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The Role and Properties of Neurons in the Medial Preoptic Area in Paternal Care of California Mice *(Peromyscus californicus)*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

Nathan Horrell

September 2019

Dissertation Committee:
Dr. Wendy Saltzman, Chairperson
Dr. Khaleel Razak
Dr. Peter Hickmott

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The Dissertation of Nathan Horrell is approved:

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ABSTRACT OF THE DISSERTATION

The Role and Properties of Neurons in the Medial Preoptic Area
in Paternal Care of California Mice *(Peromyscus californicus)*

by

Nathan Horrell

Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, September 2019
Dr. Wendy Saltzman, Chairperson

The neurobiological basis of and factors facilitating male parental behavior are not well understood in mammals. Multiple rodent species show increases in parental behavior during the transition into parenthood or after prolonged exposure to pups. For the first time, we evaluated effects of prior exposure to pups on paternal responsiveness in the biparental California mouse (*Peromyscus californicus*). Adult virgin male California mice exhibit aggressive or indifferent responses to pups more frequently than fathers, which exhibit robust paternal care. We analyzed behavioral, neural, and corticosterone responses to pups in adult virgin males that were interacting with a pup for the first time, adult virgin males that had been exposed to pups 3 times for 20 min each in the previous week, and first-time fathers. Control groups of virgins were similarly tested with a novel object (i.e., a marble). Previous exposure to pups increased paternal care: responses to pups did not differ between virgins with repeated exposure to pups and new fathers. Neither basal corticosterone levels nor corticosterone levels following acute pup or marble exposure differed among groups. Finally, Fos expression in the medial preoptic

area (MPOA) and bed nucleus of the stria terminalis was higher following exposure to a pup than to a marble. Fos expression was not, however, affected by previous exposure to these stimuli. Results suggest that previous experience with pups can increase paternal responsiveness via unknown mechanisms and implicate the MPOA in processing pup cues. In my experiment, we evaluated synaptic, intrinsic, and morphological properties of MPOA neurons in male virgins or first-time fathers using standard whole-cell recordings in a novel *in vitro* slice preparation. We measured synaptic currents in response to local electrical stimulation, and we quantified intrinsic excitability by measuring voltage changes in response to square-pulse injections of both depolarizing and hyperpolarizing current. We also analyzed the morphology of MPOA neurons. Most parameters did not differ significantly between virgins and fathers. However, we documented a decrease in synaptic inhibition in fathers. These findings suggest that the onset of paternal behavior in California mouse fathers may be associated with limited electrophysiological plasticity within the MPOA.

Table of Contents

List of Figures

List of Tables

Introduction

Some of the earliest influential work on the biology of parental care was done on parental investment and kin selection by Fisher (1930) and Hamilton (1964), respectively, but Robert Trivers' *Parental Investment and Sexual Selection* (1972) is often cited for a seminal, formal definition: parental investment is "any investment by the parent in an individual offspring that increases the offspring's chance of surviving (and hence reproductive success) at the cost of the parent's ability to invest in other offspring." Later, in Clutton-Brock's influential work *The Evolution of Parental Care* (1991), parental care is defined as "any form of parental behavior that appears likely to increase the fitness of a parent's offspring," while noting that non-behavioral aspects of physiology such as viviparity and gestation, are sometimes implied.

As with many concepts, the boundaries of what constitutes parental care are fuzzy. Burghart (2012) has criticized Clutton-Brock's definition, saying there is parental care that can decrease the offspring's fitness and/or optimal development (e.g., feeding unhealthy diets, corporal punishment) and therefore, definitions of parental care should not include considerations of fitness. Simmons and Parker (1989) and Wedell (1993) raise concerns about differentiating parental care from courtship; some behaviors that increase offspring fitness may have evolved for other reasons, like attracting a female, making the increased fitness of offspring somewhat of a by-product or spandrel. For example, in some species, male territoriality and provisioning of nuptial gifts to females may have evolved for mating, but holding the best territories and giving the most and/or highest-quality nuptial gifts may increase number, development, and survival of offspring

(Simmons and Parker, 1989; Wedell, 1993; Smiseth et al., 2012). Smiseth, Kölliker and Royle (2012) suggest such territoriality and provisioning should not be considered parental care unless there is evidence that it originated or is maintained due to beneficial effects on offspring. For this reason, they espouse a definition of parental care "that includes non-behavioral traits and excludes parental traits that incidentally increase offspring fitness" so that parental care is defined as "any parental trait that enhances the fitness of parent's offspring and that is likely to have originated and/or to be currently maintained for this function." According to this recent definition, parental care can be any trait that was naturally selected to increase the fitness of the parent's offspring: traits include parental behaviors and aspects of physiology (e.g., gamete production, viviparity and gestation). Colloquially, "parental care" gestures vaguely, and yet with great meaning and utility, at parental behavior that has positive effects on offspring.

"Parental care" can be thought of as a blanket or umbrella term referencing a variety of types of parent-offspring interactions. Kleiman (1977) may have been the first to formally describe direct and indirect forms of parental care; direct parental care involves immediate physical interaction with offspring (e.g., nursing, grooming, transporting, socializing, thermoregulating, etc.) while indirect parental care involves behaviors often exhibited in the absence of offspring, but still to their benefit (e.g., resource gathering, defense, nesting, etc.). "Alloparental care," care of young provided by adults other than the genetic parents, was coined by E.O. Wilson (1975), while early descriptions of allomaternal care are reported by Fraser Darling (1938) and Rowell, Hinde and Spencer-Booth (1964), who use the term "auntie" to refer to non-mothers

exhibiting care to offspring, with no genetic relationship necessarily implied. More recently, Rosenblatt and Mayer (1995) have posited theories of neurocircuitries mediating approach to and withdrawal from offsping while Numan (2014) has proposed theories of neurocircuitries mediating appetitive (e.g., retrieval) and consummatory (e.g., thermoregulating and grooming) offspring-directed behaviors in rodents.

The categories of behavior mentioned above, from the broadest (i.e., parental care) to the more specific (i.e., direct, indirect, alloparental, approach, withdrawal, appetitive, consummatory), are abstract types of behavior. The neural architecture and neural activity that are cause the concrete tokens of these abstract types of behavior may involve activation of quite different neural populations across taxa. For example, the indirect parental care behavior of nest building in some fish, birds, and rodents involves different motor neuron activity in different species; similarly, the direct parental care behavior of provisioning has different neural architecture and activity tokens in different species. In some taxa, the ancient drive to provide parental care evolved and proliferated into a manifold of neural architectures and behaviors, some of which may be somewhat conserved and some of which is taxon-specific

Parental care is thought to have evolved convergently in several different animal taxa (Rosenblatt and Snowdon 1996; Mank et al. 2005; Cockburn 2006). The evolutionary origin of parental care seen in contemporary mammals has been traced back hundreds of millions of years: prototypical mammary glands of ancient synapsids likely provided nutrients to and prevented desiccation of eggs >300 million years ago (Oftedal, 2002b; 2012). Mammalian ancestors started producing nutrient-dense milk for suckling

>210 million years ago (Oftedal, 2002a; 2012; Capuco and Akers, 2009). To put the evolution of lactation in temporal perspective, the ancestors of mammals started nursing before the stegosaurus and brachiosaurus existed.

Investigations into the origins of parental behavior in mammalian ancestors, beyond (proto)lactation, rely on fossil records, where a mixture of large (presumably parents) and small (presumably offspring) fossilized organisms arranged in a particular fashion (e.g., huddled), are interpreted as evidence of parental care (for review, see Botha-Brink and Modesto, 2007). Such inferences date direct parental care to >260 million years ago (Retallack et al., 2006; Botha-Brink and Modest, 2007). If that inference is true, natural selection has been changing and fine-tuning the neurocircuitry of complex parental care behaviors exhibited by mammals for hundreds of millions of years.

Parenting, regardless of the fuzziness of the general concept, multiple behavioral and physiological subcategories, diverse, idiosyncratic tokens exhibited across taxa, and murky evolutionary origins, is an ancient, innate, and necessary drive for many species, including all mammals. In contrast to the majority of extant animals, parental care is necessary for offspring survival in mammals (Smiseth et al., 2012). If all maternal and allo-maternal behavior abruptly ceased in mammals, it is likely that every mammalian species, except one, would go extinct in one generation, in no small part to the absence of lactation. The only mammalian species that could survive the cessation of maternal care is humans, due to men being able to feed formula to infants. Two species of old-world

fruit bats are known to commonly exhibit male lactation, but it is unlikely that male nursing is sufficient for offspring survival (Kunz and Hosken, 2009).

Within mammals, substantive direct parental care by males (i.e., paternal care) is exhibited by only ~10% of species, including some rodents, carnivores, and primates (Clutton-Brock, 1991). While all mammals are thought to have a common maternal ancestor, paternal mammals evolved convergently, suggesting that mechanisms of maternal behavior may be relatively conserved while those of paternal behavior have the potential to be relatively taxon-specific.

Because maternal care is necessary for survival and optimal development of all extant mammals and has been for millions of years; it is commonly referred to as a drive or basic motivation of all mammals. Neural models of ancient, innate, practically universal, mammalian drives center on the hypothalamus. Parental-care circuitry has the architecture and hormonal influence characteristic of the ancient motivations/drives (Bandler and Shipley, 1994; Sewards and Sewards, 2003; Numan and Insel, 2013; Sternson, 2013); i.e., particular drives, motivational states, or motivation-action-perception (MAP) schemas have particular hypothalamic subnuclei and hormones strongly associated with them (Sewards and Sewards, 2003). Categories of drives can be roughly broken down into ingestive and defensive self-maintenance (i.e., hunger, thirst, aggression, thermoregulation, fear) and self-propagation (i.e., sex and parental care). Particular hypothalamic subnuclei, and often hormones, are strongly associated with each drive. Somewhat labeled-line circuits are thought to exist in the hypothalamus, the activity of each being the gain control for the fundamental drives. For over-simplified

examples: the arcuate and lateral hypothalamus are associated with hunger and influenced by leptin and ghrelin, the ventrolateral preoptic area is associated with sleep and influenced by melatonin, the medial preoptic area and ventromedial nucleus are associated with sexual behavior and are influenced by gonadal hormones, the hypothalamic attack area and ventrolateral part of the ventromedial hypothalamus are implicated in aggression and influenced by gonadal hormones. Obviously, the models as described here are extremely simplistic, yet they have significant explanatory power.

In sum, thousands of investigations into the neurobiological basis of ancient, universal mammalian drives have resulted in a simplified theory of functional connectivity centered on the hypothalamus: circulating hormones and processed sensory information converge to influence hypothalamic activity in particular subnuclei that project to many nuclei, including nuclei in the limbic and reward systems, altering the salience and valence/hedonic tone of stimuli relevant to the particular drive. This functional connectivity is thought to be a large part of how a MAP schema emerges (Peterson, 2013).

The hypothalamic subnucleus most closely associated with (allo)parental care in mammals is the medial preoptic area (for review see Horrell et al., 2018; Numan and Insel, 2013; and below). In some species, males can display infanticide, indifference, or paternal care within one lifetime. The neuroplasticity that underlies the variability in the paternal drive is largely unknown. Much of my research has focused on the role and properties of neurons in the medial preoptic area in the paternal care of biparental California mouse, *Peromyscus californicus*.

Chapter 1

Effects of repeated pup exposure on behavioral, neural, and adrenocortical responses to pups in male California mice (Peromyscus californicus)

Abstract

In biparental mammals, the factors facilitating the onset of male parental behavior are not well understood. While hormonal changes in fathers may play a role, prior experience with pups has also been implicated. We evaluated effects of prior exposure to pups on paternal responsiveness in the biparental California mouse (*Peromyscus californicus*). We analyzed behavioral, neural, and corticosterone responses to pups in adult virgin males that were interacting with a pup for the first time, adult virgin males that had been exposed to pups 3 times for 20 min each in the previous week, and new fathers. Control groups of virgins were similarly tested with a novel object (marble). Previous exposure to pups decreased virgins' latency to approach pups and initiate paternal care, and increased time spent in paternal care. Responses to pups did not differ between virgins with repeated exposure to pups and new fathers. In contrast, repeated exposure to a marble had no effects. Neither basal corticosterone levels nor corticosterone levels following acute pup or marble exposure differed among groups. Finally, Fos expression in the medial preoptic area, ventral and dorsal bed nucleus of the stria terminalis was higher following exposure to a pup than to a marble. Fos expression was not, however, affected by

previous exposure to these stimuli. These results suggest that previous experience with pups can facilitate the onset of parental behavior in male California mice, similar to findings in female rodents, and that this effect is not associated with a general reduction in neophobia.

1. Introduction

In both females and males of some rodent species, parental behavior (i.e., nurturant behavior toward immature individuals) can occur outside of typical reproductive conditions via continuous or repeated exposure to infants, a process called "concaveation" or, more commonly, "sensitization." Adult, sexually naïve (i.e., virgin) female rats (*Rattus norvegicus*), for example, typically avoid pups upon first exposure, but display maternal behaviors after repeated or continuous exposure (Bridges et al., 1972; Fleming and Rosenblatt, 1974; Jakubowski and Terkel, 1985a, 1985b; Lonstein et al., 1999; Quadagno et al., 1974; Reisbick et al., 1975; Rosenblatt, 1967; Stern and Mackinnon, 1976; Wiesner and Sheard, 1933). In contrast to rats, adult virgin female house mice (*Mus musculus*) frequently exhibit maternal behavior upon their first exposure to pups and are often described as "spontaneously maternal" (Gandelman, 1973; Leussis et al., 2008; Martín-Sánchez et al., 2015; Noirot, 1969; Stolzenberg and Rissman, 2011; Stolzenberg et al., 2012); however, repeated or continuous exposure to pups can increase measures of maternal behavior in virgin females even more (Alsina-Llanes et al., 2015; Brown et al., 1999; Ehret and Koch, 1989; Ehret et al., 1987; Pedersen et al.,

2006). Virgin female prairie voles (*Microtus ochrogaster*) may attack, ignore, or care for foster pups at first exposure (Bales et al., 2007; Lonstein and De Vries, 2001), and exposure to pups in adolescence increases some aspects of maternal care in adulthood (Lonstein and De Vries, 2001), similar to rats (Stern and Rogers, 1988). In virgin female Syrian golden hamsters (*Mesocricetus auratus*), continuous exposure to pups often changes infant-directed behavior from infanticidal to maternal within a few days (Noirot and Richards, 1966; Swanson and Campbell, 1979).

Effects of repeated or continuous exposure to pups on paternal care by male rodents have received less attention than those on maternal care. Adult virgin male rats (Bridges et al., 1972; Jakubowski and Terkel, 1985b; Rosenblatt, 1967), mice (Ehret et al., 1987), and golden hamsters (Swanson and Campbell, 1979) can be sensitized to show parental care, with sensitization latencies longer than those of females. These species, however, may not be optimal models for understanding paternal behavior, as male rats, mice, and golden hamsters do not typically provide care for their offspring under naturalistic conditions. In the approximately 5–10% of rodents in which fathers provide care for their offspring in the wild (Dewsbury, 1985; Kleiman and Malcolm, 1981), almost nothing is known about the effects of prior exposure to pups.

The California mouse (*Peromyscus californicus*) is a monogamous, biparental rodent in which fathers spend as much time as mothers caring for offspring (e.g., huddling, grooming, and retrieving pups) and typically care for unrelated pups during experimental exposure, while adult virgin males may either attack, ignore, or care for experimentally presented pups (Chauke et al., 2012; de Jong et al., 2009, 2010;

Gubernick and Addington, 1994; Gubernick and Alberts, 1987; Jasarevic et al., 2013; Rosenfeld et al., 2013). Cohabitation with a younger litter increases the likelihood of males behaving paternally toward an unrelated pup in young juveniles (35–45 days of age), but not in older juveniles (55–65 days) or adults (160 days) (Gubernick and Laskin, 1994). However, whether pup exposure during adulthood alters infant-directed behavior in adult male California mice is not known.

In female rodents, one mechanism underlying the onset of maternal behavior in parturient mothers is suppression of fear-, anxiety-, and stress-related responses to infants. Inhibition of hypothalamic-pituitary-adrenal axis and neuronal responses to aversive stimuli occurs during late pregnancy and lactation, and facilitates expression of maternal care (Brunton et al., 2008; Lightman et al., 2001; Slattery and Neumann, 2008). In virgin males of some biparental rodent species, exposure to pups may dampen some stress-related responses: pup exposure decreases plasma corticosterone levels in response to a handling stressor in virgin male prairie voles (Kenkel et al., 2012). Similar stress response-dampening effects of pup exposure might occur in male California mice. In one study, repeated exposure to pups decreased males' behavioral responses to a novel-object open-field test (Bardi et al., 2011); however, other research has found few or no differences in stress response between fathers and virgin male California mice (Chauke et al., 2011, 2012; de Jong et al., 2013; Harris and Saltzman, 2013). The effects of repeated pup exposure on the acute neural and corticosterone responses to pups are unknown. The aim of this experiment was to determine the effects of repeated pup exposure on behavioral, neural and corticosterone responses to pups in adult, virgin male California

mice. To do so, we examined responses to an unfamiliar pup in virgin males that had or had not been exposed to a pup during the preceding week. To control for novelty, we also examined effects of repeated exposure to a novel object on virgin males' subsequent responses to the same object. Finally, we characterized behavioral, neural, and corticosterone responses to pups in new fathers as a positive control. We predicted that repeated pup exposure would increase paternal behavior in virgin males; alter neural responses to pups, as indicated by Fos expression, in brain regions associated with paternal care, stress and/or anxiety; decrease acute corticosterone responses to pups; and possibly decrease basal plasma corticosterone levels.

2. Methods

2.1. Animals

Fifty-three male California mice, descendants of mice purchased as adults from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA), were used. Animals were housed in 44 × 24 × 20 cm polycarbonate cages containing aspen shavings and cotton wool for nesting material, with food (Purina Rodent Chow 5001) and water available ad libitum. Colony rooms were kept on a 14:10 light:dark cycle (lights on from 0500 h to 1900 h). At 27– 33 days of age, prior to the birth of the next litter of siblings, animals were removed from their parents' cage and housed in groups of four same-sex, age-matched littermates and/or unrelated juveniles.

