). Data are medians; error bars delineate first and third quartiles. * P<0.05; ** P<0.01.

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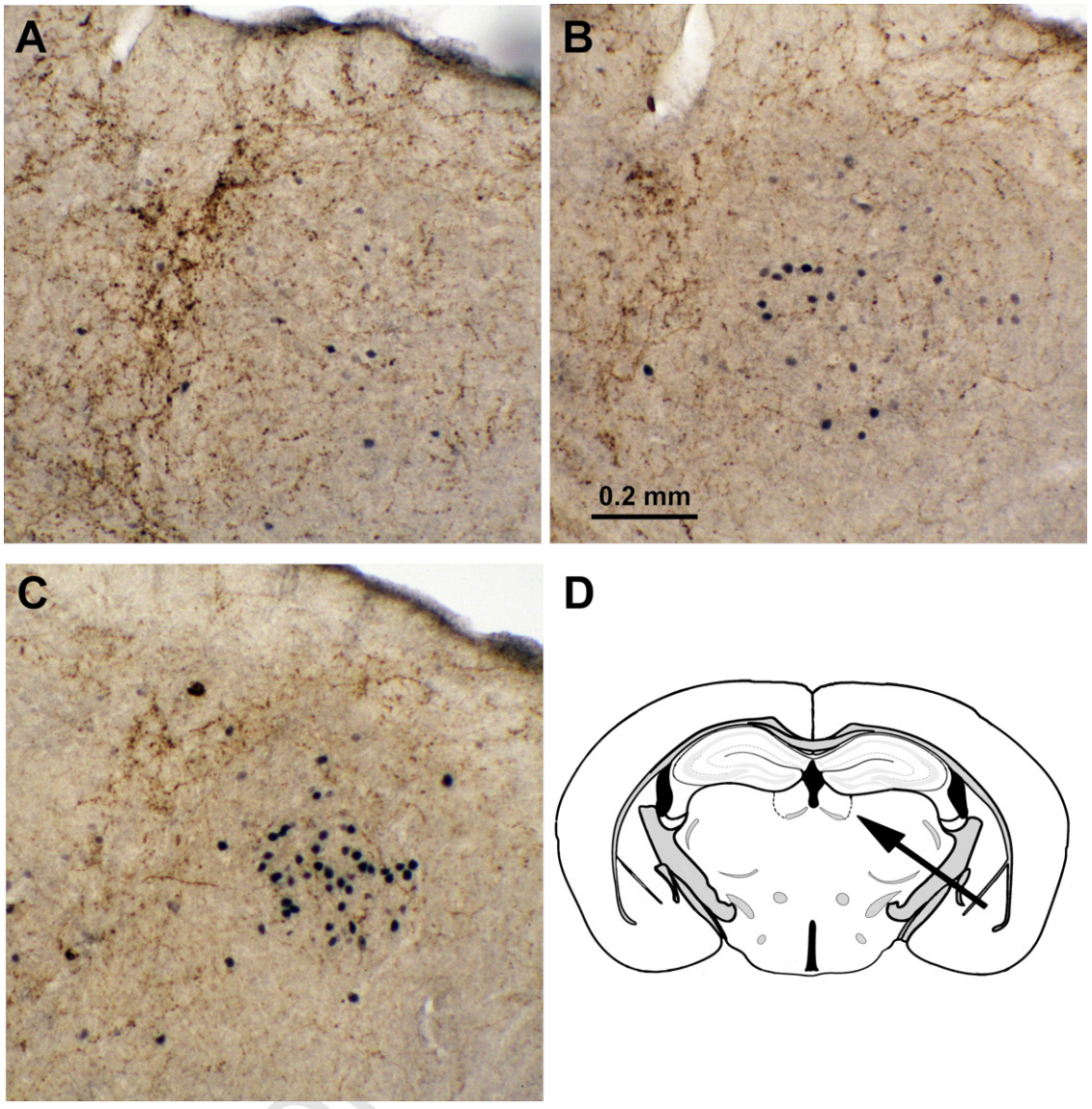

