Father absence in the monogamous California mouse impairs social behavior and modifies dopamine and glutamate synapses in the medial prefrontal cortex
Father absence in the monogamous California mouse impairs social behavior and modifies dopamine and glutamate synapses in the medial prefrontal cortex

Running title: Father absence impairs social behaviour and PFC synapses

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Abstract

The role of the father in psycho-affective development is indispensable. Yet, the neurobehavioral effects of paternal deprivation (PD) are poorly understood. Here, we examined the behavioral consequences of PD in California mice, a species displaying monogamous and biparental care, and assessed its impact on dopamine (DA), serotonin (5-HT) and glutamate (GLU) transmission in the medial prefrontal cortex (mPFC). In adult males, deficits in social interaction were observed when a father-deprived mouse was matched with a PD partner. In adult females, deficits were observed when matching a PD animal with a non-PD control, and when matching two PD animals. PD also increased aggression in females. Behavioral abnormalities in PD females were associated with a sensitized response to locomotor-activating effects of amphetamine. Following immunocytochemical demonstration of the DA, 5-HT and GLU innervations in the mPFC, we employed in vivo electrophysiology and microiontophoresis, and found that PD attenuated the basal activity of low-spiking pyramidal neurons in females. PD decreased pyramidal responses to DA in females, while enhancing responses to NMDA in both sexes. We thus demonstrate that, during critical neurodevelopmental periods, PD leads to sex-dependent abnormalities in social and reward-related behaviors that are associated with disturbances in cortical DA and GLU neurotransmission.
INTRODUCTION

Early attachment experiences with the primary caregiver forge an internal working model for subsequent adult relationships, and parental neglect is notoriously linked to neurodevelopmental and behavioral disorders that may persist through adulthood (Bowlby, 1978; Tyrka et al., 2008; Murray et al., 2012). Despite emerging evidence of the impact of parental deprivation (Helmeke et al., 2001a; Ziabreva et al., 2003a; Bredy et al., 2004; Kaffman and Meaney, 2007), the primary focus has been on mother-offspring relationships. Nevertheless, human studies have pointed out that paternal deprivation (PD) impairs psychological and mental development, and increases risk for substance abuse and personality disorders (Grossman et al., 2002; Jablonska and Lindberg, 2007; Lamb, 2010). However, the neurobiological substrate underlying PD-induced behavioral and neurobiological impairments remains unknown.

Paternal care or biparental rearing patterns are found only in a minority of mammalian species (Kleiman and Malcolm, 1981). California mouse (Peromyscus californicus) display permanent father-mother pair bond and extensive biparental investments that have accounted for increased offspring survival and growth (Yogman 1981; Gubernick and Alberts, 1987; Ribble and Salvioni, 1990; Gubernick and Teferi, 2000; Becker et al., 2005; Schradin and Pillay, 2005). California mice therefore represent a useful model for examining the behavioural and neurobiological impact of PD. Few studies in biparental rodents have suggested that PD impairs cognitive and emotional functions in offsprings, especially in low-resource availability and high-foraging demand conditions (Bredy et al., 2004; Bredy et al., 2007). This effect of PD is not surprising as all maternal offspring-directed nurturing behaviours (huddling, licking and grooming, hover-crouching, carrying and retrieving), except for parturition and lactation, are also performed by the father (Dudley, 1974; Gubernick and Alberts, 1987), which are uncompensated by the mother in PD (Dudley, 1974; Bester-Meredith and Marler, 2003; Helmeke et al., 2009; Jia et al., 2009). Paternal behaviours also include involvement in social play and enhanced attention mainly when juveniles are distressed (Wilson and Kleiman, 1974; Becker et al., 2005; Bredy et al., 2007; Lambert et al., 2013) and decreased activation of brain structures involved in emotional integration, including prefrontocortical regions (Lambert et al., 2013).
Evidence in monogamous rodents suggests that PD could compromise synaptic development in the medial prefrontal cortex (mPFC) (Helmeke et al., 2009; Pinkernelle et al., 2009). The mPFC is involved in processing socially relevant information (Insel 2003; Wolff and Sherman, 2007), and activity in its dorsomedial subregions has been linked to prosocial behaviours (Waytz et al., 2012). DA is known to interact with neurobiological systems regulating social behavior, including the neurohormones oxytocin and vasopressin (Insel, 2003; Baskerville and Douglas, 2010). Dopamine (DA) and serotonin (5-HT) receptors are highly expressed and co-localized with NMDA receptors in the mPFC, where they regulate synaptic plasticity and neural activity, as well as emotional behavior. In addition, alterations in NMDA receptor subunits have been associated with PD (Bredy et al., 2007). Sexually dimorphic effects of NMDA antagonists have been observed on social and alloparenting behavior (Kirkpatrick and Kakoyannis, 2004) and on behavioural functions associated with the reward circuit (Hönack and Löscher, 1993; D’Souza et al., 1999) and modulated by gonadal steroid hormones (D’Souza et al., 2003).

In this study, we examined social interaction and aggressive behavior, as well as the reward response to amphetamine, in adult California mice raised under sole maternal care in comparison to those raised under biparental care. In addition to providing the first description of DA, 5-HT and glutamate (GLU) innervations in the California mouse mPFC, we characterized the electrophysiological responses to DA, 5-HT and NMDA in order to correlate behavioral consequences of PD with synaptic function in the mPFC.

**MATERIALS AND METHODS**

*Animals.* Adult California mice (*Peromyscus californicus*) used for breeding (first batch) were kindly offered by Dr. Michael Meaney (McGill University, Montreal, Canada) and obtained (second batch) from the University of South Carolina Peromyscus Genetic Stock Center. All procedures were undertaken in compliance with the standards and ethical guidelines mandated by the local facility animal care committee of McGill University, the Canadian Institutes of Health Research, and the Canadian Council on Animal Care. Breeding pairs were age-matched (within a three-month age range), and no siblings were
mated. Pairs were housed in standard polycarbonate cages (26.5 cm W x 48.5 cm D x 20.3 cm H) with corncob bedding and kept under standard vivarium conditions (12:12 hr. light/dark cycle, lights on at 7:30 A.M. and temperature at 20 ± 2°C, 50–60% relative humidity) with *ad libitum* access to food and water.