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the University of California, Riverside (UCR) Institutional Animal Care and Use Committee. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. Experimental design

In adulthood (161–231 days of age), each mouse either remained housed with one of the males from its original group of four (virgin males) or was paired with an unrelated female (new fathers). Thereafter, subjects were weighed twice per week to monitor health and to habituate animals to handling.

Beginning at least 14 days (26.59 ± 1.97 days, mean ± SE) after pair formation, virgin males underwent data collection over a 10-day period (days 1–10; Table 1). On day 1, we collected a basal blood sample (see below) from each animal at 1200–1500 h. On day 3, each mouse was exposed to either an unrelated pup or a control object (pup-sized glass marble), or underwent control handling procedures without being exposed to either stimulus. Each animal subsequently underwent the same pup-exposure, marble-exposure, or handling procedures on days 5 and 7. On day 8, a second basal blood sample was collected from all animals. Finally, on day 10, 21 virgin males underwent a 60-minute exposure to an unfamiliar pup; this included the 11 males that had previously been exposed to a pup on days 3, 5, and 7 (repeated-pup condition) and 10 males that had undergone control handling procedures on the same days (single-pup condition). Similarly, 22 males underwent a 60-minute exposure to a marble on day 10, including the

12 animals that had been exposed to a marble on days 3, 5 and 7 (repeated-object condition) and the 10 remaining mice that had undergone control handling procedures on those days (single-object condition). Finally, 10 breeding males (new fathers) were tested with an unrelated pup 5–7 days after the birth of their first litter, as a positive control. Immediately after the 60-minute pup exposure on day 10, all males were decapitated, and blood and brains were collected. Brains were subsequently analyzed for Fos using immunohistochemistry, and blood was assayed for corticosterone (see below).
Males in the 5 experimental conditions did not differ in age (183.24 ± 2.37 days, mean ± SE; $F[1,47] = 2.12$, $p = 0.940$, $\eta^2 = 0.15$; one-way ANOVA) or body mass (46.49 ± 1.0 g, mean ± SE; $F[1,47] = 0.82$, $p = 0.520$, $\eta^2 = 0.06$; one-way ANOVA) at the beginning of data collection on day 1.

2.3. Pup and marble exposure

On days 3, 5, and 7, virgin males in the repeated-pup and repeated object conditions were removed from their home cage between 1200 h and 1500 h and isolated in a clean cage containing bedding, food and water. Animals were tested in new cages to allow testing of both cage mates around the same time under identical conditions. After a 10-minute habituation period, an unfamiliar, unrelated, 1- to 4-day-old pup or a marble was introduced into the corner of the cage farthest from the subject for 20 min. If a subject attacked a pup, the exposure was immediately concluded and the pup was euthanized with pentobarbital (ca. 200–300 mg/kg i.p.; Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI, USA). To control for effects of handling, subjects in the

single-pup and single-object conditions were placed in a clean cage on days 3, 5, and 7 and allowed to habituate for 10 min, after which time a gloved hand touched the bedding in the corner farthest from the subject to mimic placement of a pup or marble. Subjects then remained in the cage for an additional 20 min before being transferred back to their home cage. All exposures were videotaped. Pup and marble exposures on day 10 were conducted identically to the earlier exposures, except that animals in all 5 conditions were exposed for 1 h to either a pup (repeated-pup, single-pup, and new father conditions) or a marble (repeated-object and single-object conditions).

One hour after the beginning of the exposure, subjects were decapitated, and trunk blood and brains were collected immediately. Brains were drop fixed in 4% paraformaldehyde for 2 days before being cryoprotected in 30% sucrose and frozen in Fisher Healthcare Optimal Cutting Temperature compound until being sliced and processed for Fos immunohistochemistry as described below.

2.4. Behavioral analyses

Behavior during pup exposures and novel-object exposures was scored from videos using JWatcher software (Blumstein and Daniel, 2007) as done previously (Chauke et al., 2012; de Jong et al., 2009). For the entire duration of the 20-minute pup exposures (days 3, 5, 7), and for the first 20 min of the 1-hour exposure (day 10), we quantified total durations of sniffing, grooming, and huddling the pup, as well as latencies to perform each of these three behaviors. Grooming and huddling were summed to quantify total time engaged in paternal behavior, and latency to engage in paternal behavior was defined as the latency to groom or huddle, whichever occurred first. For novel-object exposures, latency to approach the marble and total time spent sniffing the marble were quantified.

2.5. Immunohistochemical analyses

Immunohistochemistry was performed as described previously (de Jong et al., 2009): 30 μm brains sections were incubated overnight with rabbit-anti-Fos antibody (sc-253, 1:5000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by donkey anti-rabbit second antibody (1:1500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and then stained with 3,3′-diaminobenzidine (DAB) and ammonium nickel sulfate to mark Fos-positive cells as blue-black in color. Individual brain sections were then mounted on chrome alum gelatin-coated glass microscope slides, dehydrated in ethanol, cleared in xylene, then embedded in entellan (EMS, Hatfield, PA, USA).

Because there is no brain atlas for California mice (although micrographs of Nissl-stained sections are available at brainmaps.org), The Mouse Brain in Stereotaxic Coordinates (Paxinos and Franklin, 2013) for Mus musculus was used to locate brain regions. We focused on regions of the hypothalamus and extended amygdala that have known associations with parental behaviors, stress and/or anxiety (medial preoptic area [MPOA], ventral bed nucleus of the stria terminalis [vBNST], medial amygdala [MEA], anterior hypothalamic nucleus [AHN], paraventricular nucleus of the hypothalamus [PVN], dorsal bed nucleus of the stria terminalis [dBNST]), and ventromedial hypothalamus [VMH] (Klampfl et al., 2016; Numan and Insel, 2003; Pêgo et al., 2010; Smith and Lonstein, 2008). An observer blind to the animal's condition and stimulus took digital photographs of the area with the highest density of Fos immunoreactivity for each brain region in each hemisphere at a magnification of 200× with a digital camera (Canon EOS-40D) mounted on a microscope (Leica Leitz DMRB). ImageJ software (Abramoff et al., 2004) was used to create a 200 × 200 μm square in each photograph that contained either all or the majority of immunoreactive neurons in the brain region. The numbers of Fos-positive cells within the 200 μm square were counted manually and averaged across both hemispheres to produce a measure of neural activity. Due to technical problems, usable Fos-expression data were not available for some of the animals (see Table 4 for final sample sizes).

2.6. Blood collection and corticosterone radioimmunoassay

Basal blood samples were collected from the retro-orbital sinus at 1200–1500 h, under undisturbed conditions, on experimental days 1 and 8. Animals were lightly anesthetized with isoflurane, and blood (70 μL) was collected into heparinized glass capillary tubes in b3 min from initial cage disturbance. On day 10, trunk blood was collected into 0.1 ml heparinized weighing boats after decapitation. All blood samples were centrifuged at 13,300 rpm for 12 min at 4 °C, and the plasma was separated and stored at −80 °C. Plasma corticosterone concentration was determined using a commercially available double-antibody radioimmunoassay kit (07120103; MP Biomedicals, Solon, OH) previously validated for use in California mice (Chauke et al., 2011). The assays were run in accordance with the kit instructions, except that the low end of the standard curve was extended down to 12.5 from 25 ng/ml and the samples were diluted 1:400 instead of 1:200 (dilution based on data from previous studies). The new curve ranged from 12.5 ng/ml (91–93% bound) to 1000 ng/ml (19–20% bound). A total of 3 assays was conducted. To minimize variation, samples from all experimental conditions were represented in each assay, and repeated samples from the same animal were always run in the same assay. Intra- and inter-assay coefficients of variability calculated from in-house quality-control pools were 2.23% and 3.36%, respectively.

2.7. Statistical analyses

All data (behavioral, immunohistochemical, and hormonal) were analyzed using R software (R Development Core Team, 2011) or SPSS (IBM Corp, 2013). Data were tested for normality using the Shapiro-Wilks test. Behavioral data tended to be non-normally distributed and to resist transformations to achieve normality, so between-group comparisons were analyzed using Mann-Whitney U tests or Kruskal-Wallis tests followed by Dunn's post-hoc tests (Dinno, 2016); for these analyses we used a Bonferroni-corrected critical p value of 0.0167 (=0.05/3). We refer to differences with p b 0.0167 as significant and those with 0.0167 b p b 0.05 as nominally significant. Within-group comparisons on behavioral data were analyzed with Friedman tests and Nemenyi post-hoc tests (Pohlert, 2014). Nemenyi's post-hoc test accounts for family-wise error, and no p–adjustment is required (Nemenyi, 1963; Pohlert, 2014). Effect sizes were determined for each statistical test: Cohen's d was calculated as the mean difference divided by the pooled standard deviation. Eta squared (η^2) was calculated as (SSIndependent Variable / SSTotal) and (SSInteraction / SSTotal) where appropriate. Behavioral data are presented as medians ±1st and 3rd quartiles.

Corticosterone and Fos expression data were log-transformed to meet assumptions of normality. Basal corticosterone data were analyzed by a three-way ANOVA (stimulus [pup or marble] × condition [single or repeated exposure] × day [1 or 8]). Fos data and corticosterone data from day 10 were analyzed using two-way

ANOVAs (stimulus × condition) among virgins, and planned comparisons were conducted between fathers and virgins exposed to pups using Student's t-tests. Corticosterone and Fos data are presented as the mean and 95% confidence intervals of back-transformed data.

A total of 4 virgin males (3 in the single-pup condition, 1 in the repeated-pup condition) attacked foster pups. The virgin male in the repeated-pup condition attacked a pup during each of his three 20-minute pup exposures as well as his 1-hour exposure. For these animals, latency to approach the pup was included in analyses but all other behaviors were excluded, as the pup was removed from the cage immediately after the attack. Eight virgin males (3 in the repeated-pup condition, 2 in the single-object condition, and 3 in the repeated-object condition) were asleep or inactive during presentation of the stimulus and remained asleep or inactive for the majority, if not the entirety, of one or more exposures, never investigating the stimulus. Data from these animals were not included in any analyses. All new fathers investigated and behaved paternally (i.e., huddled and/or groomed) toward pups.

3. Results

3.1. Behavioral responses to pups

3.1.1. Initial exposure to pups

During their first experimental exposure, virgins in the repeated-pup condition, virgins in the single-pup condition, and new fathers differed significantly in their behavioral responses to pups, as revealed by Kruskal-Wallis tests and Dunn's post-hoc

tests, using the Bonferroni-corrected critical p value (0.0167) (Fig. 1). Virgin males in the repeated-pup (day 3) and single-pup (day 10) conditions and new fathers (day 10; see Table 1) differed in latency to initiate paternal care ($\chi^2 = 12.14$, df = 2, p = 0.002, $\eta^2 = 0.20$): new fathers initiated paternal care more quickly than virgin males in both the repeated-pup and single-pup conditions (new fathers vs. single-pup: Z = −2.90, p = 0.002, d = 1.14; new fathers vs. repeated-pup: Z = −3.08, p = 0.001, d = 1.07; Fig. 1B), whereas the two groups of virgin males did not differ from each other, as expected (Z = 0.09, p = 0.463, d = 0.22). No significant differences were found among virgins in the repeated-pup condition, virgins in the single-pup condition, and new fathers in latency to approach pups ($\chi^2 = 5.65$, df = 2, p = 0.059, $\eta^2 = 0.17$; Fig. 1A), time spent in paternal behavior (i.e., huddling or grooming) ($\chi^2 = 4.34$, df = 2, p = 0.114, $\eta^2 = 0.18$; Fig. 1D), or time spent sniffing the pup ($\chi^2 = 4.95$, df = 2, p = 0.08, $\eta^2 = 0.22$; Fig. 1C) during their first experimental exposure

3.1.2. Final exposure to pups

In their 1-hour exposure to a pup on day 10, virgins in the repeated-pup condition, virgins in the single-pup condition, and new fathers differed significantly in latency to approach pups ($\chi^2 = 11.23$, df = 2, p = 0.004, $\eta^2 = 0.39$; Fig. 1A), latency to initiate paternal care ($\chi^2 = 9.79$, df = 2, p = 0.007, $\eta^2 = 0.14$; Fig. 1B), and duration of paternal care ($\chi^2 = 6.88$, df = 2, p = 0.032, $\eta^2 = 0.22$; Fig. 1D; Kruskal-Wallis tests). Males in the repeated-pup condition, compared to males in the single-pup condition, approached pups sooner (z = 3.34, p b 0.001), engaged in paternal care sooner (z = 2.63, p = 0.004, d =

1.68) and spent more time engaged in paternal care ($z = 2.57$, $p = 0.005$, $d = 0.95$). Similarly, new fathers, compared to virgin males in the single-pup condition, engaged in paternal care sooner ($z = -2.88$, $p = 0.002$, $d = 1.14$), approached pups nominally sooner ($z = -1.91$, $p = 0.028$, $d = 1.04$) and spent nominally more time engaged in paternal care ($z = 1.87$, $p = 0.031$, $d = 1.14$). Virgin males in the repeated-pup condition did not differ significantly from new fathers in latency to approach a pup ($z = 1.34$, $p = 0.091$, $d = 0.75$), latency to behave paternally ($z = -0.27$, $p = 0.393$, $\eta^2 = 0.22$), or duration of paternal care ($z = -0.75$, $p = 0.226$, $d = 0.03$). Time spent sniffing the pup did not differ significantly across the three groups ($\chi^2 = 5.29$, $df = 2$, $p = 0.071$, $\eta^2 = 0.32$; Fig. 1C).

3.1.3. Within-animal changes across exposures

To identify effects of repeated pup exposure within individual animals, we compared behavioral responses to pups across all four exposures in virgin mice in the repeated-pup condition using Friedman tests, with Nemenyi post-hoc tests when merited (Fig. 1). Latency to approach a pup decreased across the four exposures ($\chi^2 = 14.52$, $df = 3$, $p = 0.002$, $\eta^2 = 0.37$), with shorter latency to approach on day 10 (fourth exposure) compared to day 3 (first exposure; $p = 0.005$, $d = 0.51$) and on day 10 compared to day 5 ($p = 0.029$, $d = 1.12$). Latency to behave paternally also decreased across exposures ($\chi^2 = 11.25$, $df = 3$, $p = 0.010$, $\eta^2 = 0.17$), with a shorter latency on day 7 (third exposure) compared to day 3 ($p = 0.018$), $d = 0.48$. Duration of paternal behavior increased across exposures ($\chi^2 = 11.25$, $df = 3$, $p = 0.010$, $\eta^2 = 0.26$), with an increased duration of

paternal care on day 7 compared to day 3 ($p = 0.018$, $d = 0.76$). Duration of sniffing the pup did not change across exposures ($\chi^2 = 5.93$, $df = 3$, $p = 0.115$, $\eta^2 = 0.14$).

3.2. Behavioral responses to marbles

3.2.1. Initial exposure to marbles

During their first exposure to a marble, virgin males in the repeated-object (day 3) and single-object (day 10) conditions did not differ in latency to approach the marble ($U = 23.00$, $p = 0.242$, $d = 0.99$; Mann-Whitney U test) or duration of time spent sniffing the marble ($U = 21.00$, $p = 0.172$, $d = 0.67$; Mann-Whitney U test; Table 2).

3.2.2. Final exposure to marbles

During their final exposure, on day 10, virgin males in the single-object condition took nominally longer to approach the marble than virgin males in the repeated-object condition ($U = 13.00$, $p = 0.032$, $d = 1.34$; Mann-Whitney U test). Duration of time spent sniffing the marble during the final exposure did not differ between groups ($U = 24.00$, $p = 0.283$, $d = 0.51$; Mann-Whitney U test: Table 2).

3.2.3. Within-animal changes across exposures

To identify possible effects of repeated exposure to marbles within individual animals, we compared behavioral responses to marbles across all four exposures in virgins in the repeated-object condition. Friedman tests found no change in latency to

approach ($\chi^2 = 3.51$, df = 3, $p = 0.319$, $\eta^2 = 0.32$) or time spent sniffing the marble ($\chi^2 = 7.63$, df=3, $p=0.054$, $\eta^2 = 0.31$; Table 2).

3.3. Plasma corticosterone concentrations

To determine effects of treatment and stimulus on basal plasma corticosterone levels, a three-way mixed ANOVA (stimulus [pup or marble] × condition [single or repeated exposure] × day [1 or 8]) was conducted on log-transformed data. We found no main effects of day ($F[1,27] = 0.02$, $p = 0.896$, $\eta^2 = 0.01$), stimulus ($F[1,27] = 0.26$, $p = 0.612$, $\eta^2 = 0.01$), or condition ($F[1,27] = 3.10$, $p = 0.090$, $\eta^2 = 0.12$). Moreover, we found no significant two- or three-way interactions (day × stimulus: $F[1,27] = 0.08$, $p = 0.928$, $\eta^2 = 0.01$; day × condition: $F[1,27] = 0.006$, $p = 0.939$, η^2 b 0.01; stimulus × condition: $F[1,27] = 3.57$, $p = 0.070$, $\eta^2 = 0.12$; day × stimulus × condition: $F[1,2] = 2.53$, $p = 0.124$, $\eta^2 = 0.10$).

To examine effects of repeated exposure on the acute corticosterone response to a pup or marble, we collected trunk blood immediately after the 60-minute exposure on day 10. A two-way ANOVA on virgin males revealed no significant main effect of stimulus ($F[1,29] = 0.76$, $p = 0.392$, $\eta^2 = 0.02$) or condition ($F[1,29] = 0.14$, $p = 0.707$, η^2 b 0.01), nor a significant stimulus × condition interaction ($F[1,29] = 3.36$, $p = 0.077$, $\eta^2 = 0.10$). Planned comparisons were conducted on plasma corticosterone levels of new fathers, single-pup virgin males, and repeated-pup virgin males immediately after the 60-minute pup exposure on day 10. Acute corticosterone responses to pups did not differ between

new fathers and virgins in the repeated-pup condition ($t = 0.68$, $df = 16$, $p = 0.507$, $d = 0.24$), between new fathers and virgins in the single-pup condition ($t = 1.336$, $df = 14$, $p = 0.20$, $d = 0.20$), or between virgins in the single-pup and repeated-pup conditions ($t = 1.60$, $df = 15$, $p = 0.131$, $d = 0.01$) (Table 3).