Fig. 3. Representative photomicrographs of double-labeling of Fos (blue-black nuclear staining) and serotonin (reddish brown fibers) in the lateral habenula of isolated male California mice exposed to no stimulus (A), a glass pebble (B) or a pup (C). In (D), which is a simplification of Fig. 45 or Bregma level −1.70 mm in the mouse brain atlas (Franklin and Paxinos, 2008), the black arrow points to the location of LHb analysis. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Further analysis showed that within the DRC and the ventrally adjacent intrafascicular dorsal raphe nucleus (DRI) there were no overall significant differences in the number of 5-HT-positive neurons (DRC: χ^2=0.895, *df*=2, *P*=0.639; DRI: χ^2=0.015, *df*=2, *P*=0.993, Figs. 4 and 5), but there was an overall trend towards a pup-induced increase in Fos-LIR in both serotonergic and non-serotonergic neurons (Figs. 4 and 5). Kruskal–Wallis tests revealed that this trend reached statistical significance only for Fos expression within serotonergic neurons of the DRC (χ^2=9.711, *df*=2, *P*=0.008). Post hoc pair-wise comparisons revealed that exposure to a pup caused a significant increase in Fos-LIR in serotonergic DRC neurons compared to either exposure to a pebble ($Z_{1,13}$=2.621, *P*=0.009) or no stimulus exposure ($Z_{1,13}$=2.640, *P*=0.008).

Fos-like immunoreactivity in response to a marble stimulus compared to no stimulus under different housing conditions

Kruskal–Wallis analyses showed no overall significant differences in Fos-LIR or in 5-HT-IR in any of the brain regions analyzed among the four “non-pup” groups (the non-stimulated and pebble-stimulated family-housed and isolated males).

Fos-like immunoreactivity in response to paternal experience and litter size

Fos and serotonin expression were compared among male mice that had spent either1, 2, or 3 days with their newborn litter prior to testing and perfusion. Individuals with different

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Table 2. Number of Fos-LIR neurons per 0.2 mm^2 in all quantified brain areas (except the LHb and DRC) of adult male California mice that were housed with their female mate and pups or isolated, and were exposed for 10 min to no stimulus, a glass pebble, or one of their own pups

Housing:	With family		Isolated		
Stimulus:	None	Pebble	None	Pebble	Pup
AcbSh	89.0	92.0	93.0	110.0	96.5
	68.0/101.0	75.0/102.5	91.5/109.5	105.5/116.5	86.3/100.8
AcbC	52.0	70.0	62.0	75.0	77.0
	37.0/69.3	60.5/79.5	45.0/79.0	53.0/80.0	63.0/93.8
LSV	35.5	44.5	45.0	45.0	46.0
	30.3/43.0	26.0/49.5	24.5/49.5	40.0/51.5	36.0/50.3
STLD	54.5	40.0	59.0	54.0	66.0
	28.3/59.8	34.5/57.5	48.0/66.3	47.5/70.0	54.0/69.5
VLPO	34.0	28.0	24.0	30.5	39.5
	27.3/37.0	27.0/40.3	22.0/29.8	22.0/39.0	30.0/44.5
MPO	21.5	23.0	24.0	32.0	34.0
	15.8/23.5	18.5/28.0	19.0/46.5	23.5/47.0	28.3/52.3
STMV	26.0	29.0	40.0	35.5	41.5
	14.3/41.5	26.0/31.0	39.0/45.5	35.0/42.8	33.5/55.5
STMPM	44.0	30.5	29.0	45.5	50.0
	38.8/49.3	23.5/36.8	26.5/43.5	37.0/64.5	42.5/55.3
PaL	56.0	66.0	84.0	80.0	73.0
	34.5/79.0	39.5/84.5	71.0/89.0	62.5/98.0	54.0/95.5
BLA	21.0	13.5	27.0	24.0	20.0
	20.3/27.0	11.3/21.8	20.0/29.0	18.0/28.5	13.3/22.8
MePD	32.5	25.0	26.0	33.0	31.0
	29.8/37.5	21.5/29.5	24.0/34.5	26.8/39.5	28.5/37.0
CeL	42.5	44.0	44.0	60.5	61.5
	38.3/43.0	40.0/66.0	33.0/52.5	52.8/70.5	55.0/63.8
VLPAG	18.5	15.0	19.0	23.0	25.0
	14.3/19.0	12.0/17.0	18.0/22.0	19.5/27.5	17.0/26.0
LC	12.5	11.0	15.0	19.0	15.0
	7.3/14.8	8.0/13.5	12.0/15.0	15.0/23.0	9.0/17.5

