ABSTRACT

Title of Dissertation: NEUROENDOCRINE MECHANISMS UNDERLYING PATERNAL EXPERIENCE-INDUCED PLASTICITY OF THE HIPPOCAMPUS

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Evidence suggests that males, like females, undergo altered structure and function of the hippocampus postpartum, a brain region that regulates certain aspects of emotion, learning, and memory. These behaviors are beneficial for successful parenting. In maternal rodents, offspring contact contributes to postpartum hippocampal plasticity in both mothers and offspring. Fathers do not undergo pregnancy, parturition, or lactation, therefore, the impact of offspring on hippocampal plasticity is less clear. California mouse (*Peromyscus californicus*) fathers are highly paternal, making this monogamous species a good model of paternal care. In this species, between postnatal days 15 and 21 paternal behavior becomes more active (i.e. increased pup retrievals) to care for pups that are beginning to explore outside of the nest. I observed reduced anxiety-like behavior in fathers specifically within this temporal window. Concomitant with attenuated anxiety-

like behavior, I found that fathers maintain survival of adult born neurons in the dentate gyrus of the hippocampus. Enhanced hippocampal plasticity is not restricted to adult neurogenesis, as dendritic spine density in the dentate gyrus is increased in fathers at this same time – an effect that lasts until weaning. When permanently separated from their offspring, fathers show increased passive stress coping and reduced spine density in the DG. Taken together, these data suggest that the degree of active father-offspring interaction significantly alters hippocampal plasticity in the father. Estradiol and its receptors have been implicated in alterations to anxiety and adult neurogenesis in both males and females. I observed that estrogen receptor β (*Erβ*) mRNA expression was elevated in whole hippocampal homogenates at PND 16 in fathers. Similarly, circulating estradiol was elevated at both PND 2 and PND 16. After inhibition of Erβ with the drug tamoxifen, the number of surviving adult born neurons was suppressed in fathers alone. Taken together, these data suggest that in fathers, hippocampal plasticity occurs concomitantly with active father-offspring contact and that this plasticity, at least structural, is driven by activation of Erβ. Understanding paternal experience-induced plasticity and the mechanisms that drive it, may help to prevent deficits in paternal behavior that can disrupt offspring development and contribute to emotional dysregulation in fathers.

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Dedication and Acknowledgements

This dissertation is dedicated to my father Walter F. Hyer III for his exceptional paternal care and active father-offspring engagement. Without his guidance on hard work and dedication, I would not be the person I am today.

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Chapter 1

Introduction

Successfully rearing offspring is a strong driving force for many animal species. To facilitate offspring care, alterations to hormone levels, neural function, and neural structure often occur in parents. As females are the primary care giver in most mammalian species, a majority of research investigating alterations to behavior and neural structure in the postpartum period has focused on motherhood (for recent reviews see Bridges, 2015; Pawluski et al., 2015). However, in 6% of mammalian species, offspring care is not exclusive to the female (Kleiman and Malcolm, 1971). In this select group of mammals, males undergo a variety of species-specific changes that facilitate paternal care, leading to a biparental care structure. Biparental care likely evolved in species where high investment in smaller numbers of offspring was adaptively significant (Gubernick and Alberts, 1987). Individuals within these species, particularly the males, forego future reproductive chances by remaining with their mate and offspring to assure that their current offspring will reach reproductive age (see review, Kentner et al., 2010). Throughout hominin evolution, human males have often played a significant role in parental care (reviewed in Gettler, 2010) – similar to that observed in nonhuman biparental mammals. Despite the existence of biparental care systems in mammalian species, and the relevance to human parental care strategies, the low occurrence of paternal care in mammals has resulted in a dearth of studies focusing on fatherhood. While changes to behavior and neural structure accompanying motherhood have been, and continue to be, investigated, relatively less is known about alterations to neural

structure and function that occur with fatherhood and the mechanisms that underlie these changes.