3.4. Fos expression

Two-way ANOVAs (stimulus [pup or marble] × condition [single or repeated exposure]) were conducted on log-transformed Fos expression data from virgin males in seven brain regions: MPOA, vBNST, dBNST, MEA, PVN, VMH, and AHN. Significant main effects of stimulus were found in the MPOA ($F[1,22] = 9.18$, $p = 0.006$, $\eta^2 = 0.26$) vBNST ($F[1,22] = 15.19$, $p = 0.001$, $\eta^2 = 0.37$), and dBNST ($F[1,22] = 10.31$, $p = 0.004$, $\eta^2 = 0.34$), with all of these regions having more Fos expression in virgins exposed to pups than in those exposed to marbles (Fig. 2). None of these regions showed a significant main effect of condition or a stimulus × condition interaction. Fos expression in the MeA, VMH, PVN, and AHN did not show a main effect of stimulus, condition, or a stimulus × condition interaction (Table 4).

Planned comparisons were conducted on Fos expression among new fathers and virgin males in the single-pup and repeated-pup conditions. t-tests found no significant differences between fathers and virgins in either condition in any brain region (Table 4).

4. Discussion

The mechanisms underlying the onset of paternal behavior in rodents are not well understood in any species. This experiment is, to our knowledge, the first to investigate

the effects of brief, repeated pup exposure on paternal care in adult virgin males of a naturally biparental mammal.

Three 20-minute exposures to a pup over 8 days increased indices of paternal responsiveness in virgin male California mice. Specifically, repeated exposure decreased latency to approach the pup and latency to initiate paternal care, and increased total duration of time engaged in paternal care (i.e., grooming and/or huddling). No differences were seen between the first and second exposures for individual mice, suggesting that at least two previous exposures were necessary for sensitization. In the third exposure to a pup, virgin males showed a significant decrease in their latency to initiate paternal behavior and a significant increase in time spent behaving paternally, and during the fourth exposure, paternal behavior in virgins did not differ from that in new fathers. Many sensitization paradigms involve constant exposure to pups over several days (e.g., Rosenblatt, 1967). Our data suggest that brief contact with pups is sufficient to increase paternal behavior in virgin male California mice. To determine whether this effect might be mediated by a reduction in neophobia, we investigated the behavioral effects of repeated exposure to a pup-sized marble. No evidence of decreased neophobia was seen in the males exposed to a marble multiple times. Thus, unknown cues from pups in particular, rather than decreased neophobia in general, are likely to facilitate attraction to pups and engagement in paternal behavior in virgin male California mice.

We found no effect of prior pup exposure on either basal plasma corticosterone concentrations or corticosterone responses to pups in virgin males. Furthermore, these measures did not differ between virgin males and new fathers, nor between virgins

exposed to pups and those exposed to marbles. These results are consistent with previous findings from our lab, in which neither basal plasma corticosterone levels nor plasma corticosterone levels following a predator-odor stressor differed among virgin males, vasectomized males cohabiting with a female, and new fathers (Chauke et al., 2011). Similarly, in separate studies, measures of hypothalamic-pituitary-adrenal activity and reactivity did not differ among virgin males, males housed with a tubally ligated female, and new fathers (de Jong et al., 2013; Harris and Saltzman, 2013; Harris et al., 2013). In virgin male prairie voles, which exhibit high levels of alloparental behavior toward unrelated young, exposure to pups for 10 min prevents acute corticosterone elevations induced by handling; however, this effect is not seen after 20 min of exposure to pups (Kenkel et al., 2012). It is possible that in our study, in which blood was collected after 60 min of continuous exposure to a pup, initial corticosterone responses to stimuli differed among the groups and/or between animals exposed to pups and those exposed to marbles, but that these differences dissipated by the end of the 60-minute exposure. It is also possible that corticosterone responses to a pup or novel object were dwarfed by the response to handling and placement in a new cage, although all animals were allowed to habituate to the new cage for 10 min before being tested.

Fos expression in several brain regions previously implicated in paternal behavior – the MPOA, vBNST, and dBNST (Bales and Saltzman, 2016) – was higher in virgin males exposed to pups than in those exposed to marbles. We found no evidence, however, that Fos responses to a pup were altered by previous exposure to pups: Fos did not differ among new fathers and virgin males in either the repeated-pup or single-pup

conditions. In contrast, virgin male Mus musculus that behaved paternally in two pup tests two days apart exhibited more Fos expression in the central MPOA, as well as the rhomboid nucleus of the BNST, after pup exposure than did fathers (Tsuneoka et al., 2015).

A previous study in our lab showed Fos expression to differ between California mouse fathers and virgin males after exposure to a pup. Specifically, fathers had higher Fos expression than virgins in the MPOA, medial posteromedial division of the BNST, ventral medial division of the BNST, and caudal dorsal raphe nucleus (de Jong et al., 2009). The reason for the disparity between the findings of that study and the present one is not clear. In the earlier study, however, Fos expression was quantified 1 h after a 5-minute exposure to a pup confined in a wire mesh ball, which precluded direct contact between the adult male and the pup. Thus, male mice in both studies could engage in appetitive behavior toward pups (e.g., approach, sniff), but only the mice in the present study could engage in consummatory aspects of paternal care (i.e., direct physical interaction). The precise stimulus eliciting Fos expression may therefore have differed between the two studies, and consummatory and appetitive aspects of parental behavior may be controlled by somewhat different neural circuitry (Stolzenberg and Numan, 2011). It is also possible that in the present study, previous exposure altered Fos responses to pups in brain regions other than the ones we investigated and/or that neuronal activation in response to pups was associated with expression of an immediate-early gene(s) other than c-fos, such as egr-1 or c-jun (Kawashima et al., 2014).

In summary, this study demonstrates that in the biparental California mouse, repeated, brief exposure to a pup can increase paternal responses to pups in virgin males, similar to pup-induced paternal care in uniparental rats, mice, and golden hamsters (Bridges et al., 1972; Ehret et al., 1987; Jakubowski and Terkel, 1985b; Rosenblatt, 1967; Swanson and Campbell, 1979). On the other hand, we found no evidence that repeated exposure to pups alters basal plasma corticosterone levels, or either corticosterone or neural responses to pups. Thus, although male California mice exhibit paternal care immediately at the birth of their offspring (Gubernick and Alberts, 1987; Lee and Brown, 2002), and chemosensory cues from their mates facilitate the maintenance of this paternal care (Gubernick, 1990; Gubernick and Alberts, 1989), we found that cues from pups alone can facilitate the onset of paternal care. This suggests that cues from a female pairmate are not necessary for the onset of paternal behavior in male California mice.

The mechanisms by which pup exposure facilitates paternal care are not understood. Our findings suggest that the induction of paternal care is not mediated by changes in generalized neophobia or corticosterone response to pups in California mice. Future research investigating the effects of pup exposure on other aspects of endocrine and neural signaling may reveal the mechanisms of sensitized paternal behavior.

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Tables for Chapter 1

Table 1. Sequence of procedures conducted on adult virgin male California mice that underwent single or repeated exposure to either an unfamiliar object (marble) or an unfamiliar pup, as well as new fathers exposed to an unfamiliar pup. No procedures were conducted on days 2, 4, 6, and 9.

	Day 1	Day 3	Day 5	Day 7	Day 8	Day 10
Repeated-pup (n=11)	Baseline blood sample	20-min pup exposure	20-min pup exposure	20-min pup exposure	Baseline blood sample	60-min pup exposure; blood & brain collection
Single-pup (n=10)	Baseline blood sample	Handling	Handling	Handling	Baseline blood sample	60-min marble exposure; blood & brain collection
Repeated-object (n=10)	Baseline blood sample	20-min pup exposure	20-min pup exposure	20-min pup exposure	Baseline blood sample	60-min pup exposure; blood & brain collection
Single-object (n=11)	Baseline blood sample	Handling	Handling	Handling	Baseline blood sample	60-min marble exposure; blood & brain collection
New fathers (n=9)	---	---	---	---	Baseline blood sample (3-5 days after birth of first litter)	60-min pup exposure; blood & brain collection

Table 2. Marble-directed behaviors (medians and quartiles) for virgin males in the repeated-marble condition in their first (day 3), second (day 5), third (day 7), and fourth (day 10) exposure to a marble and virgin males in the single-marble condition on their only exposure to a marble (day 10). No differences were in marble-directed behavior were seen within the repeated object exposure condition. Virgin males in single object condition took nominally longer to approach the marble than virgin males in the repeated object exposure condition on day 10 (p=0.032).

	Repeated object exposure								Single object exposure	
	Day 3		Day 5		Day 7		Day 10		Day 10	
Behavior	Median	1st & 3rd Quartiles	Median	1st & 3rd Quartiles	Median	1st & 3rd Quartiles	Median	1st & 3rd Quartiles	Median	1st & 3rd Quartiles
Latency to approach	133.94	74.61, 145.60	34.12	28.89, 73.65	100.02	41.84, 112.92	30.19	27.87, 112.25	292.10	102.43, 486.51
Duration of sniffing	161.16	100.39, 258.01	80.59	37.25, 80.59	65.68	16.08, 187.47	68.78	36.30, 136.78	111.86	63.39, 168.99

Table 3: Plasma corticosterone levels (ng/ml; back-transformed mean and 95% confidence interval) in blood samples collected under resting conditions (days 1, 8) or immediately after 1-hour exposure to a pup or marble (day 10). Single-object, n=5-10; repeated-object, n=7, single-pup, n=10; repeated-pup, n=10; new fathers, n=10).

	Day 1: Resting		Day 8: Resting		Day 10: After exposure	
Condition	Mean	95% CI	Mean	95% CI	Mean	95% CI
Single-Pup	255.86	186.21-354.81	192.31	131.83-281.84	274.79	147.91-512.86
Repeated-Pup	266.83	158.49-446.68	306.19	208.93-446.68	323.59	173.78-602.56
Single-Object	169.82	97.72-295.12	277.33	162.18-467.74	435.51	295.12-645.65
Repeated-Object	119.83	67.61-213.80	91.50	51.29-162.18	215.97	107.15-436.52
New Fathers	---	---	202.30	141.25-288.40	400.87	229-09-691.83

Table 4: Number of fos-positive cells (back transformed means ± 95% confidence intervals) in 200x200 um sections of brain regions immediately following 1-hour exposure to a pup or a novel object (marble). Fos expression was significantly higher ($p<0.05$) in pup-exposed than marble-exposed virgins in the MPOA, vBNST, dBNST. MPOA = medial preoptic area, vBNST = ventral bed nucleus of the stria terminalis, dBNST = dorsal bed nucleus of the stria terminalis, MEA = medial amygdala, PVN = paraventricular nucleus of the hypothalamus, VMH = ventromedial nucleus of the hypothalamus, AHN = anterior hypothalamic nucleus.

	Brain Regions													
	MPOA		vBNST		dBNST		MEA		PVN		VMH		AHN	
Condition	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Single-pup n=6	13.26	10.3-17.0	15.8	11.7-21.3	27.04	19.5-37.5	18.6	16.7-20.8	17.78	11.9-26.4	13.26	8.64-20.3	14.19	10.9-18.4
Repeated-pup n=6	21.79	12.3-38.4	16.4	10.6-25.2	28.68	21.9-37.5	17.4	13.2-22.8	24.38	14.0-42.4	12.92	7.35-22.7	14.84	10.4-21.0
Single-object n=8	7.70	4.58-12.9	7.35	6.14-8.81	16.61	13.9-19.7	14.6	10.4-20.4	16.63	11.3-24.4	15.92	11.2-22.6	13.88	10.0-19.2
Repeated-object n=8	10.26	7.33-14.3	10.3	7.49-14.3	16.37	11.3-23.6	13.4	9.69-18.6	14.42	10.7-19.2	8.79	5.30-14.5	10.73	9.13-12.6
New fathers	15.27	13.0-17.8	13.8	10.9-17.6	24.11	18.8-30.9	19.9	12.9-30.8	20.04	14.6-27.4	9.84	8.03-12.0	13.27	10.2-17.2

Figures for Chapter 1

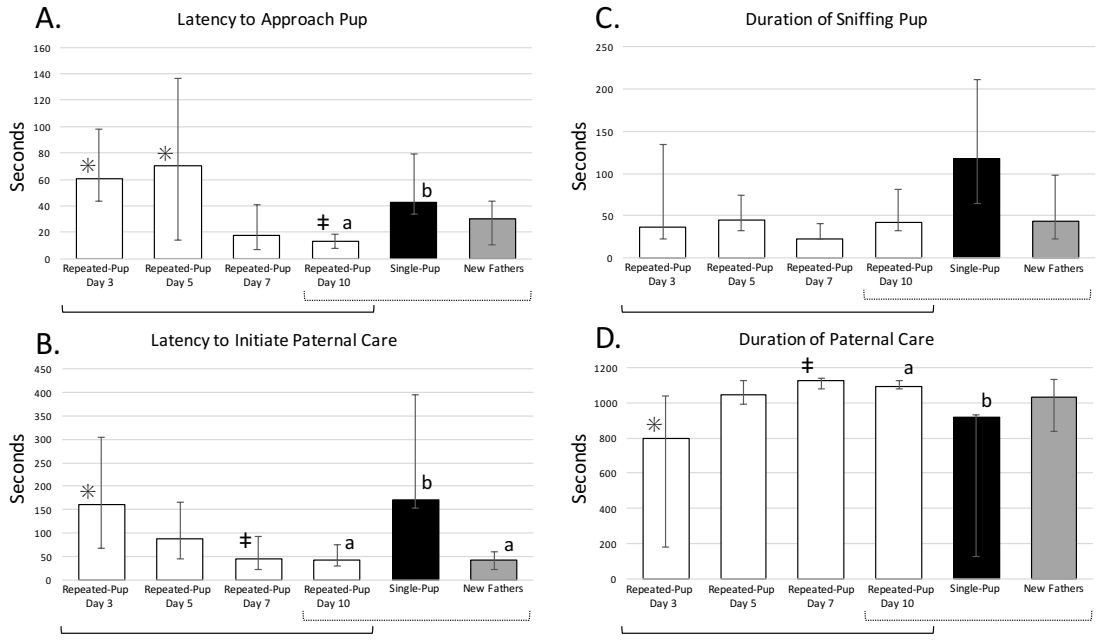

Figure 1: Pup-directed behaviors (median ± 1st and 3rd quartiles) for new fathers, virgin males in the single-pup condition (day 10) and virgin males in the repeated-pup condition in their first (day 3), second (day 5), third (day 7), and fourth (day 10) exposure to pups. Within-group comparisons of virgin males in the repeated-pup condition across the four exposures are shown in solid brackets; time points with different symbols (✳,‡) differed significantly ($p \leq 0.05$) from one another. Between-group comparisons of repeated-pup males in their fourth exposure, single-pup males, and new fathers (all on day 10) and shown in dotted brackets; groups with different letters (a,b) differed significantly ($p \leq 0.017$) from one another. A: Latency to approach a pup decreased across repeated exposures to a pup in virgin males, and was lower in previously exposed virgins and new fathers than in virgins exposed to a pup for the first time. B: Latency to initiate paternal care decreased across repeated exposures in virgin males, and was lower in previously exposed virgins and new fathers than in virgins exposed to a pup for the first time. C: Duration of time spent sniffing a pup did not change across repeated exposures in virgin males and did not differ among experimental groups. D: Duration of time spent engaging in paternal care increased across exposures to a pup in virgin males, and was higher in previously exposed virgins and new fathers than in virgin males exposed to a pup for the first time.

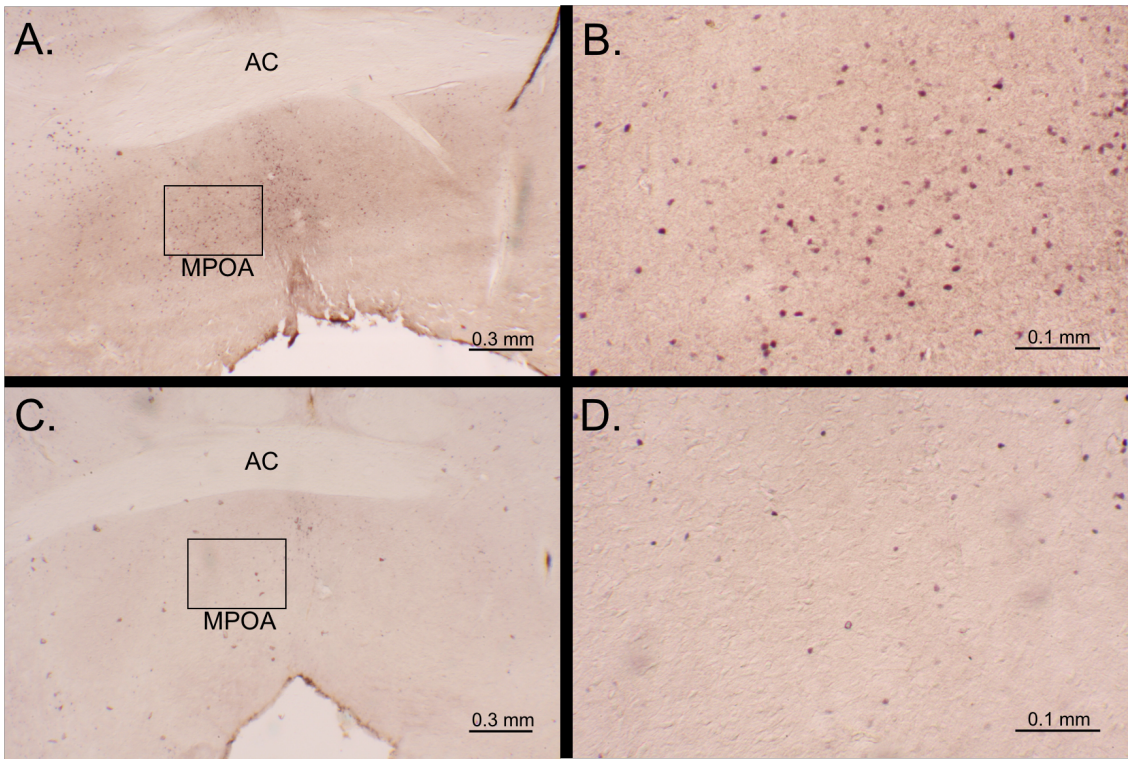

Figure 2: Photomicrographs of 30 um Fos-stained coronal sections of animals exposed to a pup (A and B) or marble (C & D). Images A. and C. are at 25x magnification showing a box containing mostly the medial preoptic area which is enhanced to 100x magnification in images B. and D. Abbreviations: AC=anterior commissure, MPOA= medial preoptic area.