Data are medians with first and third quartiles.

amounts of paternal experience were evenly spread over the five experimental groups ($F_{4,30}$=0.881, P=0.487; Table 1), but for this analysis they were re-grouped independent of housing- and stimulus condition. A Kruskal–Wallis test revealed strong trends towards overall differences in Fos-LIR in the medial preoptic nucleus (MPN; χ^2=5.741, df=2 P=0.057, Fig. 6A) and medial posteromedial bed nucleus of the stria terminalis (STMPM; χ^2=5.462, df=2, P=0.065, Fig. 6A), which appeared to indicate a decrease in Fos-expression over time. There were no effects of paternal experience on number of serotonergic neurons in the DRC or DRI.

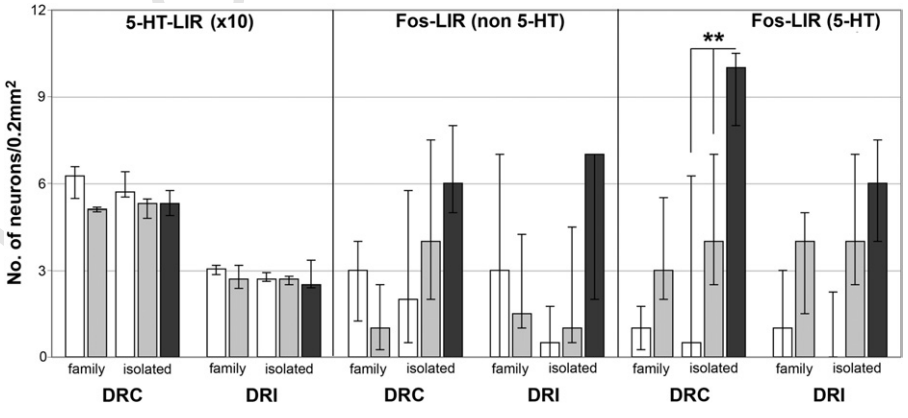

Fig. 4. Number of serotonergic neurons (10× scale on y-axis), as well as Fos-LIR nuclei in serotonergic and non-serotonergic neurons, measured in a square of 0.2×0.2 mm^2 in the caudal dorsal raphe nucleus (DRC) and the intrafascicular dorsal raphe nucleus (DRI) of male California mice that were housed with their female mate and pups (family) or isolated, and were exposed for 10 min to either no stimulus (white bars, n=6 and 7), a glass pebble (light and medium gray bars, n=7 each) or a pup (dark gray bars, n=8). Data are medians; error bars delineate first and third quartiles. ** P<0.01.