In human parents, evidence from imaging and behavior studies suggests that structural and functional plasticity in multiple brain regions are associated with the postpartum period. Both fathers and mothers show increased activation of brain regions associated with parental care such as hypothalamus, amygdala, and cingulate, following exposure to infant cues (Swain et al., 2007). Mothers experience increased gray matter in regions associated with maternal motivation and care, including the prefrontal cortex, hypothalamus, substantia nigra, and amygdala (Kim et al., 2010). These changes are associated with a positive perception of the baby and likely improve the mother's ability to respond to infant cues and enhance maternal motivation (Kim et al., 2010). Despite the beneficial increase in gray matter volume in these brain regions, the postpartum period can be accompanied by the development of mental dysfunction. Postpartum depression occurs in 10-15% of new mothers (Horowitz and Goodman, 2005; Goodman, 2007). Other psychiatric disorders, including increased anxiety (Glasheen et al., 2010), have also been characterized in mothers throughout the postpartum period. While the impact of maternal experience on emotional dysregulation has been studied, little attention has been paid to parallel consequences in fathers (Cummings et al., 2005; Paulson et al., 2006). It has been observed that 6-12% of new fathers will develop postpartum depression (Ramchandani et al., 1992; Paulson et al., 2016), however, the mechanism(s) underlying this altered emotional responsivity in fathers is unknown. Given these findings, fathers, like mothers, can be emotionally vulnerable during the postpartum period and the function of brain regions related to parenting may be underlying this vulnerability.

Evidence from fathers of biparental rodent and primate species suggests that fatherhood-induced alterations to structure and function of brain regions associated with parenting occur, however the directionality of some of these changes appear species-specific. The hippocampus is a brain region which mediates a number of ancillary behaviors associated with successful rearing of offspring such as such as anxiety (Kheirbek et al., 2013) and some forms of learning and memory (Morris et al., 1982). Like California mouse mothers, California mouse fathers show decreased adult neurogenesis in the dentate gyrus (DG) of the hippocampus at the end of the postpartum period (Glasper et al., 2011), as do prairie vole fathers (*Microtus ochragaster*; Lieberwirth et al., 2013). While plasticity of existing cell structure within the paternal hippocampus is unknown, marmoset fathers (*Callithrix jacchus)* have increased spine density in the prefrontal cortex during the postpartum period similar to that observed in maternal rodents (Leuner and Gould, 2010a), (Kozorovitskiy et al., 2006). Despite these findings, spine density and overall neuron structure in the hippocampus, as well as the trajectory of adult neurogenesis within this brain region, have not been investigated in biparental fathers of any mammalian species. New California mouse fathers show no changes in anxiety-like behavior immediately postpartum (~postnatal day (PND) 2; Chauke et al., 2012) or at the end of the postpartum period (Glasper et al., 2011), while prairie vole fathers experience an increase in anxiety-like behavior at PND 7 and depressive-like behavior at PND 9 (Lieberwirth et al., 2013). These data highlight that the postpartum period in fathers is associated with changes in functional and structural plasticity of the hippocampus. However, it is unclear to what extent these alterations

occur, how they change across the postpartum period, and the potential mechanism(s) underlying them.

Alterations in behaviors mediated by the DG are observed following parturition in female rats – including reduced anxiety-like behavior (Lonstein, 2005; Agrati et al., 2008), improved learning and memory (Kinsley et al., 1999; Macbeth et al., 2008b), and resilience to stress (reviewed in Slattery and Neumann, 2008; Macbeth and Luine, 2010). Significant alterations to new and existing neuronal structure within the hippocampus are observed in rat dams during the postpartum period. Adult neurogenesis, the birth and survival of new neurons in adulthood, is decreased in the hippocampus of maternal rats (*Rattus norvegicus)* (Darnaudéry et al., 2007; Leuner et al., 2007; Pawluski and Galea, 2007). Dendritic spine density, which reflects available sites for synapse formation (Holtmaat and Svoboda, 2009), is increased in the postpartum female rat hippocampus, specifically areas CA1 and the DG (Kinsley et al., 1999; Leuner and Gould, 2010a). Many of the neuroplastic changes during the postpartum period are directly related to mother-offspring contact (Lonstein, 2005; Boccia et al., 2007; Leuner et al., 2007; Maniam and Morris, 2010). Increased mother-infant interaction in humans increases gray matter in areas of the brain associated with emotional regulation, suggesting that contact with infants could contribute to prevention of emotional dysregulation in mothers (Kim et al., 2010). Taken together, these data suggest that, in mothers, postpartum plasticity of the hippocampus, a region associated with emotional regulation, is often dependent on offspring interaction that may prevent the development of postpartum disorders.