Chapter 2

Plasticity of paternity: Effects of fatherhood on synaptic, intrinsic and morphological characteristics of neurons in the medial preoptic area of male California mice.

Abstract

Parental care by fathers enhances offspring survival and development in numerous species. In the biparental California mouse, *Peromyscus californicus*, behavioral plasticity is seen during the transition into fatherhood: adult virgin males often exhibit aggressive or indifferent responses to pups, whereas fathers engage in extensive paternal care. In this species and other biparental mammals, the onset of paternal behavior is associated with increased neural responsiveness to pups in specific brain regions, including the medial preoptic area of the hypothalamus (MPOA), a region strongly implicated in both maternal and paternal behavior. To assess possible changes in neural circuit properties underlying this increased excitability, we evaluated synaptic, intrinsic, and morphological properties of MPOA neurons in adult male California mice that were either virgins or first-time fathers. We used standard whole-cell recordings in a novel in vitro slice preparation. Excitatory and inhibitory post-synaptic currents from MPOA neurons were recorded in response to local electrical stimulation, and input/output curves were constructed for each. Responses to trains of stimuli were also examined. We quantified intrinsic excitability by measuring voltage changes in response to square-pulse injections of both depolarizing and hyperpolarizing current. Biocytin was injected into neurons during recording, and their morphology was analyzed. Most parameters did not

differ significantly between virgins and fathers. However, we document a decrease in synaptic inhibition in fathers. These findings suggest that the onset of paternal behavior in California mouse fathers may be associated with limited electrophysiological plasticity within the MPOA.

1. Introduction

Parental care by mothers occurs in all mammalian species, while care by fathers occurs in only approximately 5–10% of mammals. Infant-directed behavior of males in biparental species can range from infanticide to avoidance to paternal care, and a male can display all three behaviors in his lifetime (Kleiman and Malcom, 1981; Woodroffe and Vincent, 1994).

The monogamous California mouse (*Peromyscus californicus*) exhibits a biparental care system, in which both parents provide extensive care to their offspring (Ribble and Salvioni, 1990; Ribble, 1991; Gubernick and Teferi, 2000). Fathers perform the same parental behaviors (except nursing) as mothers, and to a similar extent (Dudley, 1974; Gubernick and Alberts, 1987). Male California mice typically exhibit a shift in pup-directed behavior during the transition into parenthood: virgin males often exhibit infanticide or indifference when exposed to unrelated pups, whereas fathers exhibit paternal care (de Jong et al., 2009; Gubernick and Alberts, 1987). Thus, the California mouse is a useful model for investigating neural and hormonal plasticity underlying the onset of paternal care.

The neurobiological substrates of parental behavior have been examined much more extensively in females than in males. One of the brain regions most strongly implicated in maternal behavior is the medial preoptic area (MPOA) of the hypothalamus (see reviews by Numan and Insel, 2003; Numan, 2006; 2014; Dobolyi et al., 2014). In rats (*Rattus norvegicus*) and house mice (*Mus* spp.), c-fos is elevated in the MPOA after females are exposed to pups and/or engage in maternal care (Komisaruk et al., 2000; Lonstein and De Vries, 2000; Okabe et al., 2013). MPOA lesions reduce maternal care in female rodents (rat: [Noonan and Kristal, 1979; Terkel, Bridges and Sawyer et al., 1979; Numan and Callahan, 1980; Fleming, Miceli and Moretto et al., 1983; Franz et al., 1986; Lee, Clancy, and Fleming, 1999; Numan et al., 1988; Stack et al., 2002); house mouse: (Tsuneoka et al., 2013); Siberian hamster [*Mesocricetus auratus*]: (Miceli and Malsbury, 1982); California mouse: (Lee and Brown, 2002; 2007), as does infusion of GABA agonists into the MPOA of rats (Arrati et al., 2006). Moreover, kindling of the MPOA increases female rats' preference for pup-associated environments in conditioned place-preference paradigms (Morgan et al., 1999).

The MPOA is also critical for paternal care in male rodents (Bales and Saltzman, 2016; Horrell, Hickmott and Saltzman, 2016). MPOA lesions disrupt paternal care in male California mice (Lee and Brown, 2002; 2007) and disrupt or prevent sensitized allopaternal behavior in male rats and house mice (Rosenblatt, Hazelwood and Poole, 1996; Sturgis and Bridges, 1997; Tsuneoka et al., 2015). Conversely, optogenetic activation of the MPOA decreases infanticide in male mice (Tsuneoka, 2015). Additionally, c-fos expression in the MPOA increases after exposure to pups in male

house mice (Tsuneoka, 2015), prairie voles (*Microtus ochrogaster*; (Kirkparick, Kim and Insel, 1994), North American deermice (*Peromyscus maniculatus*; [Lambert et al., 2013]), and California mice (Lambert et al., 2013; Horrell et al., 2017). California mouse fathers, but not virgins, display higher levels of c-fos in the MPOA after exposure to a pup than after exposure to a control stimulus (de Jong et al., 2009).

Changes in morphology of MPOA neurons are associated with the transition into motherhood, the hormonal milieu of pregnancy, and the extent of maternal care in female rodents (Gubernick, Sengelaub and Kurz, 1993; Keyser-Marcus et al., 2001; Shams et al., 2012; Parent et al., 2017). Only one study has compared MPOA neural morphology between virgin males and fathers in any species: Gubernick et al. (1993) reported no differences in MPOA cross-sectional somal area between California mouse fathers and virgin males, with no other properties of neurons analyzed.

The electrophysiological properties of MPOA neurons have received little attention, especially in the context of parental care. Here we test the hypothesis that fatherhood increases intrinsic excitability, increases synaptic excitation and/or decreases synaptic inhibition, and alters the morphology of MPOA neurons in California mice.

2. Materials and methods

2.1. Animals

Experiments were performed on young adult California mice that were born and maintained in our breeding colony at the University of California, Riverside and descended from mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Animals were housed in 44 × 24 x 20 cm poly-carbonate cages containing aspen shavings and cotton wool for nesting material, with food (Purina Rodent Chow 5001) and water available ad libitum. Colony rooms were kept on a 14:10 light:dark cycle (lights on from 0500 h to 1900 h).

2.2. Experimental design

At 27–33 days of age, prior to the birth of the next litter of siblings, animals were removed from their parents' cage and housed in groups of three or four same-sex, age-matched littermates and/or unrelated juveniles. At sexual maturity (~3 months of age), each male mouse either remained in its group that was created at weaning (virgin males) or was paired with an unrelated female (fathers). Males were randomly assigned to the virgin and father conditions, except that those assigned to the two conditions were age-matched. Three to seven days after the birth of their first litter, fathers underwent a pup test followed by electrophysiological experiments (see below). Virgin males were tested in an age-matched fashion; age on the day of data collection did not differ between virgin males (162.1 ± 9.4 days [mean ± SE]) and fathers (175.0 ± 7.4 days [mean ± SE]; $p = 0.283$, $df = 37$). All animal procedures were consistent with the Guide for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee

of the University of California, Riverside. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.3. Pup test

At 0900–1000h, males were taken from their home cage and housed singly in a clean cage for 10 min. An unrelated, 1- to 5-day-old pup was then placed in the corner of the cage farthest from the male. The cage was video-recorded for 20 min, and the following behaviors were scored with The Observer software v. 11.5 (Noldus, Wageningen, Netherlands): latency to sniff the pup, latency to initiate paternal behavior (i.e., licking, grooming, or huddling), percent time spent sniffing the pup, percent time spent in paternal behavior, and percent time not in contact with the pup (i.e., not sniffing or engaging in paternal behavior). If a pup was attacked, the test was stopped, and the pup was immediately euthanized with pentobarbital, and the subject was as- signed a score of 0 time spent in paternal behavior.

2.4. Preparation of MPOA slices for in vitro recording

Immediately after the behavioral test, the mouse was anesthetized with isoflurane until areflexic, and the brain was rapidly removed and placed into modified artificial cerebrospinal fluid (ACSF; in mM: KCl, 2.5; NaH_2PO_4, 1; $MgSO_4$, 1.3; $CaCl_2$, 2.5; $NaHCO_3$, 26.2; D-(+)-glucose, 11; Sucrose, 196.7; 3-morpholinopropane-1-sulfonic acid (MOPS), 3.5; sodium pyruvate, 2; kynurenic acid, 3; saturated with 95%O_2/5%CO_2). Coronal slices (300 μm-thick) were cut on a vibrating microtome (VT1000, Leica Biosystems Inc., Buffalo Grove, IL, USA). Sections containing the preoptic area were

selected using easily identifiable landmarks (i.e., the anterior commissure and third ventricle) and incubated in the modified ACSF for at least 30min. Slices were then transferred into standard ACSF for at least 30 min before recording (in mM; NaCl, 119; KCl, 2.5; NaH_2PO_4, 1; $MgSO_4$, 1.3; $CaCl_2$, 2.5; $NaHCO_3$, 26.2; D-(+)-glucose, 11; saturated with 95%O_2/5%CO_2; osmolarity: 290–300 mOsm/L).

Electrophysiological data were obtained via blind whole-cell recording (Blanton, Lo Turco and Kriegstein, 1989) using patch electrodes with internal tip diameter of 1.5–2.5 μm. Patch electrodes were filled with either a Cs-based solution with QX-314 for recording synaptic currents or a K-based solution for recording intrinsic properties. The solutions consisted of (in mM): Cs-gluconate or K-gluconate, 128; CsCl or KCl, 7; QX-314, 10; EGTA, 1; HEPES, 10; Mg-ATP, 2; Na-GTP, 0.2; biocytin, 0.1-0.2%; pH 7.0–7.4; osmolarity: 290–300 mOsm/L. Electrodes had tip resistances of 3–6 MΩ. Recordings were amplified using an Axoclamp 2B amplifier (Axon Instruments, Union City, CA, USA) in voltage-clamp or current-clamp mode, digitized at 15 kHz for synaptic currents and 40 kHz for intrinsic properties (National Instruments, Austin, TX, USA), and saved to the hard disk of a personal computer (Macintosh G4) using the IgorPro (Wavemetrics Inc., Lake Oswego, OR, USA) data acquisition system. Because the central MPOA has been strongly implicated in parental care (Tsuneoka et al., 2013; 2015), neurons were obtained in that region (Fig. 1A): 609.32 ± 16.68 μm (SE ± mean) ventral to the anterior commissure and 297.86 ± 11.15 μm (SE ± mean) lateral to the midline. Location of neurons did not differ between virgins (n = 45 neurons) and fathers (n = 51 neurons) with respect to the anterior commissure ($p = 0.172$, $df = 95$) or midline ($p = 0.168$, $df = 95$).

2.5. Acquisition and analysis of data on intrinsic electrophysiological properties

Neurons were current-clamped at -70 mV, and 500-ms square pulses of positive or negative current with intensities from sub- to supra- threshold were injected at 0.2 Hz. Properties of single action potentials (APs; Fig. 1B, C) were measured using small supra-threshold injections that elicited one to a few spikes. The amplitude of single APs was measured from the inflection point between the initial passive depolarization from the current injection and the beginning of the spike to the peak of the spike. The threshold was measured from baseline to the inflection point. Action potential half-width was measured as the duration of the AP at 50% of its amplitude. Fast afterhyperpolarizations (fAHPs) were quantified at two points: as the change in voltage from the inflection point to the minimal voltage 3–5 ms and 20–25 ms later.

Spike trains were obtained by increasing the magnitude of current injections. Maximum number of APs was measured as the highest number of APs resulting from any amount of injected current. Responses to positive injections of current that elicited spike trains were quantified by plotting the magnitude of the current injection (the input) and the resulting number of APs (the output). Input/Output (I/O) plots were well fit by an exponential function, the plateau of which was the modeled maximum number of APs and tau (τ) of which provided a measure of excitability (Fig. 3 B). I/O plots were also created for average inter-spike interval (ISI) and current injected. From these I/O plots, minimum average ISI as well as tau were quantified to provide a metric of excitability independent of the maximum number of APs. Following a spike train, medium afterhyperpolarizations (mAHP) and slow afterhyperpolarizations (sAHP) were measured

at −55 mV from baseline to peak of the hyperpolarization after the AP train and 450 ms after the offset of the AP train, respectively.

Properties of subthreshold responses to negative current injection were quantified. For subthreshold potentials, the peak amplitude and the amplitude at 400 ms of the current pulse (i.e., at steady-state) were determined for each current amplitude and plotted. These I/O plots were well fit by a straight line for both peak and steady-state measures, and the slopes of the lines were used as an overall measure of hyperpolarizing potential amplitude (Fig. 4).

2.6. Acquisition and analysis of data on post-synaptic currents (PSCs)

To quantify PSCs, stimulation was applied with a bipolar parylene-coated tungsten stimulating electrode (FHC, Bowdoin, ME, USA; tip resistance ~1 MΩ; tip separation ~50 μm) placed ~200 um dorsal to the recording electrode. Neurons were clamped at the chloride reversal potential (~−55mV) to record excitatory PSCs (EPSCs) and at the glutamate reversal (~0 mV) to record inhibitory PSCs (IPSCs). Single pulses 100 μs in duration were delivered at varying intensities to attain minimal and maximal PSC amplitude. Minimal PSC amplitude was operationalized as the current response using the greatest stimulus intensity that resulted in a failure rate of > 20%. Stimulus intensity was then increased incrementally until the maximal PSC amplitude was reached. Stimulus intensity (the input) and the recorded PSC (the output) were used to generate I/O curves. These I/O curves were well fit by a single exponential function and from these exponential models, modeled maximum PSC amplitude was calculated.

Trains of PSCs were elicited by stimuli consisting of 10, 100 μs pulses at various inter-pulse intervals (IPIs) of 10, 25, 50, 100, 200, 400 ms. For trains, stimulus intensity was adjusted to evoke PSCs of approximately half the maximal PSC amplitude for that particular cell. For each train of PSCs, the paired-pulse ratio (PPR) was defined as the ratio of the amplitude of the second PSC to the first; the steady-state ratio (SSR) was defined as the ratio of the mean amplitude of the last 3 PSCs to the first.

Properties of spontaneous PSCs (sPSCs) were determined from unstimulated records of varying durations. Typically, 30–50 spontaneous events were acquired for both EPSCs (when present) and IPSCs. Spontaneous excitatory PSCs (sEPSCs) were recorded at −55mV. Spontaneous inhibitory PSCs (sIPSCs) were recorded at 0 mV. Mean amplitude and frequency of sEPSCs and sIPSCs were determined.

2.7. Acquisition and analysis of morphological data

After recordings, slices were fixed in 10% formalin overnight at 4 °C, rinsed in phosphate buffer (PB), then permeabilized in PB with 0.5% Triton X-100 and 5% goat serum for 30 min at room temperature. Slices were then incubated in a PB-based solution containing 5% goat serum and ALEXA-488 streptavidin (Molecular Probes; Eugene, OR, USA) at 0.01 mg/ml overnight at 4 °C. Sections were mounted in 90% glycerol with 4% N-propyl gallate added. Neurons were imaged using laser-scanning confocal microscopy (Zeiss 510). Images of dendrites were acquired at 10x with a 2x digital zoom. In all cases, the gain and black level were adjusted so that most of the labeled dendrites were saturated. Z-stacks were obtained for the entire depth of the cell, and 2-dimensional

projections of neuronal morphology were derived using the maximal pixel intensity at each point in the X–Y plane. Dendritic morphology was analyzed using the Sholl analysis [45,46]. In this analysis, concentric circles were overlaid on an image of a neuron at 20 μm intervals centered on the soma; the number of intersections of each circle with labeled processes was determined for the entire neuron for a measure of overall neurite complexity. Quadrantized Sholl analyses for determination of a possible bias in dorsal-ventral and medial- lateral domains were conducted: Quadrant 1 (Q1) = dorsal medial, Q2 = dorsal lateral, Q3 = ventral medial, and Q4 = ventral lateral. In addition, number of branch points in each quadrant, total number of branch points, total neurite length, length of longest neurite, number of neurites leaving the soma, soma circumference, and largest soma diameter were measured. Properties of primary, secondary, and tertiary neurites were quantified. Primary neurites were defined as neurites leaving the soma, secondary neurites were defined as the shorter neurite process after a branch point on a primary neurite, and tertiary neurites were defined as the shorter neurite process after a branch point on a secondary neurite.

2.8. Statistical analyses

All data were analyzed using SPSS (IBM Corp, 2013), Microsoft Excel, or SAS. Normality was tested using the Shapiro-Wilk test, and homogeneity of variance was tested using Levene's test. Depending on normality and homogeneity of variance, data from virgin males and fathers were compared using two-tailed Students' t-tests or Mann-Whitney U tests. Associations between behavioral and neuronal measures were evaluated

using Spearman correlations. Multiple simultaneous tests involving related data, such as those in Supplemental Table 1, increase risk of Type 1 errors. To compensate, we used the positive False Discovery Rate procedure as implemented in SAS Procedure Multtest. That procedure indicated that none of the correlations reported in Supplemental Table 1 would be considered statistically significant after controlling for multiple comparisons. We refer to the three correlations with p values < 0.05 as nominally significant.

3. Results

3.1. Pup test

Data on pup-directed behavior was available for 13 virgins and 33 fathers (Fig. 2). Fathers had a lower latency to sniff and initiate paternal behavior (licking, grooming, or huddling) than virgin males (sniff: $U = 114$, $p = 0.014$, paternal care: $U = 79$, $p = 0.001$). Additionally, fathers spent less time sniffing ($U = 118$, $p = 0.019$) more time in paternal behavior ($U = 110.5$, $p = 0.011$), and less time not in contact with pups ($U = 131.5$, $p = 0.043$).

3.2. Electrophysiology

For analysis of intrinsic properties, a total of 22 cells were patched in 15 virgin males and a total of 31 cells were patched in 25 fathers. For analysis of PSCs, a total of 22 cells were patched in 11 virgin males and 19 were patched in 12 fathers. Resting potential and input resistance did not differ between virgins and fathers in the Cs-based

solution used to measure PSCs or in the K-based solution used to measure intrinsic properties (Table 1).