**Paternal deprivation.** On post-parturition day (PND) 3, litters (1-3 pups/litter) were randomly designated to either be left with only the mother (paternal deprivation, PD) or with both parents (control, CT), as already described by Bredy et al. (2004). Fathers were then permanently removed from PD litter. Mother and pups were thereafter left undisturbed until weaning on PND30 (de Jong et al., 2012), except for routine maintenance. CT litters were left undisturbed with both parents until weaning. After weaning, a PD mouse was housed with a PD mouse and a CT mouse with another CT, in same-sex littermate pairings. Because cognitive and behavioural effects of PD can be detected as early as PND 60 (Bredy et al., 2004), we began experiments when offsprings were at least 70 days old, just at the onset of the average age of natal dispersal (in the wild) and maturity (McCabe and Blanchard, 1950; Ribble, 1992; de Jong et al., 2012). A chronological representation of procedures is depicted in figure 1.

**Behavioral assays.** The social interaction (SI) test was used to assess social behavior in same-sex dyads. The open field (OF) test was conducted for the assessment of locomotor activity and anxiety-like thigmotactic (wall following) behavior. On the day of testing, mice were acclimated for about 60 min in the behavioral room before the procedures were initiated. The apparatus was cleaned with 70% alcohol and water after each run. The behaviors were recorded, stored and analyzed as MPEG files using an automated tracking system (Videotrack, View Point Life Science, Montréal, Canada) equipped with infrared lighting-sensitive CCD cameras. The analog signals supplied by the camera were measurements of the luminosity of each point from images scanned point-by-point and line-by-line at the rate of 25 images per second. These signals were transmitted to the videotrack system and digitized on 8 bits by digital analog conversion. Before experiments, animal/image background contrast detection thresholds were calibrated by visual inspection to distinguish different behavioral patterns. Additional offline analyses were conducted by a rater who was blind to the experimental manipulations. All behavioural experiments were carried out from 2PM -7PM.
**OF and SI tests.** For the OF test, each mouse was placed at the center of a white-painted open field arena (40x40x15 cm) and left to explore the whole field for a 5 min recording. Four identical arenas were used to test 4 animals at a time. Arena assignments were counterbalanced so that all experimental groups were equally distributed to each of the arenas. Each animal underwent a single OF test. The experiment took place under standard room lighting (~350 lx) by a white lamp (100 W) suspended 2m above the arena. Locomotor activity was measured by the total distance traveled (cm). Immediately after the OF test, the SI test was carried out to analyze social behavior using a procedure modified from Whatson et al. (1974, 1976), McFarlane et al. (2008) and Cox and Rissman (2011). The test was conducted in the same arenas previously used for the OF test at a different location within the same testing room, so that animals were all habituated to the testing room. Here, we assessed changes in the social behavior of adult California mice that have been reared by both parents, in comparison to those subjected to PD, by examining the behavior of PD mice when paired with a CT or with another PD animal. The test lasted for 20 min in order to evaluate persistent changes in social behavior. On the first session, same-sex unfamiliar conspecifics were match-paired according to rearing condition (PD-PD or CT-CT). On the second session, same-sex unfamiliar conspecifics were mix-paired (PD-CT) and the total duration of active social investigation was calculated for PD (in the dyad PD-CT) and for CT (with the same dyad). No animals met more than once. Each animal was paired with a CT and a PD in a fixed order, and there was a very short delay between the first and second SI test. The frequency and duration of several components of social behavior were encoded and analyzed offline. These included social investigation, which consisted of sniffing, trailing (following the partner), mounting or crawling under or over the partner, and defensive (targeting the back or flanks) or offensive (targeting the snout) aggression. The time (latency) until the dyad first contacted each other was also measured. Avoidance (evading the partner) and passive contact or side-by-side sitting (remaining close to each other but without actively investigating the partner), as well as auto-grooming, were additionally recorded (McFarlane et al. 2008; Cox and Rissman, 2011). As the SI test has also been used to evaluate social anxiety, as well as the anxiogenic sequelae of chemical agents or genetic manipulations (Cassano et al., 2010), it is conceivable that PD-induced social
impairments observed in this test could result from a potentiation of anxiety-like states and/or secondary to motor activity impairment. We therefore also examined anxiety-like reactivity from thigmotactic behaviour measured by the frequency and total duration of central zone (30 x 30 cm) visits in the OF test (Choleris et al., 2001).

Behavioral sensitization. Since dopamine (DA) is implicated in the adverse consequences of psychostimulant exposure on pair bonding and social behaviour, and that drug reward and social behaviour may recruit overlapping DA pathways (McGregor et al., 2008; Liu, et al., 2010), we ascertained whether changes in mPFC DAergic neurotransmission would translate into an enhanced long-term sensitization to the locomotor-activating effects of amphetamine. Robust sensitizing response to the locomotor-activating effects of amphetamine that is independent of age and environmental context has been demonstrated in Peromyscus mice (Tanimura et al., 2009). Therefore, d-amphetamine sulfate (Sigma Aldrich, UK) was administered at 2 PM intraperitoneally (1.5 mg/kg) to CT and PD males and females, daily for seven days, to induce the development of locomotor sensitization. This dose is the minimal dose to optimally induce rapid-onset sensitization in both mice (Harrison and Nobrega, 2009) and rats (Bhardwaj et al., 2006). Immediately following each amphetamine injection, each animal was placed in an open field arena (40 x 40 x 15 cm) to which the animals were adequately habituated (previously used for basal locomotor testing and SI test). Locomotor activity (distance traveled, cm) on this final challenge of amphetamine was recorded for 5 min, the enhancement of which was indicative of behavioral sensitization to the psychostimulant’s effects. The daily progression of amphetamine-induced locomotor activation was also monitored. The effects of amphetamine in these animals were compared to CT and PD animals receiving once daily injections of saline. The experiments were carried out from 2PM -7PM.

Immunohistochemical detection of GLU, 5-HT and DA fibers.