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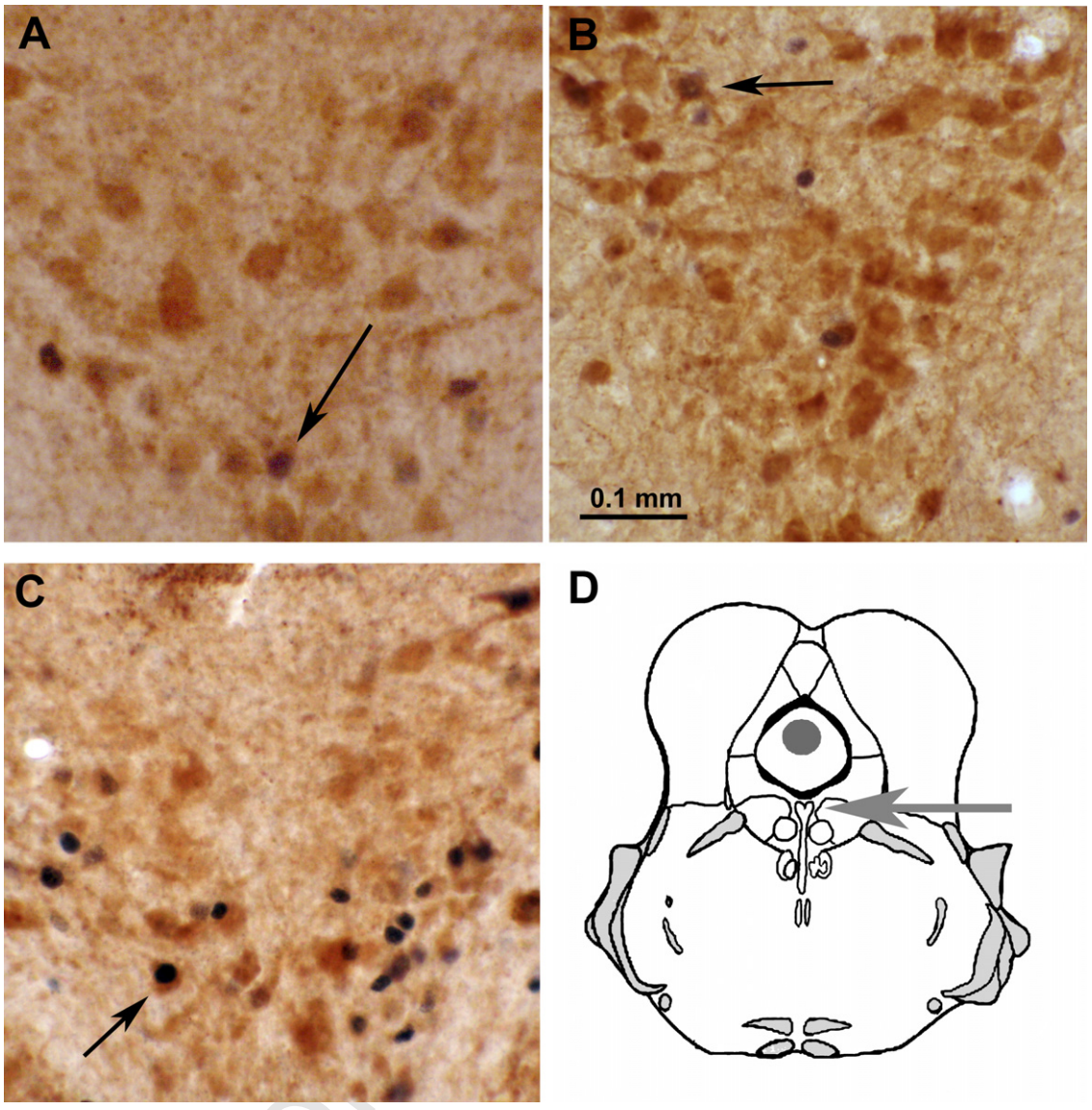

Fig. 5. Representative photomicrographs of double-labeling of Fos (blue-black nuclear staining) and serotonin (reddish brown cytoplasmic staining) in the caudal dorsal raphe nucleus of isolated male California mice exposed to no stimulus (A), a glass pebble (B) or a pup (C). Black arrows point to examples of double-labelled neurons. In (D) which is a simplification of Fig. 73 or Bregma level −5.02 mm in the mouse brain atlas (Franklin and Paxinos, 2008), the gray arrow points to the location of DRC analysis. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Fos and serotonin expression were also compared among male mice that had sired either one, two, or three pups. Individuals with different litter sizes were evenly spread over the five experimental groups ($F_{4,30}$=1.039, P=0.403; Table 1), but for this analysis they were re-grouped independent of housing- and stimulus condition. A Kruskal–Wallis test revealed an overall significant difference in Fos-LIR in the core of the nucleus accumbens (AcbC; χ^2=7.505, df=2 P=0.023, Fig. 6B) and ventral lateral septal nucleus (LSV; χ^2=11.405, df=2, P=0.003, Fig. 6B). Post hoc pair-wise comparisons revealed that Fos-LIR was lower in males siring three pups compared to two pups in the LSV ($Z_{1,23}$=2.627, P=0.009) and AcbC ($Z_{1,24}$=2.532, P=0.011), and in males siring three pups compared to one in the LSV ($Z_{1,16}$=3.254, P=0.001). There were no effects of litter size on number of serotonergic neurons in the DRC or DRI.