3.2.1. Properties of action potentials

Voltage responses to 500 ms-long square pulses of positive and negative current of various intensities were acquired while cells were current-clamped at -70 mV. APs were evoked by positive currents, and the properties of single APs (Fig. 1B, C) and trains (Fig. 1D) were examined. Each neuron exhibited one of two general spiking patterns (Fig. 3): Regular-spiking (RS), in which the inter-spike intervals (ISIs) increased gradually during the train, and Fast-spiking (FS), in which ISIs did not change during the train. RS and FS cells were further divided into initial-bursting (IB) and non-initial-bursting, based on the presence or absence of a high-frequency burst of APs at the start of the train. Measurements of properties of single APs in initial-bursting cells were done on an AP not in the burst. Percentages of neurons in these spiking categories were similar in virgins and fathers (numbers of cells: FS: 9 virgin, 13 father; IB-FS: 2 virgin, 1 father; RS: 7 virgin, 13 father; IB-RS: 4 virgin, 4 father; $p = 0.72$, Freeman-Halton extension of Fisher's exact test).

Initially, we compared all cells from virgins to those of fathers. None of the parameters extracted differed significantly between groups (Table 2).

Cells were then grouped into RS and FS groups, with IB cells subsumed into those categories, and properties of single APs (Table 3), trains of APs (Table 4), and responses to hyperpolarizing current (Table 5) were compared between virgins and

fathers. Again, we found no significant differences, though the minimum average AP ISI tended to be longer in RS of fathers than in virgins (Table 4). The peak slope/ steady-state slope tended to be greater in FS cells of fathers than in virgins (Table 5). Finally, we grouped cells by spiking patterns (FS, IB-FS, RS, IB-RS) and compared properties of single APs (Table 6), trains of APs (Table 7), and responses to hyperpolarizing current (Table 8) between virgins and fathers. IB-RS cells of fathers, compared to virgins, tended to have greater fAHP at 3–5 ms (Table 6). RS cells of fathers tended to have smaller maximum voltage change in response to hyperpolarizing current than RS cells of virgins (Table 8).

3.2.2. Properties of PSCs

Spontaneous PSCs and PSCs in response to local stimulation were characterized and compared in virgins and fathers. All cells exhibited inhibitory currents (n = 41 cells from 23 animals), while a subset of cells exhibited both inhibitory and excitatory currents (n=29 cells from 19 animals). The percentage of cells in the two categories did not differ significantly between virgins (IPSC only: n=7, IPSC+EPSC: n=15) and fathers (IPSC only: n=5, IPSC+EPSC: n=14; p=0.74, Fisher's exact test). Representative traces of excitatory and inhibitory evoked PSCs are shown in Fig. 5A. Virgins and fathers did not differ in maximal excitatory PSC amplitude, but fathers had significantly lower maximal inhibitory current than virgins (Fig. 5B, Table 9).

PPRs and SSRs of EPSCs and IPSCs did not differ at any inter-pulse interval, though we found a trend for fathers to have an increased EPSC PPR at 200-ms inter-pulse

intervals as well as a strong trend for fathers to have a lower IPSC PPR at 10-ms inter-pulse intervals, compared to virgins (Fig. 7, Table 9). Virgins and fathers did not significantly differ in amplitude or frequency of spontaneous EPSCs or IPSCs, though was a trend for fathers to have less frequent sEPSCs (Fig. 6, Table 9).

3.3. Morphology

A total of 45 neuronal morphologies were captured: 23 from 17 virgins and 22 from 17 fathers (Fig. 8). Insufficient numbers of morphologies were captured to allow for comparisons between sub-types of neurons based on spiking patterns, so tests were conducted between metrics of virgins and fathers (Table 10). No differences were found in quantified morphological properties, though we found a trend for fathers to have shorter average length of primary neurites than virgins (Table 10).

3.4. Correlations of neural and behavioral measurements

Spearman correlations were run between two key behavioral metrics (i.e., latency to engage in paternal behavior and percent time spent in paternal care) and neural properties in virgins, which exhibited more variability in paternal behavior than fathers. Latency to engage in parental behavior showed nominally significant negative correlations with AP amplitude ($r_S(14)=-0.682$, $p = 0.007$) and largest soma diameter ($r_S(7)=-0.954$, $p = 0.001$), and a nominally significant positive correlation with maximum voltage change in response to a hyperpolarizing stimulus ($r_S(14) = 0.67$, $p = 0.009$). Thus, the more readily a virgin male engaged in paternal behavior toward the pup, the larger his AP amplitude and maximal soma diameter, and the smaller his maximum voltage change

in response to a hyperpolarizing stimulus. However, after correcting for multiple comparisons, none of these correlations were statistically significant (see Supplemental Table 1 for full correlation results). Time spent in paternal care did not correlate with any neuronal properties.

4. Discussion

The MPOA plays a role in parental care in males and/or females in multiple vertebrate clades (Tsuneoka et al., 2015; Demski and Knigge, 1971; Slawski and Buntin, 1995). The identity of the cells involved in parental care, categorized by gene expression, afferent or efferent connectivity, or morphological or electrophysiological profiles, is under investigation but not well understood (e.g. Dobolyi et al., 2014; Lonstein and De Vries, 2000; Tsuneoka et al., 2013; Cservenák et al., 2013; 2017; Kuroda and Numan, 2014; Wu et al., 2014). Recently, neurons in the central MPOA have been implicated in paternal care in mice (Tsuneoka et al., 2013; Tsuneoka et al., 2015). In order to attain a thorough, unbiased survey of electrophysiological and morphological profiles of central MPOA neurons potentially involved in paternal care, we used blind, whole-cell recording to characterize all neurons in a non-specific manner in the central MPOA of both virgin males and fathers in the biparental California mouse. This experiment is among the first to characterize the electrophysiological properties of MPOA neurons in males of any species, and the first to attempt to relate these properties explicitly to paternal behavior. The MPOA of California mice likely contains a multitude of cell types. We observed four major spiking patterns in cells of the central MPOA: fast-spiking (FS), initial-bursting

fast-spiking (IB-FS), regular- spiking (RS), and initial-bursting regular-spiking (IB-RS) (Fig. 3). Initial-bursting cells characteristically fired a cluster of action potentials at stimulus onset. Regardless of presence or absence of an initial burst, cells could show accommodation (regular-spiking cells) or no accommodation (fast-spiking cells). Similar spiking patterns have been observed in neocortex, and their genetic determinants are starting to be understood (for review see Markam et al., 2004). Comparisons of intrinsic electrophysiological properties of trains and single action potentials between virgins and fathers in our study revealed no significant differences in any cell type. The numerous trends for differences in intrinsic electrophysiological properties in MPOA cells suggest that plasticity may be occurring in specific cells types categorized by connectivity or gene expression. Input resistances were similar to those reported in a nearly analogous slice preparation performed in mice by Lundius et al. (2010).

Both spontaneous PSCs and PSCs evoked by stimulating ~200 μms dorsal to the recording electrode were characterized and compared between virgins and fathers. As in rat MPOA, both excitatory (EPSCs) and inhibitory (IPSCs) currents were observed (Hoffman et al., 1994; Hoffman, Wuarin and Dudek, 1994; Karlsson, Haage, and Johansson, 1997; Karlssson, Sundgren-Andersson and Johansson et al., 1998; Haage and Johansson, 1999). Fathers exhibited lower maximal evoked IPSCs than virgins, suggesting decreased inhibition in the MPOA in fathers. Since the maximally-evoked IPSC was affected, it is likely that the overall number of inhibitory synapses onto MPOA cells was reduced. This might be caused by mating, cohabitation with a (pregnant) female, experience with pups, and/or a number of hormonal changes that occur during the

transition into fatherhood in California mice (reviewed below). Frequency of spontaneous PSCs was similar to that reported in mice by Lundius et al. (2010).

The observed reduction in inhibition is consistent with the previously documented increase in excitability of the MPOA in fathers (De Jong et al., 2009); but see Horrell et al., (2017). How exactly the reduction in inhibition affects specific behaviors is unclear, in part because the source of inhibition is unknown. Many studies on the input to the MPOA have been conducted e.g. [Simerly and Swanson, 1986; Miller and Lonstein, 2009; Rondini et al., 2010; Been and Petrulis, 2011; Northcutt and Lonstein, 2011; Sanathara et al., 2014). Unfortunately, the inhibitory inputs onto cells of the MPOA are poorly characterized and not easily separated into distinct terminal fields that would allow stimulation of identifiable axon terminals in this preparation. There are certainly inhibitory inputs from both intrinsic interneurons and from extrinsic sources, including strong projections from other parts of the hypothalamus, bed nucleus of the stria terminalis, and amygdala (e.g. Denske et al., 1975; Gardner and Phillips, 1977; Mayer, 1981; Coolen and Wood, 1998; Pardo-Bellver, et al., 2012; Shimogawa, Sakuma and Tamanouchi, 2015; Lebow and Chen, 2016; Kohl et al., 2018). Further studies using optogenetic activation of identified terminal fields in slices or in vivo could help resolve this important question.

MPOA neurons in rats exhibit inhibitory currents mediated by $GABA_A$ and glycine receptors (Hoffman et al., 1994; Hoffman et al., 1994; Karlsson, Haage and Johansson, 1997; Karlsson et al., 1997; Haage and Johansson, 1999) as well as excitatory currents mediated by AMPA and NMDA glutamate receptors and likely by T-, L-, N-, P-

and Q–type Ca^{+2} channels (Hoffman et al., 1994; Hoffman, Wuarin, and Dudek, 1994; Karlsson and Haage, 1997; Karlsson et al., 1997; Sundgren-Andersson and Johansson, 1998; Malinina, Druzin and Johansson, 2010; Tabarean, 2005; Qiu et al., 2006). Some of these currents in preoptic area neurons are acutely altered by estradiol [Qiu et al., 2006; Druzin et al., 2011; Zhang et al., 2013; Rønnekleiv et al., 2015), allopregnanolone (Haage and Johansson, 1999; Haage, Bäckström and Johansson, 2002; 2005), testosterone derivatives (Oberlander et al., 2012), capsaicin (Karlsson et al., 2005), and serotonin (Lee et al., 2008).

If and how these chemicals act on preoptic area neurons to alter paternal care in male California mice is a promising area of research. Testosterone increases paternal behavior in California mice via aromatization to estrogen, and fathers in this species have more aromatase activity in the MPOA than mated males without pups (Trainor and Marler, 2001; 2002; Trainor et al., 2003). Reports of fathers having lower circulating levels of testosterone and dihydrotestosterone (DHT) than mated males, and lower circulating DHT than virgin males, as well as the inability of DHT to restore paternal care after castration, further implicate the increase in aromatase activity and estrogen signaling in the MPOA as important for paternal care in this species (Trainor and Marler, 2002; Trainor et al., 2003); but see (Gubernick and Nelson, 1989). Furthermore, estrogen implants in the MPOA of male rats increase paternal care (Rosenblatt and Ceus, 1998). Progesterone, which is metabolized to allopregnanolone, is lower in California mouse fathers than in virgin males (Trainor et al., 2003), and progesterone antagonism increases while progesterone administration decreases paternal care in house mice (Schneider et al.,

2003). Additionally, the effects of prolactin and oxytocin on paternal care and on MPOA neurons in California mice merit investigation, as circulating levels of these peptides may change across the reproductive cycle in males of this species (Gubernick and Nelson, 1989; Gubernick et al., 1995). The possibility that hormonal effects on neural properties differ with reproductive status (e.g., virgin or father) is a potential avenue for future studies.

Some aspects of the morphology of MPOA cells (specifically, soma size and dendritic branching) of female rats are altered by pregnancy and attendant endocrine changes (i.e., progesterone withdrawal followed by elevated estrogen levels, Keyser-Marcus et al., 2001). Female California mice show a similar increase in somal area during the transition into motherhood (Gubernick et al., 1993). In a recent study of MPOA neuronal morphology in rats, mothers that licked and groomed their pups at high levels had fewer branches on primary dendrites than low-licking-grooming mothers; however, no differences were found in Sholl analysis, total dendritic arbor length, number of spines, and number of primary dendrites (Parent, et al., 2017). Only one previous study has characterized MPOA properties in male California mice. MPOA volume, number of neurons, density of neurons, and somal area did not differ among fathers, estranged fathers (fathers removed from their mate and pups 5 days postpartum for 45 days), and virgin males, according to a Golgi-Cox stain (Gubernick et al., 1993). Similarly, no major effects of fatherhood were seen in morphology of MPOA neurons in the present study. Fathers did, however, show a trend for reduction in the average length of primary neurites. Analysis of morphology of neurons of a specific cell type, such as

cells that express estrogen receptors or the neuropeptide galanin, is a promising avenue of research (Kohl et al., 2018).

Overall, the findings of this study suggest that some plasticity occurs in the MPOA during the transition into fatherhood and the onset of paternal behavior in male California mice, particularly decreased inhibition. Future studies targeting specific cell types in the MPOA, categorized on the basis of gene expression or connectivity, are needed and may reveal plasticity that facilitates parental behavior (e.g., Kohl et al., 2018; McHenry et al., 2017). Characterization and manipulation of particular inputs to the MPOA such as those from the medial amygdala, bed nucleus of the stria terminalis, periaqueductal grey, as well as hypothalamic stress and aggression centers may be of interest. Characterization of plasticity induced by hormones and neuropeptides implicated in parental behavior via signaling in the MPOA, as well as experience-dependent plasticity in the MPOA, may elucidate important mechanisms of parental care.

Conflict of interest

The authors declare no competing financial interests.

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Figures for Chapter 2

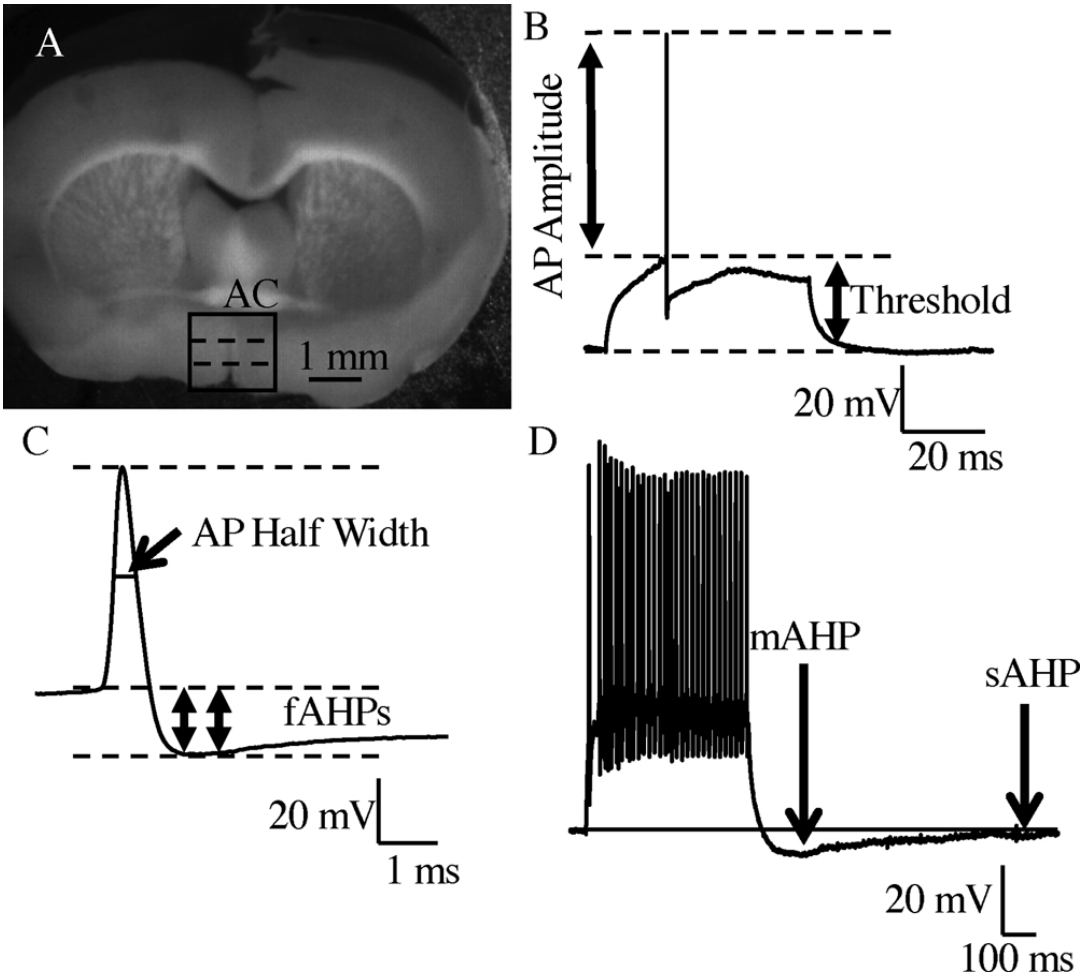

Fig. 1. Medial preoptic area of hypothalamus (MPOA) and properties of single action potentials (APs). A) Representative photomicrograph of 300 μm-thick coronal section used for recordings. The box, centered on the third ventricle, approximately delineates the MPOA. The dashed box represents the approx- imate area where recordings were made. AC: anterior commissure. B–C) Properties of single APs, including depictions of threshold, amplitude, half- width and fast afterhyperpolarizations (fAHPs). fAHPs were quantified as the largest voltage difference from baseline to 3–5 ms and 20–25 ms after the action potential crossed the baseline voltage. D) Depictions of medium after-hyperpolarization (mAHPs) and slow afterhyperpolarization (sAHP) after a train of APs. mAHP was quantified as the lowest voltage from baseline after train offset, and sAHP was quantified as voltage difference from baseline at 450 ms after stimulus offset.