Antibody staining was used to visualize, for the first time in this species, GLUergic, 5-HTergic and DAergic innervations of prelimbic and cingulated cortices. Four adult California mice (two males and two females) were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the heart with 100 ml of phosphate-buffered saline (PBS: 0.9% NaCl in 50 mM PB, pH 7.4) followed by 200 ml of fixative solution (4% paraformaldehyde in 0.1 mM phosphate buffer, PB, pH 7.4). The brain was
removed, postfixed in 4% PFA (1 hr, 4°C), and washed in PBS. 50 µm thick coronal forebrain sections were cut with a vibratome and processed free-floating as follows. Primary antibodies were: monoclonal mouse anti-tyrosine hydroxylase (TH, for DA; Sigma, St. Louis, MO, USA); polyclonal goat anti-serotonin transporter (SERT, for 5-HT; Santa Cruz Biotechnology, Santa Cruz, CA, USA); and rabbit anti-vesicular GLU transporter 2 (VGLUT2, for GLU; Synaptic Systems, Göttingen, Germany). The specificity of these commercially available antibodies has already been described in full (TH: Haycock, 1993; SERT: Huang and Pickel, 2002; VGLUT2: Takamori et al., 2001).

Secondary antibodies used for light microscopic immunocytochemistry were biotinylated goat anti-mouse, donkey anti-goat and goat anti-rabbit IgGs (Jackson Immunoresearch, West Grove, PA), to be revealed with the 3,3-diaminobenzidine (DAB) or VIP substrate kits from Vector Labs (Burlingame, CA, USA). Species-specific IgGs conjugated to Alexa Fluor-488 or 594 (Invitrogen Corporation, Grand Island, NY, USA) were used for double immunofluorescence and confocal microscopy.

For single immunoperoxidase labeling and light microscopy, all incubations were at room temperature (RT). Sections were immersed 20 min in 3% hydrogen peroxide (H$_2$O$_2$), rinsed in PBS and pre-incubated for 2h in a blocking solution (BS) of PBS containing 5% normal goat serum, 5% normal donkey serum (Vector, Burlingame, CA, USA), 0.5% gelatine, and 0.3% Triton X-100. Sections were then washed in PBS, incubated overnight with either anti-TH, anti-VGLUT2 (1:1000 in BS), or anti-SERT (1:700 in BS) antibody, rinsed in PBS, incubated 2 h with species-specific biotinylated IgGs (1:1000 in BS), and incubated for 1 h in a 1:1000 dilution of horseradish peroxidase-conjugated streptavidin (Jackson). After washes in PBS, labeling was revealed 1-2 min with the DAB (SERT) or VIP (TH, VGLUT2) peroxidase substrat kit. The reaction was stopped in distilled water, the sections rinsed in PB, air-dried on gelatine coated slides, dehydrated in ethanol, cleared in toluene and mounted with DPX (Fluka, Sigma-Aldrich, Oakville, ON, Canada). The prefrontal cortex was carefully examined at low (1.6X objective) and high (6.3X objective) magnification under a Leitz Diaplan microscope couple to an Olympus DP21 colour digital camera and software (Olympus Corporation, Tokyo, Japan).

For double immunofluorescent labelings and confocal microscopy, all sections were pre-incubated 2h in BS as above. For double GLU/DA immunofluorescence, sections
were incubated overnight with both anti-VGLUT2 and -TH primary antibodies (1:1000 in BS). After washes in PBS, sections were further incubated for 3h (light protected) with a mix of species-specific Alexa Fluor 488 (VGLUT2) and Alexa Fluor 594 (TH) conjugated IgGs (1:200 in BS). For double GLU/5-HT immunofluorescence, sections were incubated overnight with the anti-SERT primary antibody (1:700 in BS). Subsequent incubations and washes were light protected. After washes in PBS, sections were then incubated for 3h with Alexa Fluor 594 conjugated anti-goat IgGs (1:200 in BS). After washes in PBS, sections were further incubated overnight with the anti-VGLUT2 antibody (1:1000 in BS), and then 3h with Alexa Fluor 488-conjugated anti-rabbit IgGs (1:200 in BS) following washes in PBS. For both double labeling experiments, after rinses in 0.1 M PB and distilled water, sections were mounted in Vectashield medium for fluorescence (Vector) and observed with a Leica TCS-SP1 confocal microscope using sequential laser analysis (100X magnification). Images of 1024 x 1024 pixels were produced with the Leica confocal software (v2.65) and adjusted for framing and contrast with the Adobe Photoshop software.

**In vivo electrophysiology and microiontophoresis.** We aimed at determining whether PD could significantly influence DA, 5-HT and GLU neurotransmission in the mPFC. To do so, we assessed the response of mPFC pyramidal neurons to these neurotransmitters, indicating the sensitivity and function of their attendant receptors. Electrophysiological experiments were conducted between PND 70 and 105, with animals weighing 30-40 grams. The electrophysiological procedures were previously described in Bambico et al. (2010) and Gobbi (2001). Briefly, animals were anaesthetized (1.2 g/kg urethane, intraperitoneal). Additional doses were delivered during the procedure to maintain full anesthetic state. Animals were fixed to a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA), the scalp overlying the rostral medial skull was removed, and a burr hole was drilled through the skull around the area of bregma + 2.3 mm. A glass multi-barrel recording pipette (Protech International Inc., Texas, USA) was then lowered into the mPFC (0.5-2.5 mm from the brain’s surface) to record pyramidal neurons, mainly from the dorsomedial (cingulate and prelimbic) region. These neurons were identified based on their steady response to standard short pulses of NMDA (5 nA) and by large amplitudes (0.5–1.2 mV), long durations (0.8-1.2 ms), and single-action
potential patterns alternating with complex spike discharges (Barthó et al., 2004; Gobbi and Janiri, 2006; Labonte et al., 2009). Since there is no published stereotaxic atlas of the Peromyscus brain, which is considerably larger than that of Mus musculus, stereotaxic coordinates were approximated by integration of coordinates from the mouse (Paxinos and Franklin, 2007) and the rat (Paxinos and Watson, 2007) stereotaxic atlases. The location of the last recording was verified histologically in each animal by Pontamine sky blue microinjections. The recording barrel was filled with Potamine sky blue, and adjusted to a signal impedance of 0.5-4 MΩ by breaking the tip. A two molar NaCl solution acted as an automatic balance and was leaked against a 50 MΩ impedance. Drug barrels were filled as follows: DA (50mM in 200 mM NaCl, pH 4-5) was ejected against an impedance of 150 MΩ, retained with a -5 nA current, and ejected as a cation. NMDA (50mM in 200 mM NaCl, pH 8) was ejected against an impedance of 125 MΩ, retained with a 10 nA current, and ejected as an anion. 5-HT (50mM in 165 mM NaCl, pH 4) was ejected against an impedance of 150 MΩ, retained with a -5 nA current, and ejected as a cation. When a neuron displaying a stable firing activity was encountered, the spontaneous firing rate was recorded for at least 5 minutes and the first minute was excluded from analysis to avoid artifacts. Each drug was then ejected for at least 30 seconds, and then withdrawn to re-establish the baseline. Upon drug current withdrawal, the firing rate normally reverts to baseline. Drug-induced changes in firing rate (percentage increase/decrease from pre-drug/baseline activity) was calculated as the difference between the mean firing rate during the ejection phase and the baseline mean firing rate (at least 30 minutes prior to ejection) divided by the baseline mean firing rate. Neuronal responses were sampled across 4-6 drug currents. All electrophysiological recordings were conducted from 2 to 10 PM. Forty-eight animals (33 males + 15 females) underwent electrophysiological procedures. Spontaneous/basal pyramidal firing rate data were obtained from all of these animals (4-7 electrode descents per animal = 307 neurons recorded from males and 196 neurons from females). Some animals representing each group (6-8 animal per group) were used for iontophoretic drug applications. Six to nineteen neurons were recorded per group. Two to three transmitters (1 drug current response each) were tested per neuron. Since animals tested aged from PND 70 to PND 105, a correlational analysis between firing rate and age of animals was done, as well as
maximal excitatory/inhibitory response to drugs and age, without reaching any significant correlations. We therefore did not proceed running covariate analysis. Moreover, extensive studies in our lab suggest that monoaminergic firing activity within this age range is not significantly influenced by age in mice (Gobbi et al., 2001, Bambico et al., 2010)