My dissertation addresses the gaps in the literature on postpartum hippocampal plasticity in fathers. Specifically, I investigated 1) the extent to which paternal

experience-induced functional and structural plasticity of the hippocampus is modified postpartum, 2) the extent to which offspring contact drives hippocampal plasticity in fathers, 3) the temporal specificity of alterations to DG plasticity across the postpartum period in fathers, and 4) a possible neuroendocrine mechanism underlying paternal experience-induced plasticity of this brain region. Completion of this work provides insights on paternal experience-induced hippocampal plasticity and how neuroendocrine factors contribute to postpartum hippocampal alterations in fathers of a biparental species. By characterizing hippocampal plasticity during the paternal postpartum period and the mechanism(s) underlying these changes, my dissertation research will add to our knowledge of how the hippocampus is altered in the postpartum period in fathers and possibly shed light on how disruptions to hippocampal function could potentially lead to postpartum emotional dysfunction.

The California mouse (P. californicus) as a model system

The dearth of information on postpartum plasticity in fathers is primarily a result of merely 6% of mammalian species exhibiting paternal care in the wild (Kleiman and Malcolm, 1971). While early work did utilize appropriate models for paternal care such as the California mouse (Horner, 1947; Dudley, 1974), mainstream research focused on species that unnaturally exhibit paternal care and classified it as "maternal care" performed by the male (Brown, 1993) or described lab-artifact paternal care in non-paternal species (Horner, 1947). This has led to many studies comparing paternal and maternal behavior – often assuming the two are parallel and driven by similar mechanisms (Stolz et al., 2005). However, despite the comparison to the hormonal control of maternal behavior, it is becoming apparent that paternal care, and its initiation, is powered by different mechanisms than maternal care. Furthermore, it is evident that these mechanisms vary across males of biparental species (Wynne-Edwards and Timonin, 2007; Saltzman and Ziegler, 2014). These species-specific patterns of hormonal regulation of paternal care contribute to the difficulty in isolating mechanisms underlying paternal behavior.

Paternal care in the California mouse

California mice (*P. californicus*) are a socially and sexually monogamous, biparental species that provides an ideal model of paternal care (Dudley, 1974; Gubernick & Nelson, 1989; Gubernick & Alberts, 1987; Ribble & Salvioni, 2013). California mouse fathers exhibit all aspects associated with parental care including nest building, pup retrieval, grooming, and huddling, with the exception of nursing (Dudley, 1974). Offspring survival is largely dependent on the presence of the father – in varying

experimental conditions the removal of the father decreases offspring survival by nearly 40% (Gubernick et al., 1993; Cantoni and Brown, 1997; Rosenfeld et al., 2013). Given that California mouse fathers display all aspects of paternal care, aside from nursing, and the necessity of the father's presence for offspring survival, it is evident that this species provides an appropriate model for the study of fatherhood. Since the 1970s, paternal care, the hormonal and neural mechanisms underlying this care, and the subsequent effects of fatherhood on other aspects of behavior have been a topic of research interest in this species (Dudley, 1974; Gubernick et al., 1994; Wynne-Edwards and Timonin, 2007; Glasper et al., 2011; Lambert et al., 2011; Saltzman and Ziegler, 2014). This literature provides a foundation on which further studies can be designed to investigate paternal experience-induced plasticity.