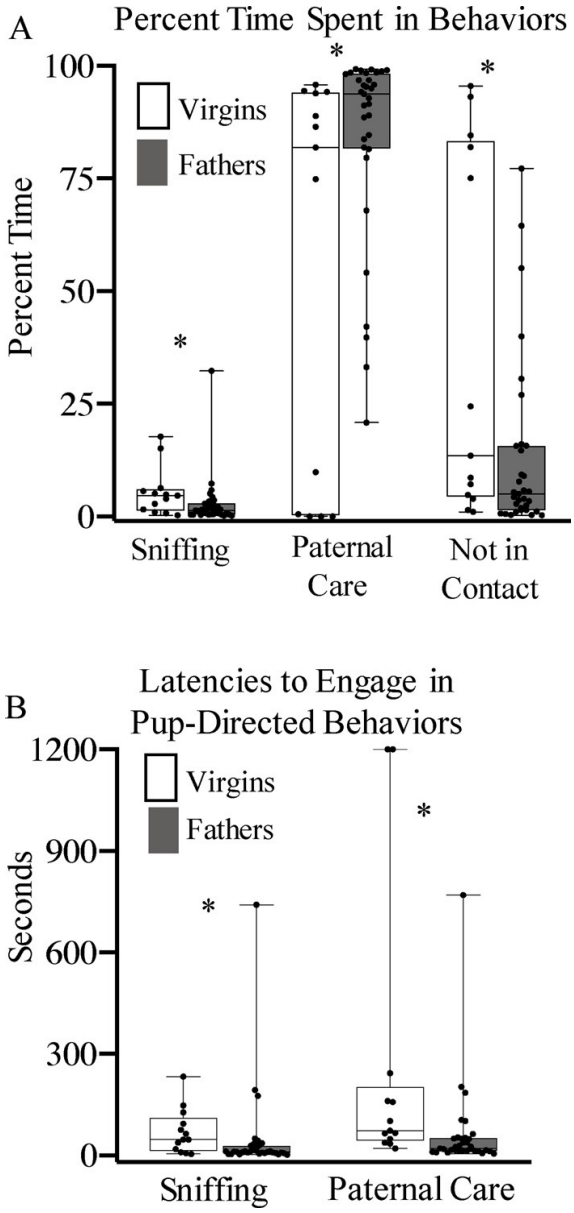

Fig. 2. Infant-directed behavior of virgin males (n = 13) and fathers (n = 33). A) Box and whisker plots of time spent in each behavior. Fathers spent sig- nificantly more time in paternal behaviors (licking, grooming, and/or huddling pup), less time sniffing, and less time not in contact with pups compared to virgins. B) Latency to engage in pup-directed behaviors. Fathers approached pups and initiated paternal care significantly more rapidly than did virgins. * $P < 0.05$.

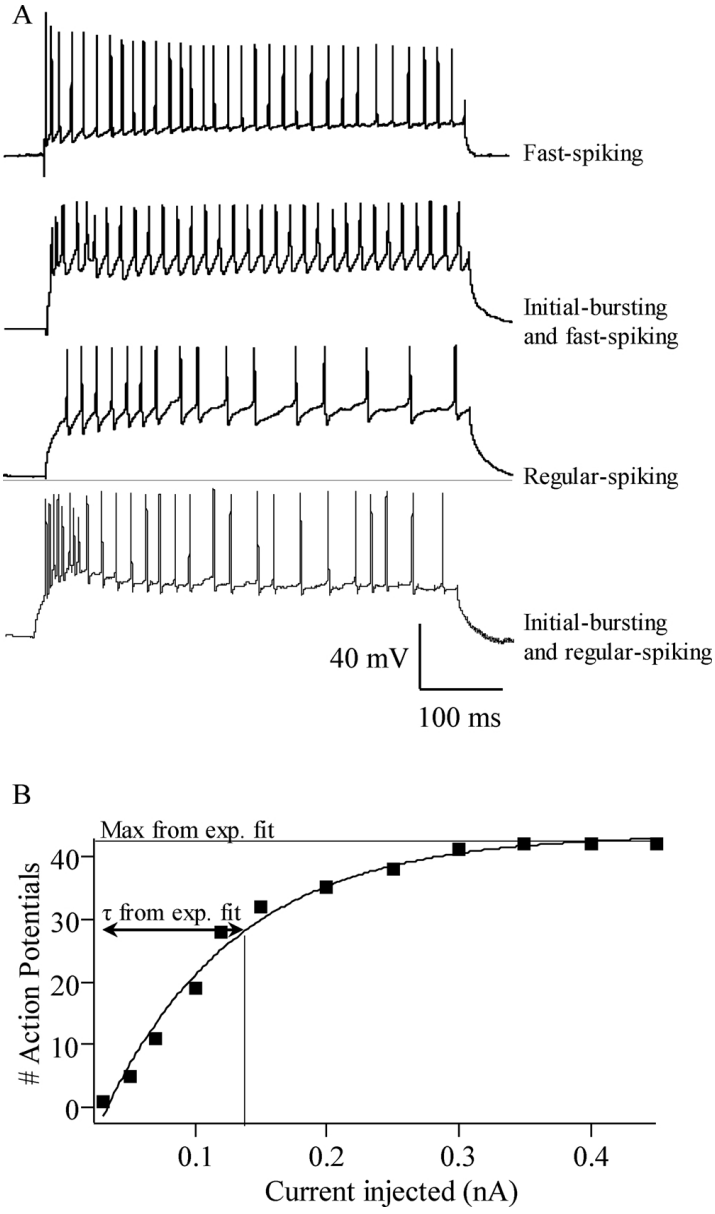

Fig. 3. Representative action potential (AP) trains of different cell types in the central medial preoptic area. A) Fast-spiking (FS), in which inter-spike intervals (ISIs) do not change during the train, and regular-spiking (RS), in which ISIs increase gradually during the train. RS and FS cells could also be initial-bursting (IB), in which cells fired a short burst of APs at high frequency followed by an increased ISI. B) Example of an input/output plot depicting the number of APs evoked by discrete amounts of depolarizing current (black squares). An exponential function was fitted to the data (black line); from this line, τ and a modeled maximum number of APs were determined.

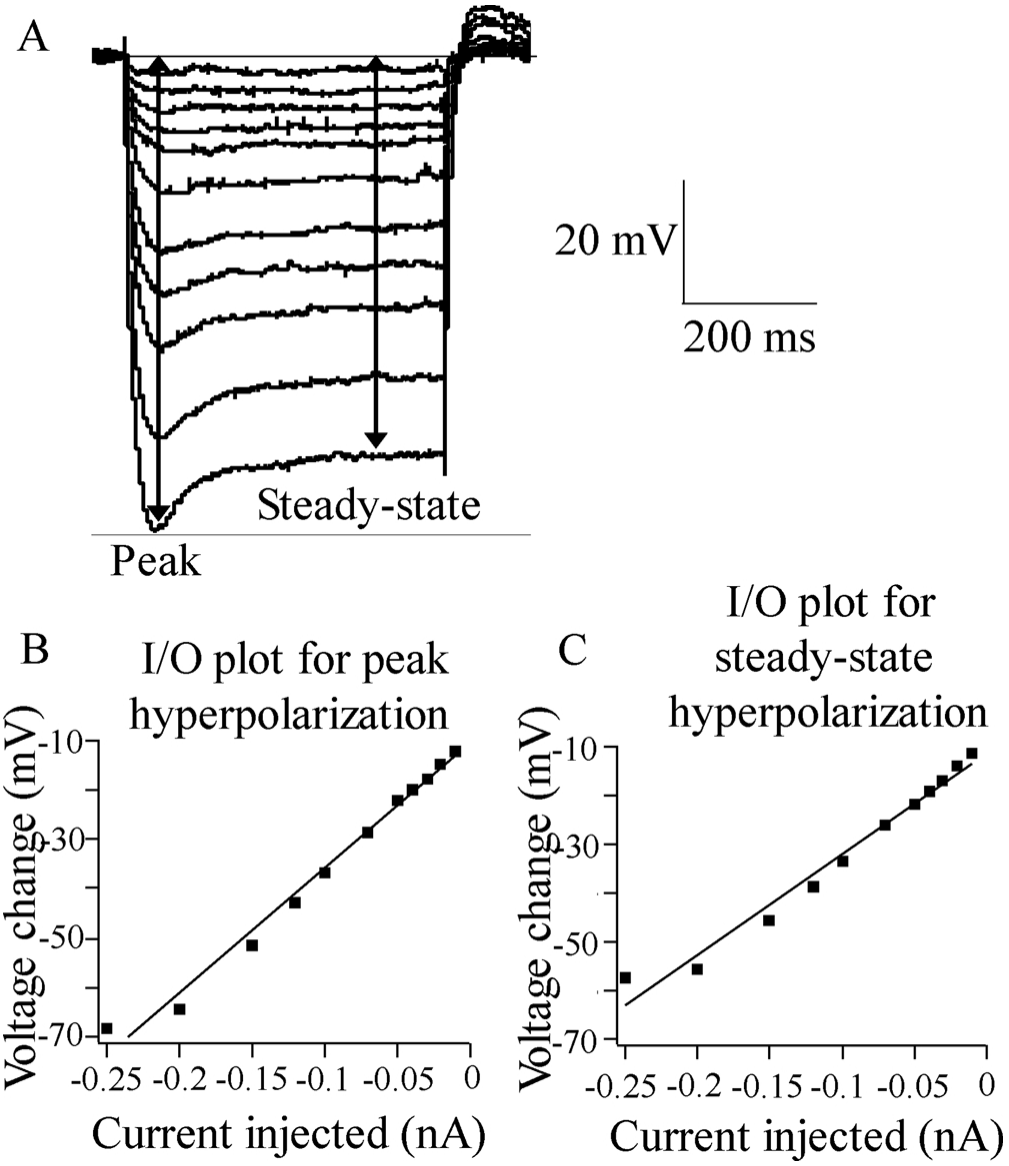

Fig. 4. Properties of responses to hyperpolarizing current injections. A) Example of voltage responses to hyperpolarizing current steps of 500ms duration. Responses at maximal hyperpolarization (peak) and at 400ms (steady-state) were quantified for each step. B–C) Examples of input/output plots for discrete amounts of hyperpolarizing current and resulting voltage changes from baseline to peak hyperpolarizing voltage response (B) and to the response at 400 ms (i.e., at steady-state, C). The slopes of lines of best fit were quantified.

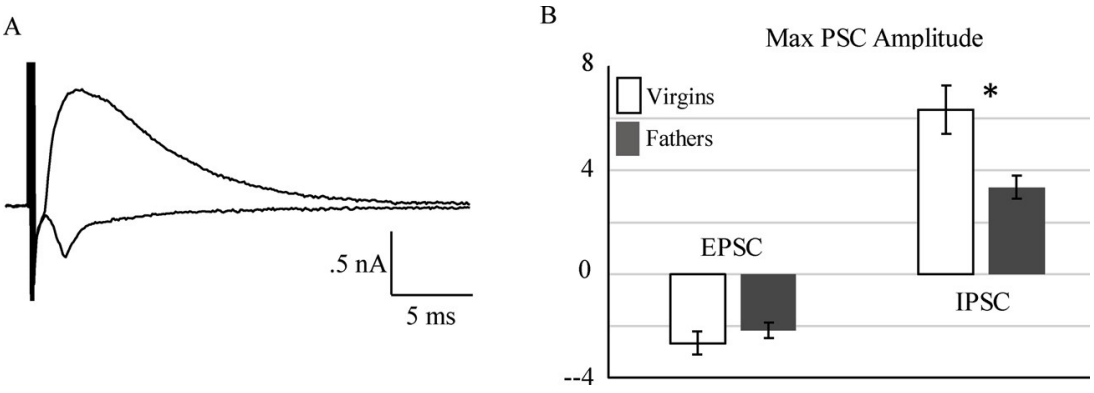

Fig. 5. Evoked postsynaptic currents (PSCs) in virgin males and fathers. A) Representative traces of an excitatory postsynaptic current (bottom) and inhibitory postsynaptic current (top). B) Maximal PSCs in cells from virgins (white, n = 22) and fathers (gray, n = 19). See Table 9 for full analysis of PSCs.

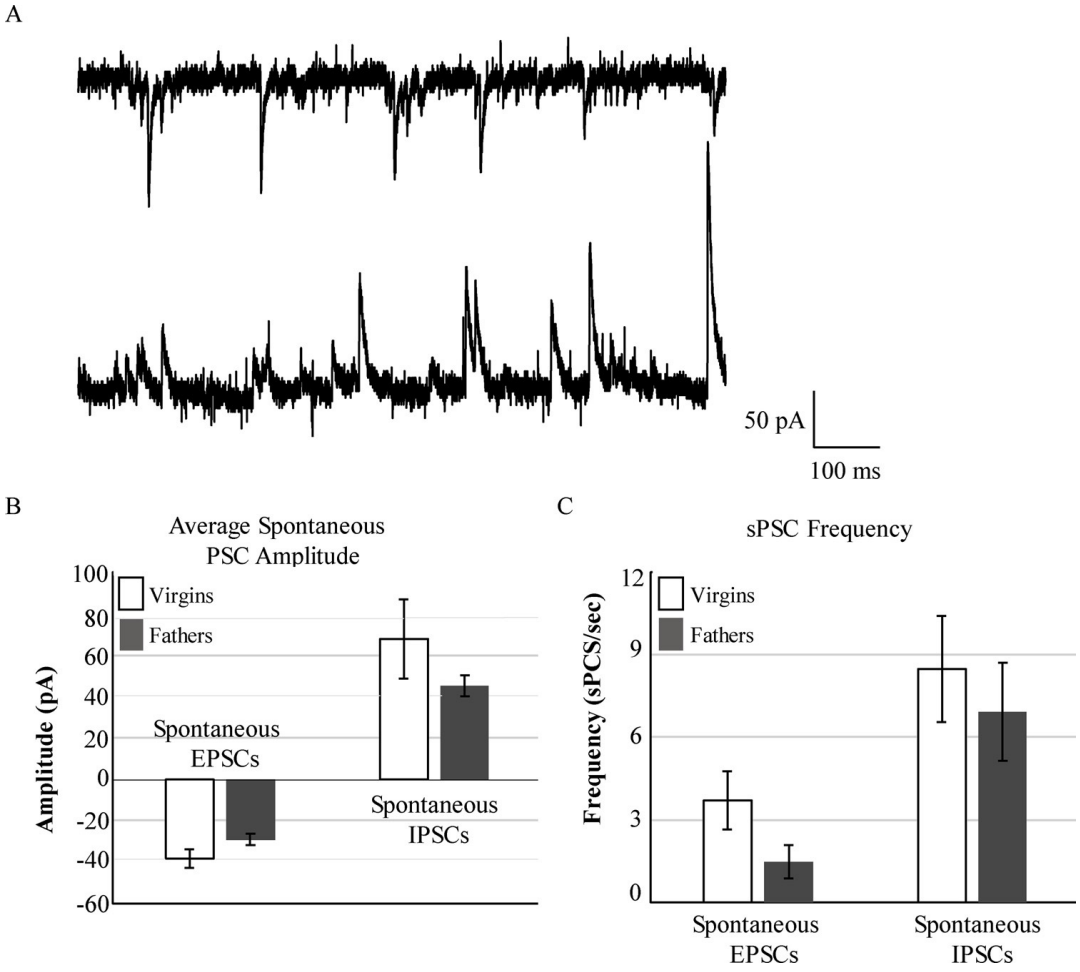

Fig. 6. Spontaneous postsynaptic currents (PSCs) in virgin males and fathers. A) Representative traces of spontaneous ex- citatory postsynaptic current (top) and inhibitory postsynaptic currents (bottom). B) Average amplitude of spontaneous postsynaptic currents (sPSCs) of cells from virgins (white; excitatory sPSCs: n = 10; inhibitory sPSCs: n=21) and fathers (gray; excitatory sPSCs: n = 5; inhibitory sPSCs: n = 13). C) Frequency of sPSCs in cells from virgins (white; excitatory sPSCs: n = 10; inhibitory sPSCs: n=21) and fathers (gray; excitatory sPSCs: n = 6; inhibitory sPSCs: n = 13).

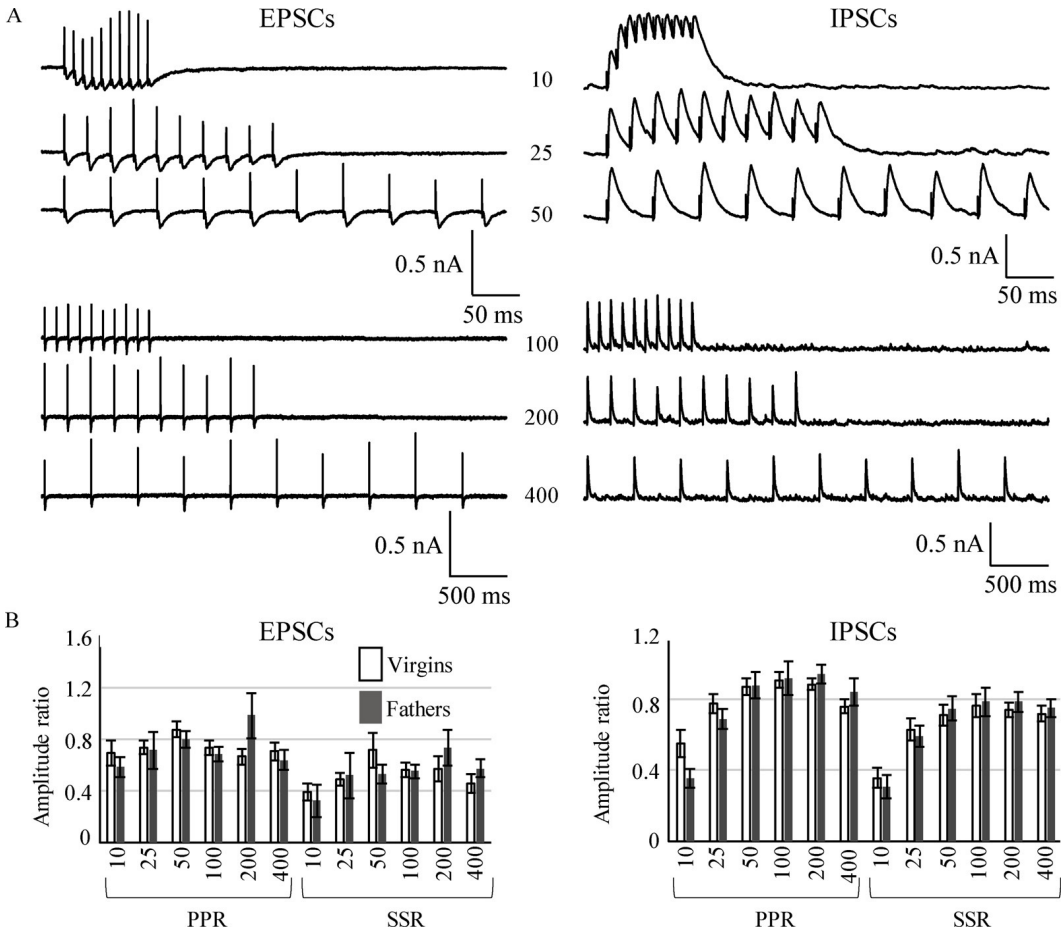

Fig. 7. Postsynaptic currents (PSCs) evoked by trains of stimuli at various interpulse intervals (IPIs). A) PSCs in response to stimulation at various IPIs (indicated between traces in ms). Cell were voltage-clamped at 0mV to iso- late inhibitory postsynaptic currents and at −55mV to isolate excitatory postsynaptic currents. Note change in scale from top three traces to bottom three traces. B) Paired-pulse ratios and steady-state ratios of PSCs evoked at various IPIs. No differences were ob- served between virgin males (white) and fathers (gray) at any IPI. See Table 9 for full analysis of PSCs.