Statistical analyses. Data are presented as mean ± standard error of the mean (SEM). All data were analyzed using Sigma Plot version 11 (Systat Software, Inc., San Jose, California) and SPSS version 17 (SPSS Inc., Chicago, Illinois) softwares. Following testing for assumptions of data distribution normality (Shapiro-Wilk and Kolmogorov-Smirnov tests) and for variance homogeneity, data sets were accordingly submitted to one-way or two-way analyses of variance (ANOVA) with pairing scheme (match-paired vs. mix-paired) and rearing condition (PD vs. CT) as between-group factors, when assumptions were satisfied. Otherwise, the nonparametric counterpart of these tests were carried out. Electrophysiological data were submitted to Student’s t-test or Mann-Whitney U-test for between-group comparisons or to one-way or two-way mixed design ANOVA (rearing condition x current) when accounting for repeated microiontophoretic current ejections. Tukey’s honestly significant difference (HSD) test was used for multiple post-hoc comparisons. Probability value of p ≤0.05 was considered statistically significant.

RESULTS

PD impairs dyadic social interactions in both sexes

Figure 2 shows that among males, a dramatic reduction of social investigation was observed only when a PD male was matched with another PD male (p<0.01), but not when matched with a CT male (Two-way ANOVA: pairing, F(1,64)=6.574, p=0.013; rearing, F(1,64)=16.240, p<0.001; interaction, f(1,64)=10.570, p=0.002; Fig. 2A). The first mutual contact of PD-PD pairs had significantly longer latencies in comparison to CT-CT or CT-PD pairs (p<0.05) (Kruskal-Wallis, followed by the Mann-Whitney U test, p=0.011; Fig. 2C). In females, however, a reduction of the total time spent in social investigation was measured in all PD animals, regardless of whether they were paired with another PD female (PD-PD) or a non-deprived control (PD-CT) (Two-way
ANOVA: pairing, $F_{(1,74)}=0.376$, $p=0.542$; rearing, $F_{(1,74)}=176.188$, $p<0.001$; interaction, $F_{(1,74)}=1.358$, $p=0.258$; Fig. 2B). Furthermore, the latency to the first mutual contact was prolonged in both CT-PD and PD-PD female pairs in comparison to CT-CT pairs, although achieving significance in PD-PD pairs only ($p<0.05$) (Kruskal-Wallis, $df=2$, $p=0.028$; Fig. 2D).

The reductions in the duration of social investigation observed in all affected animals were not due to increased avoidance or active movement away from the partner (Figs. 2E,F). Rather, passive contact with the partner (physical proximity devoid of any active, investigational behavior) was particularly increased in males (Figs. 2G,H) (PD-PD vs. CT-CT or vs. PD-CT, $p<0.001$; Two-way ANOVA: pairing, $F_{(1,64)}=7.842$, $p=0.007$; rearing, $F_{(1,64)}=19.997$, $p<0.001$; treatment, $F_{(1,64)}=19.042$, $p<0.001$) and females (PD-PD vs. CT-CT or vs. PD-CT, $p<0.05$; Two-way ANOVA: pairing $F_{(1,74)}=0.619$, $p=0.434$; rearing, $F_{(1,74)}=116.821$, $p<0.001$; interaction, $F_{(1,74)}=1.008$, $p=0.319$).

**PD increases aggressive behavior in PD female dyads**

Assessment of offensive and defensive behaviors in antagonistic encounters revealed that PD females in a matched PD-PD pair exhibited dramatically longer duration episodes ($p<0.001$) of aggressive behavior than any other pairs (Fig. 3; Two-way ANOVA: pairing, $F_{(1,74)}=3.584$, $p=0.062$; rearing, $F_{(1,74)}=11.399$, $p=0.001$; interaction, $F_{(1,74)}=6.893$, $p=0.011$).

**PD does not affect anxiety-like thigmotaxis and locomotor activity in the open field, but induces sensitization to the locomotor-activating effect of amphetamine in females**

Contrary to the hypothesis that social behavioural impairments in the SI test may be confounded by anxiety-like reactivity, we found no significant effects of PD in the anxiety-like thigmotactic behavior and locomotor or ambulatory activity (distance traveled) in the open field (OF) test (Figs. 4A,B). At best, there was a trend toward a lower locomotor activity in PD males compared to CT males (Tukey’s test, $p=0.068$; Fig. 4A) and an attenuation of the central zone duration (thigmotaxis) in PD compared to CT mice (Two-way ANOVA: rearing, $F_{(1,40)}=3.66$, $p<0.063$; Fig. 4B). However, when PD was associated with psychostimulant amphetamine treatment (Figs. 4C,D), a significant enhancement of ambulatory activity was observed only in females most detectable on the
7th day of locomotor testing (p=0.029), indicating a sensitized response of the DA system in a sex-specific manner (Fig. 4D).