Correlations between behavior and Fos/serotonin expression

To investigate whether variability in behavior during the stimulus exposure were related to variability in Fos- or 5-HT expression, correlations between the amount of time spent interacting with (sniffing and touching) the pup (n=8) or the pebble (n=14; isolated and family-housed males exposed to a pebble were pooled) and the number of Fos-LIR or 5-HT-IR neurons in all quantified brain areas

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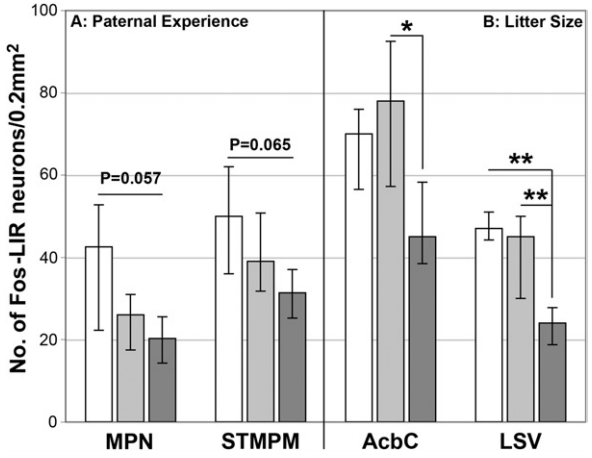

Fig. 6. Number of Fos-LIR nuclei measured in a square of 0.2×0.2 mm^2 in (A) the medial preoptic nucleus (MPN) and medial posteromedial bed nucleus of the stria terminalis (STMPM) of male California mice with one day (white bars, $n=13$), 2 d (medium gray bars, $n=15$) or 3 d (dark gray bars, $n=6$) of paternal experience, or in (B) the core of the nucleus accumbens (AcbC) and ventral lateral septal nucleus (LSV) of male California mice siring one pup (white bars, $n=10$), two pups (medium gray bars, $n=18$) or three pups (dark gray bars, $n=6$). For these analyses, mice from different housing- and stimulus groups were pooled. Data are medians; error bars delineate first and third quartiles. * $P<0.05$; ** $P<0.01$.

were calculated. However, no significant positive or negative correlations were found between any of the behavioral and neuroanatomical parameters within each experimental group.

In addition, no significant correlation was found between the number of Fos-LIR neurons in the two brain areas showing a stimulus-induced effect, the LHb and DRC, in any of the three groups of isolated male mice (no stimulus: $\rho=0.232$, $P=0.656$, $n=6$; pebble: $\rho=-0.257$, $P=0.623$, $n=7$; pup: $\rho=0.709$, $P=0.079$, $n=7$). Since the pup group showed a trend towards a positive correlation, further analyses were performed to calculate the correlation of Fos-LIR in the LHb with Fos-LIR in serotonergic and non-serotonergic neurons in the DRC within this experimental group. However, neither correlation was significant (LHb/serotonergic DRC: 0.327, $P=0.474$, $n=7$; LHb/non-serotonergic DRC: 0.556, $P=0.195$, $n=7$).

DISCUSSION

The present experiment was conducted to clarify which brain areas are associated with parental responses towards a pup, compared to responses to a control stimulus (a glass marble roughly similar in shape and size to a newborn pup) or no stimulus in adult, paternally experienced male California mice.

Male mice tested with one of their own pups in their home cage all interacted paternally with the pup, showing typical parental behaviors such as huddling, licking and occasionally kyphosis. Male mice interacted significantly less with a pebble stimulus, but some mice licked, bit, nudged and/or handled the pup-sized pebble. Despite the clear behavioral differences between mice receiving a pup stimulus and mice receiving a pebble stimulus, only two brain areas showed clear pup-induced increases in Fos-LIR in California mouse fathers: the LHb and the DRC.

The LHb has been shown previously to express Fos in response to pup-exposure in female rodents (Lonstein et al., 1998; Kalinichev et al., 2000; Komisaruk et al., 2000). In addition, lesion of the LHb has repeatedly been found to cause significant deficits in maternal behavior, especially in pup-retrieval scores (Corodimas et al., 1992, 1993; Felton et al., 1998). The LHb has strong connections with the medial and lateral preoptic area, medial bed nucleus of the stria terminalis and medial amygdala (Felton et al., 1999), indicating that this brain area is part of an important pathway controlling maternal and, possibly, paternal care. Interestingly, several authors have suggested that it is actually the removal of the pup, rather than exposure to the pup, that activates the LHb. In primiparous maternal female rats, exposure to a litter following 2 days of isolation activated the LHb only when pups were removed prior to perfusion, and not when they remained with the female until perfusion (Stack and Numan, 2000). Other researchers also reported increased Fos-LIR in the LHb of female rats in response to maternal interactions followed by separation (Lonstein et al., 1998; Smith and Lonstein, 2008). On the other hand, some investigators found increased Fos-LIR in the LHb in response to continuous maternal interactions up to the moment of perfusion (Kalinichev et al., 2000; Komisaruk et al., 2000), and in the present experiment the LHb was not activated following 4 h of isolation from the female mate and pup, without the short bout of pup exposure (Fig. 2). The new findings prompted us to revisit the brain tissue collected in a previous experiment, which was stained for Fos-LIR using the same immunohistochemical techniques as used in the present study (de Jong et al., 2009). Analysis of the LHb showed that exposure to a pup in a mesh ball strongly increased Fos-LIR in fathers compared to both (1) exposure to an empty ball, and (2) virgin males exposed to a pup in a ball, with values resembling those in the present experiment (Table 3). Together, the two datasets indicate that the LHb is activated by relatively brief exposure to a pup, in a manner independent of actual parental behaviors but highly dependent on parental experience.