Hippocampal plasticity in California mice

Changes in hippocampal functional plasticity have been investigated in the California mouse at multiple time-points across the postpartum period resulting in a variety of results. California mouse fathers show improved spatial memory at about PND 15 (Franssen et al., 2011). However, both fathers and mothers show no changes in recognition memory three weeks later at weaning (Glasper et al., 2011). Studies on anxiety-like behavior have shown no alterations on the elevated plus maze (EPM) immediately postpartum (Chauke et al., 2012), but have shown reduced anxiety-like behavior in the open field task in both multiparous (multiple litters) fathers and pup-exposed virgins after 3 days of pup contact (Bardi et al., 2011). However, any anxiolytic effect is lost by the late postpartum period as primiparous (first time) fathers do not exhibit reduced anxiety-like behavior in the novelty suppressed feeding task at PND 35

(Glasper et al., 2011). Taken together, these data, although somewhat limited, indicate that the postpartum period is accompanied by changes to hippocampally-mediated behaviors in California mouse fathers.

Neural circuitry of paternal care in California mice

In addition to these behavioral studies, evidence has shown that California mouse fathers experience postpartum neuroplasticity in the hippocampus and a number of other brain regions associated with parenting. In the hippocampus Glasper and colleagues (2011) found that 3-week survival of adult born neurons in the DG, measured 4 weeks postpartum, was suppressed in California mouse fathers and mothers. Lambert and colleagues (2011) observed increased nestin-immunoreactivity (ir), a marker for multipotent neural stem cells, in areas CA2 and CA3 of the hippocampus in California mouse fathers, suggesting neural growth in these regions. To characterize regional activity in the brains of California mouse fathers, multiple studies have used expression of the immediate early gene c-fos. Compared to controls, paternal males had lower c-fos-ir in the medial amygdala (MeA) and the paraventricular nucleus (PVN), areas involved in anxiety and stress reactivity. C-fos expression within the medial preoptic area (MPOA) is enhanced in fathers after contact with a novel pup through a mesh barrier. Interestingly, males that have mated with a female will show longer contact time with the pup contained in the mesh barrier compared to virgins – suggesting that mating can drive some aspects of paternal motivation but fatherhood itself may be necessary to alter neurochemistry (de Jong et al., 2009). C-fos expression is also altered in fathers following separation, and subsequent reunion, with their offspring. Separation for 24 hours on PND 11 enhances c-fos-ir in the MPOA (Lambert et al., 2011). After only 3

hours of separation on ~PND 2, fathers have more c-fos-ir in the lateral habenula and dorsal raphe nucleus (de Jong et al., 2010). As these regions are associated with parental care (MPOA) and emotional processing (lateral habenula and dorsal raphe) it is possible that this circuit contributes to paternal motivation following separation (de Jong et al., 2010). However, following exposure to a distressed pup, California mouse fathers show less c-fos expression in brain regions associated with emotionality, such as the insula and anterior cingulate, compared to virgins, suggesting a reduced stress response (Lambert et al., 2013). While these aspects of fatherhood-induced functional and structural plasticity have been characterized using this species, the extent and mechanistic control of these changes is unknown.

Maternal care circuitry and the hippocampus

The work investigating brain regions underlying paternal care in the California mouse has many parallels with research on the neural circuitry underlying maternal behavior. In 1974, Michael Numan identified the MPOA as a primary target within the hypothalamus for maternal behavior. When this region was lesioned, rat dams showed nearly a total lack of maternal care behaviors (Numan, 1974). Prolactin infusions into the MPOA initiated maternal care in steroid-primed female rats (Bridges et al., 1990) indicating that this region is both necessary and sufficient for initiating maternal care behaviors. Maternal care, specifically pup retrievals, are similarly disrupted with lesions to the bed nucleus of the stria terminalis (BNST; Numan and Numan, 1996). Connections between the MPOA and BNST are a primary part of the maternal care circuitry, as when axons between these regions are severed, maternal care is impaired (Numan et al., 1990). Projections from these regions target the ventral tegmental area (VTA; Numan and