GLU, DA, and 5-HT fibers coexist in the California mouse mPFC.

Following single immunoperoxidase labeling for photon microscopy and double immunofluorescence for confocal microscopy, the three transmitter systems (GLU, DA and 5-HT) were detected in the California mouse forebrain (Fig. 5). As illustrated in Fig. 5A (left), we demonstrate that VGLUT2 immunoreactivity is strong throughout the forebrain and denser in neocortical layers III-VIa, a distribution also observed in the mPFC (Fig. 5A, middle). At high magnification, VGLUT2 immunofluorescence appears punctate, which is characteristic of axon terminal labeling (Fig. 5A, right). TH immunoreactivity appeared particularly strong in the striatum, the major target of mesencephalic DAergic projections, and much more diffuse in the cerebral cortex (Fig. 5B, left). Despite being present in all cortical layers, TH-positive fibers appeared denser in neocortical layer VI, particularly in the mPFC (Fig. 5B, middle). As detected following anti-SERT immunohistochemistry, 5-HT fibers were found throughout the forebrain (Fig. 5C, left), particularly in the neocortex where they formed a denser network within layer IV. In the mPFC, however, SERT-positive axons were uniformly distributed (Fig. 5C, middle). At high magnification, both DA and 5-HT fibers (Figs. 5B,C, right) appeared as varicose axons intermingled with GLUergic nerve endings. Noteworthy is the fact that no co-localization was observed between the three markers in the mPFC.

PD-induced behavioral impairments are associated with altered glutamatergic transmission in the mPFC

The basal discharge activity of mPFC (cingulate and prelimbic) pyramidal neurons is illustrated in figure 6 (left). The recorded neurons were subdivided into low-spiking (firing rates < 20 Hz) and high-spiking (firing rates > 20 Hz) according to previously established criteria (Barthó et al., 2004; Gobbi and Janiri, 2006). The high-spiking group corresponds to putative interneurons; the low-spiking group corresponds to putative pyramidal neurons (Swadlow, 2003; Barthó et al., 2004; Gobbi and Janiri, 2006) that were further tested in the microiontophoresis experiments. In male PD mice, when
compared to CTs, no significant alterations in basal firing rates of both low-spiking and high-spiking neurons were found (Fig. 6). However, low-spiking pyramidal neurons from PD females showed significantly lower firing rates than in CT females (t=329, p=0.008).

In order to assess the functional integrity of GLU neurotransmission in the mPFC, we evaluated the sensitivity of pyramidal neurons to NMDA that was administered microiontophoretically (Gobbi and Janiri, 2006). Under control conditions, the responses to increasing microiontophoretic infusion of NMDA were higher in female than in males (Fig. 7, upper panel). However, in comparison to their respective CTs, the excitatory response of mPFC pyramidal neurons to increasing NMDA currents was significantly enhanced in both PD males (Two-way ANOVA: rearing $F_{(1,100)}=9.680$, $p=0.002$; current, $F_{(3,100)}=0.595$, $p=0.620$; interaction, $F_{(3,100)}=0.453$, $p=0.982$) and PD females (Two-way ANOVA: rearing $F_{(1,36)}=18.661$, $p<0.001$; current, $F_{(3,36)}=7.234$, $p<0.001$; interaction, $F_{(3,36)}=0.066$, $p=0.977$) (Fig. 7, lower panel).

**PD did not affect serotonergic neurotransmission in the mPFC**

In order to further explore the neurobiological substrates of the above-described behavioral deficits, we investigated whether 5-HT transmission in the mPFC was altered by PD. Microiontophoretic application of 5-HT in the mPFC mostly produces inhibitory neuronal responses that rely on 5-HT$_{1A}$ receptor activation (Labonte et al., 2009). We assessed the inhibitory responses of 5-HT and found neither between-group (CT vs. PD), nor sex differences (Fig. 8), suggesting that PD-induced behavioral impairments do not depend on 5-HT transmission in the mPFC.

**PD-induced behavioral deficits are associated with abnormal synaptic processing of dopamine in the mPFC**

In males, low DA currents (5-30 nA) slightly decreased baseline mPFC pyramidal activity, but with no significant difference between CT and PD males (Fig. 9). In females, however, microiontophoretic injections of DA produced a current-dependent increase in pyramidal activity. Most interestingly, significantly lower excitatory responses were recorded in mPFC neurons of PD females compared to CT females (Two-way ANOVA: rearing, $F_{(1,42)}=6.825$, $p=0.012$; current, $F_{(3,42)}=4.272$, $p=0.01$; interaction, $F_{(3,42)}=0.849$, $p=0.475$; Fig. 9).
DISCUSSION

In the present study, we demonstrate that paternal deprivation (PD) in California mice, particularly during critical developmental periods, leads to impaired social and behavioral functions in adults. PD-induced behavioral impairments were more pronounced in females and associated with modifications in dopamine (DA) and glutamate (GLU) neurotransmission in the mPFC, an area long propounded as essential to normal psycho-affective and social development.

PD particularly increased initial mutual contact latencies and aggressive episodes in females. Social investigation was also impaired, as indicated by significant reductions in body sniffing, trailing and crawling-over. Our data suggest that these social impairments were neither a result of alterations in impulse control nor from increased anxiety, since anxiety-like behaviors and locomotor activation in the OF test were unaffected by PD. Social deficits were also unlikely to have resulted from a dysfunctional switching to evasive behavior because PD failed to influence active social avoidance, while dramatically increasing passive contact. PD animals, notably females, were also sensitized to the locomotor-activating effects of amphetamine, suggesting that DA neurotransmission might contribute to impairments in social behavior in female PD mice. This is of particular interest since, by contrast, repeated maternal separation, that mimics early life deprivation, has shown controversial and equivocal outcomes on different measures of stress, cognition, social behavior and hypothalamic-pituitary-adrenal axis regulation (Millstein and Holmes, 2007; Hulshof et al., 2011).