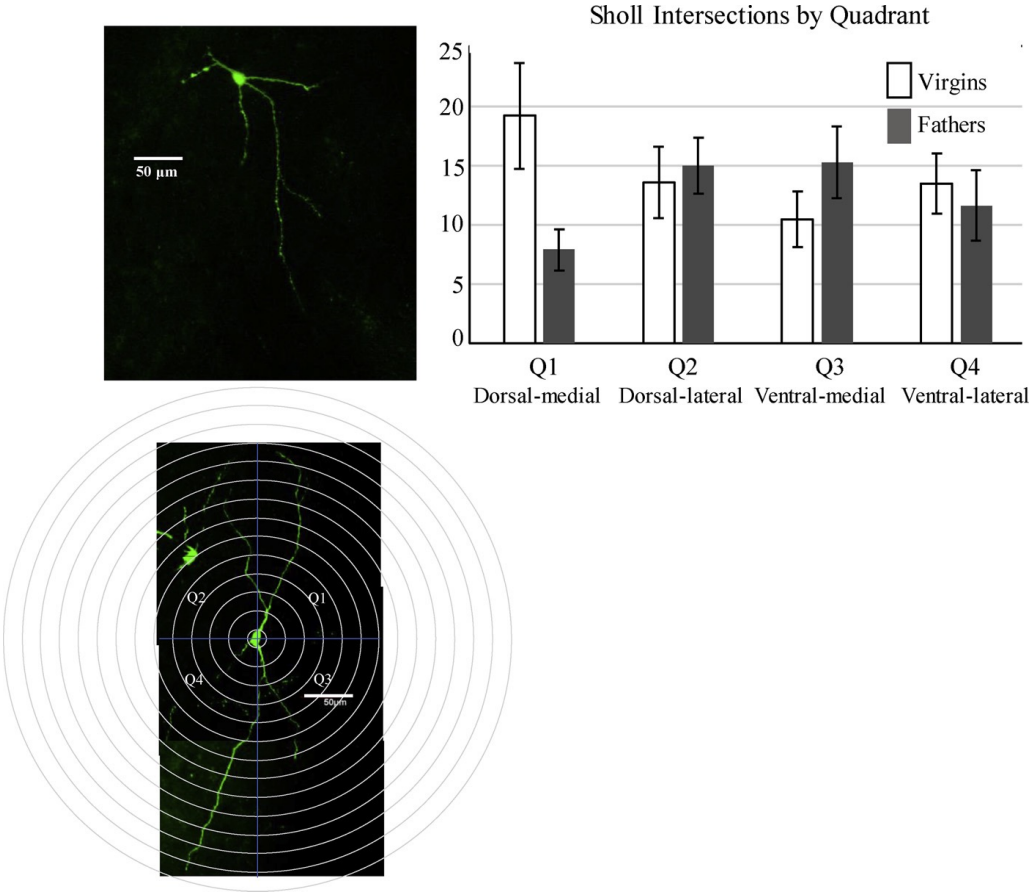

Fig. 8. Examples of MPOA neurons filled with biocytin during whole-cell recordings. Quadrantized Sholl analysis revealed no differences in morphology between cells from virgin males (white, n = 23) and fathers (gray, n = 22). See Table 10 for more detailed morphological analyses.

Tables for Chapter 2

Table 1
Resting potential and input resistance of neurons from virgin males and fathers in Cs-based and K-based solutions.

		Virgins			Fathers			Analysis			
Property	Unit	N	Mean	SE	N	Mean	SE	Test	df	t	P-value
Resting potential											
Cs-based solution	mV	22	−44.50	1.09	19	−45.95	1.32	t-test	39	0.85	0.40
K-based solution	mV	23	−50.17	1.72	32	−44.63	3.66	t-test	53	0.23	0.23
Input resistance											
Cs-based solution	MΩ	22	519.45	40.08	19	567.47	48.10	t-test	49	−0.77	0.44
K-based solution	MΩ	23	648.56	60.00	31	812.35	77.66	t-test	52	−1.57	0.12

A Cs-based solution with QX-314 was used for recording synaptic currents, and a K-based solution was used for recording intrinsic properties (see Materials & Methods). N = number of cells.

Table 2
Properties of action potentials and responses to hyperpolarizing current in all cell types in virgin males and fathers.

		Virgins			Fathers			Analysis			
Measure	Unit	N	Mean	SE	N	Mean	SE	Test	df	Stat.	P-value
Properties of single APs											
Threshold	mV	22	29.29	1.80	31	27.74	1.64	t-test	51	0.63	0.53
Amplitude	mV	22	40.89	3.92	31	45.64	2.79	t-test	51	−1.02	0.31
Half-width	ms	22	0.92	0.09	32	0.85	0.05	MW	–	324.0	0.76
fAHP at 3-5 ms	mV	22	−11.24	1.24	31	−10.46	1.11	t-test	51	−0.46	0.64
fAHP at 20-25 ms	mV	22	−10.74	1.29	31	−8.68	0.97	MW	–	289.0	0.35
Properties of trains of APs											
Maximum # of APs	#	21	45.86	2.57	32	52.53	4.61	MW	–	335.0	0.99
Maximum APs from exp. fit	#	21	80.13	12.96	31	76.27	9.66	MW	–	295.0	0.57
Tau from AP exp. fit	nA	21	0.40	0.15	32	0.24	0.04	MW	–	334.0	0.97
Minimum average AP ISI	ms	21	10.32	0.83	32	12.26	1.06	MW	–	279.0	0.30
Minimum ISI from exp. fit	ms	21	16.06	3.31	32	12.18	1.44	MW	–	312.0	0.66
Tau from ISI exp. fit	ms	21	0.03	0.01	32	0.39	0.32	MW	–	287.0	0.37
mAHP	mV	20	−3.56	1.05	30	−3.35	0.70	MW	–	273.5	0.60
sAHP	mV	19	−2.20	0.38	30	−2.31	0.36	t-test	47	0.20	0.84
Responses to hyperpolarizing current											
Maximum voltage change	mV	21	−90.74	6.76	28	−76.46	5.50	t-test	47	−1.65	0.10
Slope of peak hyperpolarization	—	21	45.19	6.06	30	40.30	4.30	MW	–	313.0	0.97
Slope of steady-state hyperpolarization	—	21	38.95	6.13	30	34.89	3.91	MW	–	301.0	0.79
Peak slope/steady-state slope	—	21	1.14	0.04	30	1.18	0.03	MW	–	250.0	0.21

N = number of cells, AP = action potential, ISI = inter-spike interval, fAHP = fast afterhyperpolarization, mAHP = medium afterhyperpolarization, sAHP = slow afterhyperpolarization, exp. = exponential, Stat. = test statistic, MW = Mann-Whitney U test.

Table 3
Properties of single action potentials in fast-spiking and regular-spiking cells in virgin males and fathers.

		Virgins			Fathers			Analysis			
Property	Unit	N	Mean	SE	N	Mean	SE	Test	df	t	P-value
Fast-spiking											
Threshold	mV	11	26.16	2.02	14	24.22	1.77	t-test	23	0.73	0.48
Amplitude	mV	11	43.44	5.50	14	49.17	4.93	t-test	23	−0.77	0.45
Half-width	ms	11	0.77	0.11	14	0.79	0.09	t-test	23	−0.12	0.90
fAHP at 3-5 ms	mV	11	−11.79	2.01	14	−11.27	1.79	t-test	23	−0.19	0.85
fAHP at 20-25 ms	mV	11	−12.49	2.22	14	−8.80	1.18	t-test	23	−1.56	0.13
Regular-spiking											
Threshold	mV	11	32.43	2.76	17	30.63	2.43	t-test	26	0.48	0.64
Amplitude	mV	11	38.34	5.74	17	42.74	3.05	t-test	26	−0.74	0.47
Half-width	ms	11	1.06	0.15	17	0.91	0.04	t-test	26	1.12	0.25
fAHP at 3-5 ms	mV	11	−10.68	1.53	17	−9.79	1.41	t-test	26	−0.42	0.68
fAHP at 20-25 ms	mV	11	−8.99	1.20	17	−8.58	1.51	t-test	26	−0.19	0.85

N = number of cells, fAHP = fast afterhyperpolarization.

Table 4
Properties of trains of action potentials of fast-spiking and regular-spiking cells in virgin males and fathers.

		Virgins			Fathers			Analysis			
Property	Unit	N	Mean	SE	N	Mean	SE	Test	df	Stat.	P-value
Fast-spiking											
Maximum APs	#	10	48.2	4.38	15	62.93	7.13	MW	–	57.5	0.33
Maximum APs from exp. fit	#	10	62.09	7.39	14	74.13	10.43	MW	–	65.0	0.77
Tau from AP exp. fit	nA	10	0.19	0.05	15	0.19	0.04	MW	–	75.0	1.00
Minimum average AP ISI	ms	10	9.85	1.42	15	9.77	1.22	t-test	23	0.42	0.97
Minimum ISI from exp. fit	ms	10	19.60	6.58	15	11.68	1.50	MW	–	58.0	0.35
Tau from ISI exp. fit	ms	10	0.03	0.01	15	0.03	0.004	MW	–	74.0	0.96
mAHP	mV	10	−2.70	1.77	14	−2.87	0.68	t-test	22	0.10	0.92
sAHP	mV	9	−1.66	0.50	14	−2.11	0.47	t-test	21	0.64	0.53
Regular-spiking											
Maximum APs	#	11	43.73	2.94	17	43.35	5.21	MW	–	76.0	0.41
Maximum APs from exp. fit	#	11	96.53	23.24	17	78.04	15.68	MW	–	76.0	0.41
Tau from AP exp. fit	nA	11	0.60	0.27	17	0.29	0.07	MW	–	89.0	0.83
Minimum average AP ISI	ms	11	10.74	0.98	17	14.46	1.51	t-test	26	−1.82	0.08*
Minimum ISI from exp. fit	ms	11	12.85	2.06	17	12.62	2.40	t-test	26	0.07	0.95
Tau from ISI exp. fit	ms	11	0.04	0.01	17	0.70	0.60	MW	–	65.0	0.18
mAHP	mV	10	−4.43	1.15	16	−3.76	1.18	MW	–	63.0	0.37
sAHP	mV	10	−2.69	0.53	16	−248	0.54	t-test	24	−0.27	0.79

N = number of cells, AP = action potential, ISI = inter-spike interval, fAHP = fast afterhyperpolarization, mAHP = medium afterhyperpolarization, sAHP = slow afterhyperpolarization, exp. = exponential, Stat. = test statistic, MW = Mann-Whitney U test.
* = $0.10 > p > 0.05$.

Table 5
Responses to hyperpolarizing current in fast-spiking and regular-spiking cells in virgin males and new fathers.

		Virgins			Fathers			Analysis			
Property	Unit	N	Mean	SE	N	Mean	SE	Test	df	Stat.	P-value
Fast-spiking											
Maximum voltage change	mV	10	−89.31	9.44	14	−76.50	8.47	t-test	22	−1.00	0.33
Slope of peak hyperpolarization	—	10	42.86	10.44	14	34.95	5.36	MW	–	63.0	0.68
Slope of steady-state hyperpolarization	—	10	40.87	11.04	14	30.19	5.02	MW	–	60.0	0.56
Peak slope/steady-state slope	—	10	1.09	0.04	14	1.20	0.06	MW	–	40.0	0.08*
Regular-spiking											
Maximum voltage change	mV	11	−92.04	10.05	14	−76.41	7.35	t-test	23	−1.27	0.21
Slope of peak hyperpolarization	—	11	42.49	7.15	16	44.98	6.48	t-test	25	−0.15	0.88
Slope of steady-state hyperpolarization	—	11	37.20	6.55	16	39.01	5.84	t-test	25	−0.20	0.84
Peak slope/steady-state slope	—	11	1.19	0.07	16	1.16	0.03	MW	–	88.0	1.00

Responses to 500 ms-long square pulses of positive and negative current of various intensities were acquired while cells were current-clamped at -70 mV (see Material and Methods). N = number of cells, Stat. = test statistic, MW = Mann-Whitney U test.

* = $0.10 > p > 0.05$.

Table 6
Properties of single action potentials for fast-spiking, initial-bursting + fast-spiking, regular-spiking, and initial-bursting + regular-spiking cells in virgin males and fathers.

		Virgins			Fathers			Analysis[a]			
Property	Unit	N	Mean	SE	N	Mean	SE	Test	df	Stat.	P-value
Fast-spiking											
Threshold	mV	9	25.90	2.41	12	23.43	1.98	t-test	19	0.80	0.43
Amplitude	mV	9	42.55	6.36	12	48.89	5.37	t-test	19	−0.76	0.45
Half-width	ms	9	0.86	0.11	12	0.79	0.10	t-test	19	0.42	0.68
fAHP at 3-5 ms	mV	9	−11.73	2.40	12	−10.80	2.01	t-test	19	−0.30	0.77
fAHP at 20-25 ms	mV	9	−13.21	2.53	12	−8.73	1.39	t-test	19	−1.66	0.11
Initial-bursting + fast-spiking											
Threshold	mV	2	27.30	—	2	28.97	—	—	—	—	—
Amplitude	mV	2	47.45	—	2	50.83	—	—	—	—	—
Half-width	ms	2	0.4	—	2	0.78	—	—	—	—	—
fAHP at 3-5 ms	mV	2	−12.05	—	2	−14.14	—	—	—	—	—
fAHP at 20-25 ms	mv	2	−9.25	—	2	−9.20	—	—	—	—	—
Regular-spiking											
Threshold	mV	6	32.88	4.66	13	29.39	3.03	t-test	17	0.64	0.53
Amplitude	mV	6	42.27	8.39	13	42.74	3.51	t-test	17	−0.06	0.95
Half-width	ms	6	1.06	0.24	13	0.89	0.05	MW	–	30.5	0.46
fAHP at 3-5 ms	mV	6	−12.45	1.86	13	−9.36	1.76	t-test	17	−1.07	0.30
fAHP at 20-25 ms	mV	6	−7.55	1.57	13	−9.32	0.97	t-test	17	1.00	0.33
Initial-bursting + regular-spiking											
Threshold	mV	4	31.83	3.89	4	34.67	2.71	MW	–	6.0	0.56
Amplitude	mV	4	26.79	5.65	4	42.73	7.15	t-test	6	−1.75	0.13
Half-width	ms	4	1.19	0.12	4	0.96	0.06	t-test	6	1.82	0.12
fAHP at 3-5 ms	mV	4	−6.29	0.94	4	−11.18	1.98	t-test	6	2.23	0.07*
fAHP at 20-25 ms	mV	4	−10.02	2.01	4	−6.21	6.11	t-test	6	−0.59	0.57

N = number of cells, fAHP = fast afterhyperpolarization, Stat. = test statistic, MW = Mann-Whitney U test.
[a] Properties of initial-bursting + fast-spiking cells were not analyzed statistically due to small sample sizes.
* = 0.10 > p > 0.05

Table 7
Properties of trains of action potentials for fast-spiking, initial-bursting + fast-spiking, regular-spiking, and initial-bursting + regular-spiking cells in virgin males and fathers.