The LHb has recently been shown to be strongly activated in rhesus monkeys upon perceiving either an unexpected aversive stimulus or the absence of an expected reward (Matsumoto and Hikosaka, 2007; Hikosaka et al., 2008), and removal of a pup may fall into either one of these categories. It is possible that fathers have learned to associate pup cues with positive reinforcement, which would be consistent with the more general role of the LHb in reward-driven memory retrieval (Tronel and Sara, 2002; Zhang et al., 2005). Taken together, the specific combination of (presumably stressful) isolation from the family, followed by brief exposure to a pup and, finally, removal of that pup activates the LHb in paternally experienced male Calfiornia mice, but it is not yet clear which (combination of) component(s) is ultimately responsible.

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Table 3. Number of Fos-LIR neurons per 0.2 mm^2 in the LHb and DRC of adult male California mice, comparing results from a previous experiment (de Jong et al., 2009) with the present results

	LHb median—quartiles		DRC median—quartiles	
Previous experiment				
Virgins				
Empty Ball	17.00	16.75	18.00	12.50
		20.00		21.25
Pup in Ball	16.00	10.50	10.00	7.75
		19.00		13.00
Paired males				
Empty Ball	14.00	8.00	16.00	8.50
		24.50		21.25
Pup in ball	24.00	16.50	20.00	14.25
		37.00		28.00
Fathers				
Empty ball	19.50	8.75	26.50	24.00
		31.50		28.75
Pup in ball	44.00*†	38.50	28.50*	25.75
		45.00		29.75
Present experiment				
Fathers				
No stimulus	20.00	8.56	4.00	2.00
		21.38		7.25
Pebble	17.00	10.44	7.00	2.25
		21.63		9.00
Pup	39.00#	36.00	16.00#	13.00
		45.75		19.00

In the previous experiment, fathers, paired males and virgin males were exposed for 5 min to either a mesh ball containing an unfamiliar pup, or an empty mesh ball. In the present experiment, fathers were isolated and exposed for 10 min to either no stimulus or a freely accessible pebble or pup. Data are medians with first and third quartiles.

* Significantly different from corresponding virgin male group ($P < 0.05$).

† Significantly different from corresponding empty ball group ($P < 0.05$).

Significantly different from no stimulus and pebble groups ($P < 0.05$).

Brief exposure to a pup, but not a pebble, within the home cage also increased Fos-LIR in both serotonergic and non-serotonergic neurons within the caudal dorsal raphe nucleus. An intact serotonin system, especially the dorsal raphe nucleus, has been shown to be required for the expression of maternal care and suckling-induced prolactin release in female mice and rats (Barofsky et al., 1983; Lerch-Haner et al., 2008), but it has not before been directly implicated in male parental care. Fos expression in the DRC has generally been associated with increased anxiety and with unpredictable and inescapable stressors (Grahn et al., 1999; Lowry, 2002; Maier and Watkins, 2005), and it is certainly possible that either the pup stimulus itself and/or the sudden removal of the pup, perhaps exacerbated by pre-existing isolation-induced stress, caused enough stress in adult males to activate this area in the present experiment. Interestingly, the DRC also reacts to accoustic stressors (Evans et al., 2009), and the loud and persistent vocalizations of a distressed California mouse pup (Vieira and Brown, 2002; de Jong et al., unpublished observation) may have activated the stress-sensitive DRC in the paternal male California mice in this study.