An interesting facet of our data is that under the testing conditions adopted (same-sex dyad; neutral, non-home cage environment) the observed social impairments were more profound in females. PD females exhibited most behavioral abnormalities in the SI test regardless of their pairing with another PD or CT female. By contrast, PD males showed reduced social investigation and increased passive contact only when matched with another PD mouse. These observations appear to be consistent with those observed among human patients with social cognitive and personality disorders (such as antisocial personality disorder), whereby symptoms may be maintained by mutually reinforcing social interactions (Russel and Hersov, 1983). We also observed dramatically greater episodes of aggressive behavior in match-paired PD-PD females than in other pairings,
suggesting that females are more sensitive to PD. It has been reported that paternal behaviors occurring later during the pre-weaning stage, such as pup retrieving and grabbing but not huddling and grooming, positively correlate with aggressiveness (Bester-Meredith et al., 1999; Bester-Meredith and Marler, 2001, 2003; Frazier et al., 2006). The fact that the observed increase in offensive behavior was exhibited only by PD-PD matched females suggests that these behaviors were most likely aggressive rather than play-fighting. In keeping with these findings, Trainor and co-workers (2010, 2011, 2013) showed that, under a significant stress load, female California mice unlike their male counterparts show a decrease in social interaction and an increase in aggressive behaviour. On the other hand, Frazier et al. (2006) found that experimentally increasing paternal retrieval behavior leads to increased territorial aggression, with shorter attack latencies in both male and female offsprings in the resident-intruder paradigm. However, this effect was within a normal range of variation and was not observed in a neutral environment. Decreased paternal grooming also led to enhanced levels of corticosterone.

It is also conceivable that PD-induced female aggression and social deficits are exacerbated by a lack of social play stimulation in the absence of the father. Social play, including play-fighting (boxing/wrestling and pinning), which peaks before early puberty, contributes to the development of adult social competence in rodents (Bell et al., 2009; Bell et al., 2010). Although, both the father and the mother may participate in social play (Wilson and Kleiman, 1974), fathers exhibit greater propensities for it than mothers, as demonstrated in rodents (Guerra et al., 1999; Becker et al., 2005) and primates (Redican and Mitchell, 1973). Parental absence or low parental licking and grooming experience results in altered play behavior throughout the weaning period, but appears to drive male rodent offsprings to compensate in social play by increasing initiations and thus solicit more playful interactions (Becker et al., 2005; Parent and Meaney, 2008).

Some effects observed in offsprings may not necessarily be directly related to the absence of the father, but indirectly to the stress imposed on the mother by the father’s absence (Gubernick and Alberts, 1987; Gubernick and Teferi, 2000). Our data do not rule out this possibility. We note, however, that from the delivery to the weaning we did not observe any alteration in the mothers’ overt behaviour that might be consistent with stress-induced effects (data not published, behavior was observed daily for 30 minutes,
comparing gross behavior in single mothers vs. coupled mothers). These effects may therefore occur under conditions of low resource availability and high-foraging demand, which have been shown to impact parenting strategies and offspring development (Bredy et al., 2007). Remarkably, as mentioned before, California mouse females do not typically compensate for mate (father) absence with increased maternal care (Dudley, 1974; Bester-Meredith and Marler, 2003), suggesting that the impact of altered maternal behavior over the PD is minimal.

Overall, the observed PD-induced behavioral deficits are consistent with epidemiological studies in children raised without a father, highlighting an increased risk for deviant behavior and criminal activity, substance abuse, impoverished educational performance and mental illness. Accordingly, the direct engagement of the father with the child, including playful interaction, has been found to reliably predict later positive childhood outcomes, intellectual and linguistic competence, or decreases in adolescent delinquency even after accounting for the mother’s contributions (Coley and Medeiros, 2007; Sarkadi et al., 2008; Cabrera et al., 2009; Lamb 2010). A positive father-child interaction in early life or during adolescence has been documented to influence children’s prosocial development, predicting later popularity, social skills, peer relationship competence and spousal relationship adjustment (Gottman et al., 1997; Lieberman et al., 1999; Parke et al. 2004; Rah and Parke, 2008; Lamb, 2010). The notion that females, compared to males, are more profoundly affected by low paternal care has also been supported by a stronger association with adult antisocial personality traits (Reti et al., 2002).

Our anatomical investigations revealed dense innervations of GLUergic, 5-HTergic and DAergic fibers in the prelimbic and cingulate regions of the mPFC. 5-HTergic fibers are uniformly dense throughout the mPFC, while DAergic fibers are most dense in layer VI. This suggests that the activity of mPFC pyramidal neurons is greatly regulated by GLU, DA and 5-HT transmission via their postsynaptic cognate receptors expressed on these neurons. A significant perturbation in the signaling of these transmitters is a parsimonious explanation for the observed aberrations in the sensitivity of these receptors examined here. Indeed, our electrophysiological findings suggest that impairments in social behavior and potentiation of aggressive behavior are underpinned
by modifications in the function of both DA and GLU, but not of 5-HT, synapses in the mPFC. The lack of effect on 5-HT is consistent with the lack of effect on anxiety-like behaviours, in which 5-HT plays a major modulatory role. On the other hand, cortical DAergic and GLUergic systems are engaged in a close functional crosstalk, and evidence for a role of DA on social behavior and aggression has accumulated over the years (Baskerville and Douglas 2010). Since DA and NMDA receptors also exert organizational functions in brain development, aberrations of these transmitter systems at any critical time point, following early life stress and/or parental neglect, could persistently perturb their integrity in adulthood and consequently their behavioral response to social stimuli (Frederick and Stanwood, 2009; Baskerville and Douglas, 2010).