Smith, 1984). When axons posterior to the VTA were bilaterally sectioned, pup retrieval, nursing, and nest building were disrupted. VTA projections synapse onto brainstem nuclei to drive the motor aspects of maternal behaviors (Numan and Numan, 1991). These findings indicate that the maternal circuit is shaped by the connections between the MPOA, BNST, VTA, and brainstem. (VTA; Numan and Smith, 1984). When axons posterior to the VTA were bilaterally sectioned, pup retrieval, nursing, and nest building were disrupted. VTA projections synapse onto brainstem nuclei to drive the motor aspects of maternal behaviors . These findings indicate that the maternal circuit is shaped by the connections between the MPOA, BNST, VTA, and brainstem. (VTA; Numan and Smith, 1984). When axons posterior to the VTA were bilaterally sectioned, pup retrieval, nursing, and nest building were disrupted. VTA projections synapse onto brainstem nuclei to drive the motor aspects of maternal behaviors . These findings indicate that the maternal circuit is shaped by the connections between the MPOA, BNST, VTA, and brainstem. (VTA; Numan and Smith, 1984). When axons posterior to the VTA were bilaterally sectioned, pup retrieval, nursing, and nest building were disrupted. VTA projections synapse onto brainstem nuclei to drive the motor aspects of maternal behaviors . These findings indicate that the maternal circuit is shaped by the connections between the MPOA, BNST, VTA, and brainstem. (VTA; Numan and Smith, 1984). When axons posterior to the VTA were bilaterally sectioned, pup retrieval, nursing, and nest building were disrupted. VTA projections synapse onto brainstem nuclei to drive the motor aspects of maternal behaviors . These findings indicate that the maternal circuit is shaped by the connections between the MPOA, BNST, VTA, and brainstem. (VTA; Numan and Smith, 1984). When axons posterior to the VTA were bilaterally sectioned,

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While the MPOA, BNST, and VTA, and the connections between them, form the circuitry underlying direct maternal care behaviors (for reviews see Numan and Sheehan, 1997; Kinsley and Lambert, 2008) the hippocampus plays a role in ancillary maternal care behaviors. The structure of this brain region is modified in the maternal postpartum period. Dendritic spine density is enhanced in area CA1 (Kinsley et al., 2006; Pawluski and Galea, 2006) and the DG (Leuner and Gould, 2010a) in maternal rats. Dendritic length and branching of the CA1 region are suppressed in rat dams (Pawluski and Galea, 2006). Similarly, maternal rats experience a decrease in adult neurogenesis in the early- (Leuner et al., 2007) and mid- (Pawluski and Galea, 2007) postpartum periods. These alterations in hippocampal structure are accompanied by changes in behaviors mediated

by this brain region. Anxiety-like behavior is reduced in pregnant (Macbeth et al., 2008a) and lactating (Agrati et al., 2008) female rats. Pregnant female rats have improved spatial memory compared to virgin females (Macbeth et al., 2008a) and after multiple litters recognition memory is enhanced (Macbeth et al., 2008b). Reductions in anxiety and improvements in memory performance, while not direct maternal care behaviors, are nonetheless beneficial for offspring survival as they can improve aspects such as pup retrieval or resource acquisition. Taken together, these findings indicate that the hippocampus is altered in the maternal postpartum period and these alterations are accompanied by changes in behaviors that may benefit maternal rats in the postpartum period (for review see Kinsley and Lambert, 2008).

The above studies have laid the groundwork for studying postpartum plasticity in the California mouse. Postpartum changes are exhibited in the hippocampus in California mouse fathers suggesting that this brain region is responsive to fatherhood. As the hippocampus mediates some aspects of learning, memory, and emotional regulation, as well as HPA axis responsivity, appropriate function of this brain region is of particular importance for facilitating offspring care and the well-being of the father. As hippocampal plasticity associated with motherhood is heavily driven by hormonal mechanisms (Lonstein, 2005; Leuner et al., 2007; Macbeth et al., 2008b), it is possible that the above findings are also being influenced by neuroendocrine factors. Despite the scarcity of information on hormonal regulation of fatherhood-induced hippocampal plasticity, the hormonal mechanisms underlying paternal care have been studied extensively. In addition to a number of mammalian species, the California mouse has been used as a model for investigating the hormonal control of paternal care. Below, I

review this literature. Specifically, I discuss hormonal mechanisms underlying paternal behavior, and how these neuroendocrine systems can alter behaviors mediated by the hippocampus, which are indirectly related to paternal care but that influence reproductive success.