Re-analysis of Fos-LIR in the DRC in brain tissue from a previous experiment, in order to match the location and size of the area analyzed in the present study more closely, showed a similar activation pattern as reported previously (de Jong et al., 2009): fathers had increased Fos-LIR in the DRC compared to virgin males following exposure to either a pup in a ball or an empty ball (Table 3). In our previous report, we tentatively interpreted those findings as an increased reaction to stress in fathers, induced either by separation from the mate and pups, or by hypersensitivity to the novel environment in which we tested the mice. In that study, Fos-LIR in the DRC was generally higher compared to the present experiment, which can probably be explained by the test condition (novel cage vs. home cage), and this may have interfered with the results in that experiment. In the present experiment, neither separation from mate and pups nor exposure to a novel object (pebble) affected Fos-LIR in the DRC as compared to control procedures, and we can therefore conclude that it was specifically the exposure to a pup that caused the activation.

Interestingly, the LHb and raphe nuclei have strong reciprocal connections with each other, and the LHb is known to exert part of its effects through serotonergic neurons in the dorsal raphe (Varga et al., 2003; Lecourtier and Kelly, 2007). In the present study the number of Fos-LIR neurons in the LHb was not correlated with the number of Fos-LIR serotonergic neurons in the DRC or DRI in any of the experimental groups. Nonetheless, a functional role for the LHb-DR connection in the observed behavioral responses cannot be excluded.

Brief exposure to a pup did not change the level of Fos expression in paternally experienced male California mice in several brain areas that are consistently associated with parental care, including the MPN and medial posteromedial and medioventral bed nucleus of the stria terminalis (STMPM and STMV) (Kirkpatrick et al., 1994; Numan and Numan, 1994; Kalinichev et al., 2000; Lonstein and De Vries, 2000). We did find, however, that, independent of housing and stimulus conditions, Fos-expression in the MPN and STMPM was higher in males with one day versus 3 days of pup experience. It is possible that long-term exposure to pups (either experimentally or, in the present experiment, after birth of a litter) initially causes a strong increase in Fos-expression in these areas, which slowly declines over time, as has been shown in primiparous female rats (Stack and Numan, 2000). The ongoing paternal experience in the nest might habituate the MPN and STMPM to pup cues, preventing increases in Fos expression in response to short bouts of pup exposure. Similar effects have been found in male Japanese quail, which show reduced immediate early gene expression in response to sexual stimuli following extensive sexual experience (Can et al., 2007). In a previous experiment (de Jong et al., 2009), Fos-LIR was increased in the STMV and STMPM, but not the MPN, of male California mice with 3 days of paternal experience compared to virgin males. A

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5-min exposure to distal pup cues did not further increase Fos-LIR in the STMV and STMPM, but it did increase Fos-LIR in the MPN in fathers. The combined data of the previous and present experiments are difficult to interpret, but it is possible that perceiving cues from an unfamiliar pup (as used in the previous experiment) overrides the habituation of the MPN in paternally experienced California mice whereas cues from one of their own pups do not. Another possibility is that cues from an inaccessible pup (as used in the previous experiment) overrides the habituation whereas an accessible pup does not, although opposite results have been reported in female rats (Calamandrei and Keverne, 1994; Lonstein et al., 1998).

The ventral lateral septum and the core of the nucleus accumbens were not activated by housing or stimulus condition, but they did appear to be less activated in male California mice that had sired large litters (three pups) compared to smaller litters (one or two pups). This effect was particularly strong in the ventral lateral septum, an area that is known to show differences in Fos expression as well as neuropeptide (vasopressin and oxytocin) innervation and receptor expression in response to paternal experience in voles (Kirkpatrick et al., 1994; Wang et al.,1994; Parker et al., 2001). It is unclear at this point how and why litter size would affect Fos expression in this area; however, this presents an interesting subject for future studies.

CONCLUSION

In conclusion, a short-lasting bout of pup exposure followed by removal of the pup caused a strong activation of the LHb and DRC in paternally experienced male California mice. These brain areas are most likely activated by an aversive, stressful aspect of the test, putatively the distal sensory cues indicating the presence of a distressed pup and/or the sudden removal of the pup. Further research is needed to determine which aspects of a pup stimulus are responsible for LHb and DRC activation, and whether these brain areas are responsible for the decision to behave paternally towards a pup.

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