PD-induced reductions in mPFC pyramidal response to DA, which were evident in females, occurred in parallel with significant deficits in social behavior and increases in aggressive behavior, and more notably with an enhanced sensitization to amphetamine. The concordance of these behavioral and neurobiological abnormalities supports the fact that the mPFC may serve as a central junction where DA signals conveying stress adaptation, social motivation and reward are simultaneously processed, and that disruption of one pathway may disturb the others (Aragona et al., 2007). The decreased basal and DA-evoked pyramidal excitability in PD females points to a functionally hypoactive mPFC that may be argued to undermine both prefrontal facilitation of prosocial behaviour and inhibitory control over drug-seeking behavior. Such hypoactive mPFC may have been brought about by a desensitization/downregulation of pyramidal D1 receptors and/or upregulation of pyramidal D2 receptors, thus disturbing the balance of D1 and D2 activity that exert opposite effects on neural excitability (Tseng and O'Donnell 2004). Indeed, pharmacological activation of D1 receptors in the prefrontal cortex, but not in the striatum, enhances social cognition (Di Cara et al., 2007; Loiseau and Millan, 2009), while intra-mPFC infusion of D1 antagonists increases nucleus accumbens-dependent stress response (Doherty and Gratton, 1992). Likewise, early social isolation, as well as psychostimulant challenge, have been found to increase i) D2 receptor expression in mPFC (Han et al., 2012), ii) D2 intracellular activation with greater effects in females (Sun et al., 2010), and iii) impairment in D2 function as
observed in prefrontal cortex of psychostimulant-sensitized rats (Kroener and Lavin, 2010). In studies employing the social defeat paradigm, in vivo electrophysiological recordings revealed an increased discharge activity in DAergic neurons of the ventral tegmental area (VTA) from stress-vulnerable animals, an effect also associated with a socially withdrawn phenotype (Krishnan et al., 2007; Cao et al., 2010). Interestingly, PFC DAergic transmission between male and female CTs is clearly different, and that PD in females decreases DA receptor sensitivity to the level of male CTs. This difference between males and females might be explained by sex-dependent variations in D1 and D2 receptor density in the mPFC, as suggested by D1-like receptor binding being greater in females than in males in the mPFC of monogamous prairie voles, while D2-like receptor binding being greater in males (Smeltzer et al., 2006).

Finally, we noted that the excitatory response of mPFC neurons to NMDA was significantly enhanced in PD animals. This response profile likely reflects a compromised integrity of pyramidal neuronal synapses because the basal firing rate of the low-firing subpopulation of mPFC pyramidal neurons were significantly reduced in females, which appeared particularly vulnerable to PD in the SI test. Indeed, sensitized GLUergic activity or upregulation of NMDA receptor subunits in the mPFC are induced after glucocorticoid administration, following chronic stress (Martin and Wellman, 2011) or by acute or chronic psychostimulant exposure (Russo et al., 2010). Changes in NMDA receptors also correlate with synaptic remodeling and dendritic atrophy in the mPFC of stressed animals (Martin and Wellman, 2011). The hypothesis that neuroplastic changes may result from PD is additionally supported by findings of significant reductions in apical spine length and number of pyramidal neurons in the PFC of Octodon degus, another monogamous murid species (Ovtscharoff et al., 2006; Helmeke et al., 2009). The sex-dependent impact of PD on mPFC behavioural and neurophysiological integrity seems to diverge from recent findings regarding the lack of sexual dimorphism in the PFC of monogamous rodents (Kingsbury et al., 2012), although dimorphism in chemo-architecture cannot be ruled out. As such, California mice may be far from displaying sex differences in emotional reactivity and in response to stress (Kingsbury et al., 2012). However, some monogamous species like the prairie vole do exhibit sexually dimorphic structural, hodological or functional features of the PFC (Kingsbury et al., 2012), as may be the case...
with California mice. Indeed, female California mice relative to males exhibit glucocorticoid hyper-responsiveness (Trainor et al., 2010, 2011, 2013). This could be attributed to extra-hypothalamic structures modulating the neuroendocrine response to stress. Indeed, along with the hippocampus, the prelimbic and cingulate mPFC are known to inhibit the hypothalamic-pituitary adrenal axis and negatively regulate corticosterone release (Radley et al., 2006), and that this brain stress axis is particularly sensitive in female California mice, an area that could be a subject of further investigation.

In summary, our results emphasize the importance of father during critical neurodevelopmental periods, and that father absence induces impairments in social behavior that persist to adulthood, deficits which are associated with sex-dependent dysregulation of neurotransmission in the mPFC.
Statement of disclosure and conflicts of interest

The authors do not have any conflicts of interest or any financial involvements or other activities to disclose, which may potentially bias the conduct, interpretation or presentation of this work.

Acknowledgment

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**Figure legends**

**Figure 1. Experimental design.** The chronogram shows a simplified organization of events pooled from separate experiments for 2 different batches of animals obtained from 2 groups of breeding pairs. After mating that commenced on Day 1, a month was allotted to obtain a sufficient number of offsprings that were subsequently assigned at PND3 to the father-deprived (PD) group or the biparental (CT) group (maximal age range = 30 days). The offsprings were weaned at PND30, and then housed in same-sex pairs. Upon reaching a sufficient number of animals, the open field (OF) and social interaction (SI) tests were conducted on Day 70 (animals at PND70-PND100). Some of these animals were used for electrophysiological experiments from Day 72 to Day 105 (animals were at PND72 to PND105). Some animals that did not undergo the OF and SI tests were also used for electrophysiology. Prior exposure to the OF, SI and similar behavioural tests does not produce detectable changes in monoaminergic neural activity as we have previously demonstrated (Bambico et al., 2010). Another group was used for the amphetamine (amph) sensitization experiments (PND72-PND102), which was not further tested for electrophysiology.

**Figure 2. Paternal deprivation (PD) led to marked impairments in several aspects of adult social behavior, as measured in the social interaction (SI) test.** Father-deprived (PD) male and female mice showed significant reductions in the time spent investigating another PD mouse (sniffing, trailing and crawling-over; stacked bars: black, gray and white, respectively) (A, B). Female PD mice also exhibited this reduction in social investigation (B) when interacting with non-deprived (control, CT) mice. The latency to first contact was also significantly increased in PD-PD pairs of both sexes (C, D); female CT-PD pairs showed a slight (non-significant) increase in the latency to first contact. There was no change in the time spent actively avoiding contact across parings and between sexes (E, F) but passive contact was increased in females and in PD males (G,H). Bars represent mean duration (in sec) ± standard error of mean (SEM). Male CT (paired with male PD), n=21; male PD (paired with male CT), n=18; male CT (paired with male CT), n=21; male PD (paired with male PD), n=8; Female CT (paired with female PD), n=25; female PD (paired with female CT), n=28; female CT (paired with female CT), n=15; female PD (paired with female PD), n=10. * p<0.05, ** p<0.01.
Figure 3. Paternal deprivation (PD) influenced aggressive behaviour in a sex-dependent manner. PD significantly increased episodes of aggressive behaviour only in female PD mice partnered with another female PD mouse (B). There was no difference observed in male mice (A). Bars represent mean duration (in sec) ± standard error of mean (SEM). Male CT (paired with male PD), n=21; male PD (paired with male CT), n=18; male CT (paired with male CT), n=21; male PD (paired with male PD), n=8; Female CT (paired with female PD), n=25; female PD (paired with female CT), n=28; female CT (paired with female CT), n=15; female PD (paired with female PD), n=10. ** p<0.01.