		Virgins			Fathers			Analysis[a]			
Property	Unit	N	Mean	SE	N	Mean	SE	Test	df	Stat.	P-value
Fast-spiking											
Maximum APs	#	8	50.88	5.06	13	62.23	7.73	MW	–	40.0	0.39
Maximum APs from exp. fit	#	8	62.80	9.13	12	76.00	11.67	t-test	18	−0.82	0.43
Tau from AP exp. fit	nA	8	0.14	004	13	0.21	0.05	MW	–	36.0	0.25
Minimum average AP ISI	ms	8	8.79	1.56	13	9.85	1.30	MW	–	47.0	0.72
Minimum ISI from exp. fit	ms	8	20.16	8.31	13	11.98	1.61	MW	–	45.0	0.61
Tau from ISI exp. fit	ms	8	0.03	0.01	13	0.03	0.005	MW	–	48.0	0.77
mAHP	mV	8	−2.47	2.13	13	−2.80	0.86	t-test	19	0.174	0.86
sAHP	mV	7	−1.81	0.60	13	−2.02	0.50	t-test	18	0.25	0.80
Initial-bursting + fast-spiking											
Maximum APs	#	2	37.5	—	2	61	—	—	—	—	—
Maximum APs from exp. fit	#	2	59.23	—	1	35.35	—	—	—	—	—
Tau from AP exp. fit	nA	2	0.35	—	2	0.07	—	—	—	—	—
Minimum average AP ISI	ms	2	14.05	—	2	9.25	—	—	—	—	—
Minimum ISI from exp. fit	ms	2	17.37	—	2	9.701	—	—	—	—	—
Tau from ISI exp. fit	ms	2	0.04	—	2	0.03	—	—	—	—	—
mAHP	mV	1	−7.3	—	1	−3.9	—	—	—	—	—
sAHP	mV	1	−2.2	—	1	−3.3	—	—	—	—	—
Regular-spiking											
Maximum APs	#	6	46.83	4.17	13	43.61	6.27	t-test	17	0.33	0.75
Maximum APs from exp. fit	#	6	111.04	38.08	13	73.39	15.87	MW	–	26.0	0.25
Tau from AP exp. fit	nA	6	0.62	0.39	13	0.25	0.06	MW	–	34.0	0.66
Minimum average AP ISI	ms	6	10.38	1.41	13	13.94	1.71	t-test	17	−1.31	0.21
Minimum ISI from exp. fit	ms	6	11.23	1.93	13	13.01	3.10	t-test	17	−0.37	0.72
Tau from ISI exp. fit	ms	6	0.04	0.01	13	0.84	0.79	MW	–	37.0	0.86
mAHP	mV	6	−5.73	1.78	13	−3.01	0.79	t-test	17	−1.63	0.12
sAHP	mV	6	−1.84	0.76	13	−2.54	0.63	t-test	17	0.65	0.53
Initial-bursting + regular-spiking											
Maximum APs	#	4	42.5	3.78	4	42.5	10.25	t-test	6	0.00	1.0
Maximum APs from exp. fit	#	4	92.24	28.36	4	93.16	46.89	MW	–	7.00	0.77
Tau from AP exp. fit	nA	4	0.72	0.50	4	0.42	0.24	MW	–	7.00	0.77
Minimum average AP ISI	ms	4	13.32	1.26	4	16.13	3.55	t-test	6	−1.01	0.35
Minimum ISI from exp. fit	ms	4	16.81	3.47	4	11.35	2.20	MW	–	4.00	0.25
Tau from ISI exp. fit	ms	4	0.03	0.02	4	0.23	0.15	MW	–	2.00	0.83
mAHP	mV	4	−3.01	1.80	4	−6.01	4.32	t-test	6	0.64	0.55
sAHP	mV	4	−3.97	0.51	4	−2.28	1.16	MW	–	5.00	0.39

N = number of cells, AP = action potential, ISI = inter-spike interval, fAHP = fast afterhyperpolarization, mAHP = medium afterhyperpolarization, sAHP = slow afterhyperpolarization, exp. = exponential, Stat. = test statistic, MW = Mann-Whitney U test.

[a] Properties of initial-bursting + fast-spiking cells were not analyzed statistically due to small sample sizes.

Table 8
Responses to hyperpolarizing current for fast-spiking, initial-bursting + fast-spiking, regular-spiking, and initial-bursting + regular-spiking cells in virgin males and fathers.

		Virgins			Fathers			Analysis[a] *:			
Property	Unit	N	Mean	SE	N	Mean	SE	Test	df	Stat.	P-value
Fast-spiking											
Max voltage change	mV	8	−94.01	10.91	13	−74.16	8.79	t-test	19	−1.41	0.18
Slope of peak hyperpolarization	—	8	46.96	12.69	13	34.99	5.79	MW	–	43.0	0.52
Slope of steady-state hyperpolarization	—	8	46.0	13.26	13	30.54	5.41	MW	–	39.0	0.35
Peak slope/steady-state slope	—	8	1.04	0.02	13	1.19	0.07	t-test	19	−1.72	0.10
Initial-bursting + fast-spiking											
Max voltage change	mV	2	−70.5	—	1	−107	—	—	—	—	—
Slope of peak hyperpolarization	—	2	26.45	—	1	34.4	—	—	—	—	—
Slope of steady-state hyperpolarization	—	2	20.15	—	1	25.6	—	—	—	—	—
Peak slope/steady-state slope	—	2	1.29	—	1	1.34	—	—	—	—	—
Regular-spiking											
Max voltage change	mV	6	−105.32	14.91	12	−74.06	9.13	t-test	14	−1.90	0.08*
Slope of peak hyperpolarization	—	6	42.60	7.81	12	43.98	8.15	t-test	16	−0.11	0.92
Slope of steady-state hyperpolarization	—	6	34.62	6.69	12	37.67	7.18	MW	–	34.0	0.85
Peak slope/steady-state slope	—	6	1.24	0.11	12	1.17	0.04	MW	–	32.0	0.71
Initial-bursting + regular-spiking											
Max voltage change	mV	4	−69.63	10.31	4	−82.28	13.33	t-test	6	0.75	0.48
Slope of peak hyperpolarization	—	4	36.43	14.31	4	47.99	10.34	MW	–	4.00	0.25
Slope of steady-state hyperpolarization	—	4	31.18	11.62	4	43.03	10.31	t-test	6	−0.76	0.47
Peak slope/steady-state slope	—	4	1.16	0.05	4	1.13	0.03	t-test	6	0.51	0.63

[a] Properties of initial-bursting + fast-spiking cells were not analyzed statistically due to small sample sizes. N = number of cells, Stat. = test statistic, MW = Mann-Whitney U test.
* = $0.10 > p > 0.05$.

Table 9
Properties of post-synaptic currents in virgin males and fathers.

		Virgins			Fathers			Analysis			
Property	Unit	N	Mean	SE	N	Mean	SE	Test	df	Stat.	P-value
Single evoked PSCs											
EPSC min	nA	15	−0.32	0.04	14	−0.25	0.02	MW	–	79.5	0.26
EPSC max	nA	15	−2.67	0.46	14	−2.17	0.31	MW	–	95.5	0.68
IPSC min	nA	22	0.33	0.04	19	0.26	0.03	t-test	39	0.13	0.14
IPSC max	nA	22	6.36	0.93	19	3.35	0.45	MW	–	111.5	0.01**
Paired-pulse ratio of ePSCs											
Inter-pulse interval											
10 ms	—	11	0.87	0.12	8	0.73	0.10	t-test	17	0.84	0.42
25 ms	—	11	0.92	0.07	8	0.89	0.18	t-test	17	0.14	0.89
50 ms	—	11	1.09	0.07	8	1.00	0.08	t-test	17	0.87	0.40
100 ms	—	11	0.92	0.07	8	0.86	0.07	t-test	17	0.58	0.57
200 ms	—	10	0.83	0.07	8	1.23	0.22	t-test	16	−1.85	0.08*
400 ms	—	10	0.88	0.08	8	0.80	0.10	t-test	16	0.65	0.53
Steady-state ratio of ePSCs											
Inter-pulse interval											
10 ms	—	11	0.49	0.08	8	0.40	0.16	MW	–	29.0	0.22
25 ms	—	11	0.61	0.06	8	0.65	0.21	MW	–	30.0	0.25
50 ms	—	11	0.89	0.17	8	0.66	0.09	t-test	17	1.09	0.29
100 ms	—	11	0.70	0.07	8	0.69	0.06	t-test	17	0.14	0.89
200 ms	—	10	0.71	0.12	8	0.92	0.17	t-test	16	−1.00	0.33
400 ms	—	10	0.57	0.09	7	0.72	0.09	t-test	15	−1.11	0.28
Paired-pulse ratio of iPSCs											
Inter-pulse interval											
10 ms	—	20	0.55	0.08	14	0.35	0.05	t-test	32	1.93	0.06*
25 ms	—	20	0.77	0.05	15	0.69	0.06	MW	–	112.0	0.21
50 ms	—	20	0.87	0.05	15	0.88	0.08	t-test	33	−0.10	0.92
100 ms	—	20	0.91	0.04	15	0.92	0.10	t-test	33	−0.09	0.93
200 ms	—	20	0.89	0.04	15	0.94	0.05	t-test	33	−0.88	0.39
400 ms	—	20	0.76	0.04	15	0.84	0.08	t-test	33	−1.03	0.31
Steady-state ratio of iPSCs											
Inter-pulse interval	Unit										
10 ms	—	20	0.36	0.06	15	0.31	0.07	MW	–	132	0.55
25 ms	—	20	0.63	0.06	15	0.59	0.06	t-test	33	0.42	0.68
50 ms	—	20	0.71	0.06	15	0.74	0.07	t-test	33	0.45	0.65
100 ms	—	20	0.76	0.07	15	0.79	0.08	t-test	33	−0.21	0.83
200 ms	—	20	0.74	0.04	15	0.79	0.06	t-test	33	−0.67	0.51
400 ms		20	0.72	0.04	15	0.75	0.05	t-test	34	−0.47	0.64
Spontaneous PSCs											
sEPSC frequency	Hz	10	3.71	1.07	6	1.49	0.60	MW	–	14.0	0.08*
sEPSC amplitude	nA	10	−0.39	0.05	5	−0.29	0.03	t-test	13	−1.34	0.20
sIPSC frequency	Hz	21	8.46	1.94	13	6.92	1.79	t-test	32	0.54	0.59
sIPSC amplitude	nA	21	0.68	0.19	13	0.45	0.05	t-test	32	0.92	0.36

N = number of cells, PSC = post-synaptic current, EPSC = excitatory post-synaptic current, IPSC = inhibitory post-synaptic current, sEPSC = spontaneous excitatory post-synaptic current, sIPSC = spontaneous inhibitory post-synaptic current, Stat. = test statistic, MW = Mann-Whitney U test.

* = $0.10 > p > 0.05$.

** = $p < 0.05$.

Table 10
Morphological properties of medial preoptic area cells in virgin males and fathers.

		Virgins (N = 23 cells)		Fathers (N = 22 cells)		Analysis (df = 43)		
Sholl Analysis	Unit	Mean	SE	Mean	SE	Test	Stat.	P-value
Q1 (dorsal medial) intersections	#	19.17	4.47	7.86	1.76	MW	190.0	0.15
Q2 (dorsal lateral) intersections	#	13.52	3.01	14.95	2.37	MW	198.5	0.22
Q3 (ventral medial) intersections	#	10.43	2.39	15.23	3.04	MW	186.0	0.13
Q4 (ventral lateral) intersections	#	13.43	2.53	11.59	2.98	MW	232.5	0.64
Q1 + Q3 (medial) intersections	#	29.61	4.47	23.09	3.52	MW	211.0	0.34
Q2 + Q4 (lateral) intersections	#	26.96	4.56	26.55	4.17	MW	224.0	0.41
Medial/lateral intersections	–	1.41	0.19	2.73	1.27	MW	177.0	0.08*
Q1 + Q2 (dorsal) intersections	#	32.70	5.49	22.82	2.95	MW	232.0	0.63
Q3 + Q4 (ventral) intersections	#	23.87	3.60	26.82	3.82	MW	208.5	0.31
Dorsal/ventral intersections	–	1.75	0.32	1.52	0.49	MW	203.5	0.26
Total intersections	#	56.61	7.56	49.64	5.48	MW	246.5	0.88
Branch points								
Branches in Q1	#	0.96	0.28	0.45	0.13	MW	209.0	0.26
Branches in Q2	#	0.83	0.27	0.73	0.20	MW	225.0	0.48
Branches in Q3	#	0.56	0.18	0.86	0.23	MW	213.0	0.31
Branches in Q4	#	0.65	0.16	0.68	0.21	MW	235.0	0.65
Total Branches	#	3	0.43	2.64	0.51	MW	230.5	0.61
Properties of primary neurites								
Number of primary neurites	#	3.17	0.24	3.63	0.23	MW	211.0	0.33
Average length of primary neurites	µm	271.62	27.58	212.48	17.70	t-test	1.79	0.08*
Longest length of primary neurites	µm	416.62	47.71	371.25	30.82	t-test	0.79	0.43
Total length of primary neurites	µm	903.42	123.49	793.47	86.65	MW	244.0	0.84
Properties of secondary neurites								
Number of secondary neurites	#	2.78	0.39	2.09	0.31	MW	218.5	0.43
Average length of secondary neurites	µm	102.37	19.91	97.88	12.91	MW	223.0	0.50
Longest length of secondary neurites	µm	172.95	29.89	153.94	26.66	MW	245.0	0.86
Total length of secondary neurites	µm	359.31	72.89	253.46	53.34	MW	238.0	0.73
Properties of tertiary neurites								
Number of tertiary neurites	#	0.39	0.14	0.32	0.17	MW	220.5	0.33
Average length of tertiary neurites	µm	28.38	11.60	6.22	3.01	MW	207.5	0.17
Longest length of tertiary neurites	µm	28.69	11.66	7.45	3.92	MW	207.5	0.17
Total length of tertiary neurites	µm	35.35	13.80	12.30	7.24	MW	210.5	0.20
Other morphological properties								
Total neurite length	µm	1296.4	178.02	1055.69	123.99	MW	244.0	0.84
Largest soma diameter	µm	18.81	1.48	17.73	0.65	MW	250.0	0.95
Soma circumference	µm	55.41	4.03	54.06	2.40	MW	246.0	0.87

Q = quadrant, Stat. = test statistic, MW = Mann-Whitney U test.
* = $0.10 > p > 0.05$.

Supplemental Table 1. Correlations of selected behavioral and neuronal measures in virgin males.

	Latency to engage in paternal care			Percent time in paternal care		
	r_s	P-value	N	r_s	P-value	N
Input resistance in K-based solution	-0.09	0.78	15	0.11	0.69	15
Threshold to AP	-0.01	0.98	14	0.08	0.79	14
AP amplitude	-0.68	0.007*	14	-0.17	0.57	14
AP half-width	0.34	0.23	14	0.35	0.23	14
fAHP at 3-5 msec	0.38	0.18	14	0.04	0.9	14
Maximum number of APs	0.02	0.96	13	0.15	0.62	13
Minimum average AP ISI	0.09	0.76	13	-0.12	0.71	13
Minimum average AP ISI	0.06	0.84	13	0.41	0.17	13
Tau from ISI exponential fit	0.13	0.68	13	0.04	0.91	13
mAHP	-0.01	0.96	13	-0.14	0.69	13
sAHP	0.25	0.41	13	0.23	0.47	13
Maximum voltage change in response to hyperpolarizing current	0.67	0.009*	14	-0.12	0.67	14
Input resistance in Cs-based solution	-0.48	0.23	8	0.48	0.23	8
EPSC min	0.49	0.22	8	-0.54	0.17	8
EPSC max	-0.14	0.75	8	0.50	0.21	8
IPSC min	-0.67	0.07	8	-0.15	0.72	8
IPSC max	-0.27	0.52	8	-0.25	0.56	8
sEPSC frequency	-0.30	0.51	7	0.40	0.37	7
sEPSC amplitude	-0.28	0.54	7	0.05	0.92	7
sIPSC frequency	-0.07	0.86	8	0.29	0.49	8
sIPSC amplitude	0.16	0.70	8	-0.08	0.87	8
Total Sholl intersection	-0.01	0.98	7	0.03	0.95	7
Total branches	0.40	0.37	7	-0.22	0.64	7
Total neurite length	-0.27	0.60	6	0.21	0.69	6
Largest soma diameter	-0.95	0.001*	7	0.84	0.17	7

Spearman correlations between key infant-directed behaviors and neuronal properties in virgin males. After correcting for false discovery rate, none of the correlations were statistically significant. * = nominal significance, $p < 0.05$. N = number of cells, AP = action potential, ISI = inter-spike interval, fAHP = fast afterhyperpolarization, mAHP = medium afterhyperpolarization, sAHP = slow afterhyperpolarization, PSC = post-synaptic current, EPSC = excitatory post-synaptic current, IPSC = inhibitory post-synaptic current, sEPSC = spontaneous excitatory post-synaptic current, sIPSC = spontaneous inhibitory post-synaptic current. See methods for full description of metrics.

Conclusion

Parental care is an intrinsic drive seen in every mammalian species and has been evolving for hundreds of millions of years. Many mammalian species exhibit plasticity in infant-directed behavior throughout their lifetime, often increasing parental responses to infants during the transition into parenthood. In females, this is in no small part mediated by the hormonal changes during pregnancy, parturition, and lactation while in males, cues from the mate, environment, or offspring may play a larger role. Despite a substantive amount of research done in recent decades, much of the neural plasticity that underlies changes in infant-directed behavior has yet to be discovered (for review see Horrell et al., 2018).

In a process commonly referred to as "sensitization," prolonged exposure to infants increases measures of parental behavior in multiple rodent species (reviewed in Chapter 1). We conducted the first sensitization experiment in California mice and investigated potential mechanisms by measuring fos expression and plasma corticosterone levels in adult virgin males that were interacting with a pup for the first time, adult virgin males that had been exposed to pups 3 times for 20 min each in the previous week, and new fathers. Previous exposure to pups decreased virgins' latency to approach pups and initiate paternal care, and increased time spent in paternal care. Responses to pups did not differ between virgins with repeated exposure to pups and new fathers. Neither basal corticosterone levels nor corticosterone levels following acute pup or marble exposure differed among groups. Finally, fos expression in the medial preoptic area, ventral and dorsal bed nucleus of the stria terminalis was higher following exposure to a pup than to a marble. Fos expression was not, however, affected by previous

exposure to these stimuli. Thus, mechanisms of sensitization may not be mediated by changes in corticosterone levels in response to pups. This research, in accord with decades of other studies (reviewed above), implicates the MPOA in parental care behavior.

The neurons in the MPOA likely undergo plasticity during the transition into parenthood in ways that increase parental care in mammals. This hypothesis is difficult to test. Some types of evidence that support this hypothesis are 1) measures of neural activity increase in the MPOA in response to pup cues, 2) decreasing activity of MPOA neurons decreases measures of parental care, 3) increasing activity of MPOA neurons increases measures of parental care, 4) neurons in the MPOA have receptors for hormones which change during pregnancy and are implicated in parental care behavior, 5) and plasticity has been observed in various properties of MPOA neurons during the transition into parenthood (see Horrell et al., 2018 for recent review). However, the MPOA is also involved in sexual behavior, thermoregulation, aggression, sleep and other behaviors, making direct causal connections between observed plasticity in neural properties and observed plasticity in infant-directed behavior difficult to establish.

To investigate plasticity in the MPOA which may contribute to plasticity in paternal behavior in the biparental California mouse, we evaluated synaptic, intrinsic, and morphological properties of MPOA neurons in adult males that were either virgins or first-time fathers. This was the first electrophysiology done in California mice and the first whole-cell electrophysiology done in the context of paternal care. We used standard whole-cell recordings in a novel in vitro slice preparation. Synaptic and intrinsic

electrophysiological properties, as well as morphological properties were quantified and compared between virgins and fathers. Though most parameters did not differ significantly between virgins and fathers, we did document a decrease in synaptic inhibition in fathers. These findings suggest that the onset of paternal behavior in California mouse fathers may be associated with limited electrophysiological plasticity within the MPOA.

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