Hormonal regulation of paternal care.

Early studies on mammalian paternal care described virgin males that cared for pups after repeated exposure as exhibiting maternal behaviors. These data were derived from a species that does not normally exhibit paternal care in the wild (*Rattus norvegicus*; Rosenblatt, 1967). To better characterize paternal care, subsequent work has focused on the underlying mechanisms of this behavior in a variety of naturally biparental mammals. Paternal mammals that have been investigated include, but are not limited to, cotton topped tamarins (*Saguinus oedipus*; Ziegler et al., 2004; Zahed et al., 2010), common marmosets (Dixson and George, 1982; Ziegler and Sosa, 2016), Djungarian hamsters (*Phodopus campbelli*; Brooks et al., 2005; Ma et al., 2005), prairie voles (Insel et al., 1995; Lieberwirth et al., 2013), the California mouse (Gubernick and Alberts, 1987; Glasper et al., 2011), and humans (Fleming et al., 2002; Delahunty et al., 2007; Kim et al., 2014). Evidence from these species shows that despite lacking pregnancy, parturition, and lactation, the hormonal changes experienced by the father parallel changes in the female. However, the hormonal milieu that induces and maintains paternal care in mammals is not consistent across biparental species. These discrepancies are likely a result of convergent evolution of mammalian paternal care that developed in specific ecological niches (Wynne-Edwards and Timonin, 2007; Saltzman and Ziegler, 2014). Below, I outline some of the primary hormones associated with paternal care and the differing roles they play across paternal mammalian species.

Gonadal Steroids

The gonadal steroid estrogen can act to initiate and/or maintain paternal care. Concentrations of circulating estradiol in male *P. campbelli* hamsters are as high as those

measured in fertile, adult females (Schum and Wynne-Edwards, 2005). In males of two non-human primate species, cotton-topped tamarins (Ziegler et al., 2004) and marmosets (Nunes et al., 2000), peripheral estradiol levels are higher as parturition approaches but then returns to baseline after the birth of offspring. This suggests that estradiol may help initiate the onset of paternal care in these species. In California mice, estradiol promotes paternal care. Initial work found that castration reduced paternal care in California mouse males but replacement with testosterone reinstated care behaviors (Trainor and Marler, 2001). As testosterone is converted to estradiol via aromatase, it was likely that testosterone's effect on paternal care was due to estradiol. When gonadectomized California mouse fathers received either testosterone or estradiol replacement, huddling and pup grooming behaviors were increased – an effect that was blocked by the aromatase inhibitor fadrozole (Trainor and Marler, 2002). The effect of estradiol on paternal care in the California mouse appears to occur through aromatization of testosterone specifically in the MPOA, as this brain region exhibits increased aromatase activity 2-3 weeks postpartum (Trainor et al., 2003). Like California mouse fathers, castration impairs paternal behavior in prairie voles, indicating that testosterone is important for facilitating paternal care in multiple biparental species (Wang and De Vries, 1993). However, it is unknown if the conversion to estradiol is always necessary for testosterone's effect on paternal behavior.