Figure 4. Basal locomotor activity (distance traveled in cm) and in thigmotaxis (total duration spent in the central zone) were not affected by PD. However, the distance that male father-deprived (PD) mice traveled was slightly less than that traveled by male non-deprived (control, CT) (p=0.068). (A). Male and female PD mice also showed slight, non-significant attenuations in the total time of central zone visits (slight thigmotaxis, B). Male CT, n=17; Female CT, n=11; Male PD, n=7; Female PD, n=9. In testing for differences in the sensitized response to a psychostimulant, amphetamine was injected daily for 7 days. Immediately after the 7th treatment, locomotor activity (distance traveled in cm) in the OF was increased in comparison to control condition (saline injection) (C, D); paternal deprivation (PD) enhanced this locomotor-activating effect of amphetamine only in females (D). Bars represent mean distance traveled (cm) or mean duration (in sec) ± standard error of mean (SEM), n=6-8. * p<0.05, ** p<0.01.

Figure 5. Immunohistochemical detection of GLU, DA and 5-HT innervations in the California mouse forebrain and mPFC. Single immunohistochemical labelings (left panels) revealed with DAB (brown precipitate) or VIP (purple precipitate) demonstrate the coexistence of VGLUT2-positive (A), TH-positive (B) and SERT-positive (C) nerve fibers in the California mouse forebrain. Whereas 5-HT innervation (SERT+) appeared diffuse in the mPFC (C, middle), GLUergic (VGLUT2+) fibers were denser in cortical layers III-Via (A), and DAergic (TH+) fibers denser in layer VI (B). As illustrated for layer V of the mPFC (PrL) following double immunolabeling at the confocal level.
(A,B,C, right panels), GLU (green), DA (red) and 5-HT (red) axons appeared intermingled and not overlapping.

**Figure 6. Effect of paternal deprivation (PD) on the basal firing activity of medial prefrontal cortex (mPFC) pyramidal neurons.** Right panel: Graph showing the distribution of basal neuronal firing rates of recorded mPFC pyramidal neurons obtained from PD and control (CT) males (triangle) and females (circle). Neurons were segregated based on firing rate as low-spiking (20 Hz, below dotted line) and high-spiking (above dotted line). Only PD females had significantly lower mean low-spiking rates in comparison to female CTs. Left panel: Stereotactic location of recorded pyramidal neurons in the dorsomedial mPFC of CT males (unshaded triangles), PD males (shaded triangles), CT females (unshaded circles) and PD females (shaded circles). Cg1, cingulate area 1; PrL, prelimbic; IL, infralimbic; MO, medial orbital; DP, dorsal peduncular cortex. Bottom numbers represent plate coordinates in mm anterior to bregma (Paxinos and Franklin, 2007). Male CT, n=196; Male PD, n=111; Female CT, n=81; Female PD, n=115. ** p<0.01.

**Figure 7. Effect of paternal deprivation (PD) on the postsynaptic receptor-mediated excitatory response to N-methyl-D-aspartate of mPFC pyramidal neurons.** Upper left inset: Stereotactic location of the recording sites in the mPFC (cingulate and prelimbic regions, layer V-VI; 0.25-0.75 mm lateral to midline, boxed area) and a representation of the complex spike waveform of pyramidal neurons (black bar=10 ms duration, scaled). Top panels: Line graphs showing between-group difference (father-deprived, PD: black circles; non-deprived controls, CT: white squares) in the response of mPFC pyramidal neurons (ordinate, percent increase in excitatory response) to increasing microiontophoretic currents of NMDA (abscissa), with PD increasing excitatory response in both males (left) and females (right). Bottom panels: Representative integrated firing rate histograms showing the activity of mPFC pyramidal neurons (ordinate, spikes/10 s) plotted against time (abscissa). The response to microiontophoretic currents (values on top of horizontal bars) of NMDA is significantly increased by PD in both males (PD compared with CT) and females (PD compared with CT). N=6-22/current data point. ** p<0.01.
Figure 8. Effect of paternal deprivation (PD) on the postsynaptic receptor-mediated excitatory response to serotonin (5-HT) of mPFC pyramidal neurons. Upper left inset: Stereotactic location of the recording sites in the mPFC (cingulate and prelimbic regions, layer V-VI; 0.25-0.75 mm lateral to midline, boxed area) and a representation of the complex spike waveform of pyramidal neurons (black bar=10 ms duration, scaled). Top panels: Line graphs showing an absence of between-group difference (father-deprived, PD: black circles; non-deprived controls, CT: white squares) in the response of mPFC pyramidal neurons (ordinate, percent increase in excitatory response) to increasing microiontophoretic currents of 5-HT (abscissa), observed in both males (left) and females (right). Bottom panels: Representative integrated firing rate histograms showing the activity of mPFC pyramidal neurons (ordinate, spikes/10 s) plotted against time (abscissa). The response to microiontophoretic currents (values on top of horizontal bars) of NMDA was not changed by PD in both males (PD compared with CT) and females (PD compared with CT). N=4-8/current data point.

Figure 9. Effect of paternal deprivation (PD) on the postsynaptic receptor-mediated excitatory response to dopamine (DA) of mPFC pyramidal neurons. Upper left inset: Stereotactic location of the recording sites in the mPFC (cingulate and prelimbic regions, layer V-VI; 0.25-0.75 mm lateral to midline, boxed area) and a representation of the complex spike waveform of pyramidal neurons (black bar=10 ms duration, scaled). Top panels: Line graphs showing between-group difference (significant attenuation after PD) in the response of mPFC pyramidal neurons to increasing microiontophoretic currents of DA occurring in females (right) but not in males (left). Bottom panels: Representative integrated firing rate histograms showing the significantly blunted response of mPFC pyramidal neurons to microiontophoretic currents of DA in PD females (compared to CT) but not in males. N=4-21/current data point ** p<0.01.
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130x80mm (300 x 300 DPI)
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176x146mm (300 x 300 DPI)