Other steroid hormones, including testosterone alone and progesterone, have mixed effects on paternal care across biparental species. Peripheral testosterone often increases as parturition approaches yet is generally decreased in the postpartum period. This pattern is evident in marmosets (Ziegler et al., 2009), cotton-topped tamarins

(Ziegler et al., 2004), and Mongolian gerbils (Brown et al., 1995). In Djungarian hamsters, testosterone actually increases as parturition approaches and lower levels of testosterone are associated with reduced pup retrieval – an example of impaired paternal care, suggesting that testosterone facilitates paternal care in this species (Reburn and Wynne-Edwards, 1999). Finally, the sex steroid progesterone is rarely measured in males as it is more closely associated with pregnancy (reviewed in Nelson, 2011; Bales and Saltzman, 2016). What little work has been done on progesterone in males shows that the effects on paternal care are mixed. California mouse fathers have lower circulating progesterone (Trainor et al., 2003) as well as lower receptor mRNA expression in the bed nucleus of the stria terminalis (BNST; Perea-Rodriguez et al., 2015) than non-fathers. On the other hand, Djungarian hamsters have elevated progesterone levels before, during, and after birth of offspring (Schum and Wynne-Edwards, 2005). Overall, the data on gonadal steroid hormones suggest that estradiol is largely a facilitator of paternal care. Testosterone and progesterone appear to have mixed effects on paternal care, the directionality of which is species-dependent.

Neuropeptides

The neuropeptides oxytocin (OT) and arginine vasopressin (AVP) have been traditionally associated with bonding and maternal care (reviewed in Numan and Young, 2015), yet their roles in paternal care, like that of steroid hormones, are inconclusive. Multiparous prairie vole fathers have more OT-ir neurons in the PVN compared to virgin males (Kenkel et al., 2014). However, early postpartum neither *OT* gene expression or OT receptor binding is altered in prairie vole fathers compared to virgins (Wang et al., 2000). OT increases in California mouse males post copulation but returns to baseline

levels in the postpartum period, suggesting OT's role in this species is possibly for social bonding alone (Gubernick et al., 1995). More recent evidence supports this conclusion as California mouse fathers have reduced OT mRNA expression in the BNST, but not the MPOA or MeA, compared to virgin males on ~PND 3 (Perea-Rodriguez et al., 2015). AVP correlates positively with paternal care in California mouse fathers (Bester-Meredith and Marler, 2003) and *AVP* gene expression is elevated in prairie vole fathers early postpartum (Wang et al., 2000). Additionally, vasopressin receptor (*V1a*) expression is increased in the prefrontal cortex of marmoset fathers (Kozorovitskiy et al., 2006). Taken together, these data indicate that OT and AVP are often associated with increased paternal care. However, their role in promoting social bonding may be more prominent (for review see Beery, 2015).

Prolactin (PRL) has been associated with paternal care in a number of biparental species. Djungarian hamsters show increased circulating PRL shortly postpartum compared to the non-paternal *Phodopus sungorus* (Reburn and Wynne-Edwards, 1999). Marmoset fathers perform 70% of the offspring carrying, an important aspect of parental care in this species (Kozorovitskiy et al., 2006), which is accompanied by elevated circulating PRL levels (Dixson and George, 1982). When treated with bromocriptine, a dopamine antagonist that inhibits PRL release, marmoset fathers' carrying rate is decreased (Roberts et al., 2001). In California mouse fathers, plasma PRL levels are elevated 2 days postpartum compared to expectant fathers and virgins, suggesting that pup exposure is necessary for the observed increase (Gubernick and Nelson, 1989). Despite these associations between prolactin and paternal care, whether or not prolactin has a direct influence on paternal behavior remains unclear (Wynne-Edwards and

Timonin, 2007). As multiple studies have shown either weak or no link between prolactin and paternal care (Roberts et al., 2001; Brooks et al., 2005; Ziegler et al., 2009), it is likely that prolactin is important for initiating paternal care through promoting father-offspring contact but its role in maintaining paternal care is less important (Bales and Saltzman, 2016; Storey and Ziegler, 2016).

Adrenal Steroids

The data on adrenal steroid hormones and their impact on paternal care are somewhat limited. Interestingly, circulating glucocorticoids appear to be no different between non-fathers and fathers shortly postpartum in California mice (Chauke et al., 2012; Harris and Saltzman, 2013). In fact, fathers appear relatively glucocorticoid-resistant as they do not respond to acute stressors with increased corticosterone in either the early or mid-postpartum periods (Harris et al., 2012). However, when subjected to a chronic unpredictable stress paradigm for 7 days, California mouse fathers show increased corticosterone levels and disrupted paternal care (Harris et al., 2013). In contrast, Djungarian hamster fathers have an increase in cortisol levels just prior to the birth of pups that declines early postpartum (Reburn and Wynne-Edwards, 1999). Finally in male prairie voles, no change is evident in circulating corticosterone from the time they are housed with a same-sex pair-mate, pair-bonded with a female, a first-time father, or after three litters (Campbell et al., 2009). Generally, these findings indicate that corticosterone is not associated with paternal care in the biparental species that have been investigated.

Many parallel alterations in hormones levels are observed between human fathers and non-human mammals. Human fathers experience a decrease in testosterone early

postpartum which returns to baseline in the late postpartum period (Storey et al., 2000) Higher estradiol levels are evident pre-birth and last till one month postpartum in human males (Berg and Wynne-Edwards, 2001). As parturition approaches, PRL is increased in human males. Couvade symptoms, sympathetic pregnancy symptoms observed in men with pregnant partners, are associated with higher PRL levels and increased responsiveness to infant crying (Storey et al., 2000). The human equivalent to corticosterone, cortisol, is elevated as parturition approaches, mirroring the elevation observed in human mothers (Storey et al., 2000). Despite this, the rise is only temporary, as glucocorticoids return to baseline during the postpartum period (Fleming et al., 2002).

The above research on the endocrine mechanisms underlying paternal care paints a mixed picture. These discrepancies make it challenging to isolate possible neuroendocrine factors that may be contributing to fatherhood-induced neuroplasticity. Animal models can be used to determine neuroendocrine mechanisms driving fatherhood-induced behavioral and structural plasticity, which may inform us about mechanisms underlying alterations in human fathers. However, it is important to consider a model that closely mirrors the changes that we know occur in human males when they become fathers. The above review indicates that California mouse fathers have many parallel endocrine changes to human fathers. Both California mouse fathers (Trainor and Marler, 2002) and human fathers (Storey et al., 2000) experience a decline in testosterone shortly after parturition. In the California mouse, testosterone is converted to estradiol in the brain to facilitate paternal care (Trainor and Marler, 2002). Estradiol is similarly elevated in human fathers in the early postpartum period (Berg and Wynne-Edwards, 2001). Males of both species have elevated prolactin levels that may be a result of

offspring exposure (California mouse: Gubernick and Nelson, 1989; humans: Storey et al., 2000). Importantly, California mouse males are both socially and sexually monogamous (Gubernick and Nordby, 1992). A single male and female pair inhabit a specific territory without residing in social groups – providing a strong social structure to facilitate extensive paternal care (Gubernick and Alberts, 1987; Gubernick and Nordby, 1992). These data suggest that the California mouse is an appropriate model for understanding the endocrine mechanisms underlying paternal care and specifically, how paternal care may shape hippocampal plasticity. The California mouse has been used as a model to investigate fatherhood-induced functional and structural plasticity of the hippocampus, providing a framework from which new studies can be structured to better understand paternal-experience induced plasticity in this brain region.

The role of the hippocampus in learning and memory, emotional regulation, and stress

The hippocampus is a brain region that is crucial for a number of behavioral functions, including certain forms of learning, memory, emotional regulation, and stress reactivity. Many aspects of these behaviors are altered in the postpartum period. These alterations often facilitate parental care and reproductive success. While the hypothalamus is traditionally the main target of interest for investigating the neural mechanisms underlying parental care (Rosenblatt et al., 1996; Keyser-Marcus et al., 2001; Stolzenberg and Champagne, 2016), the hippocampus has also been a region of interest as it mediates ancillary behaviors associated with successful parenting (Love et al., 2005; Leuner et al., 2010a; Pawluski et al., 2015). Below I review evidence that highlights the role of the hippocampus in a number of behaviors, all of which are important for effective parental care.

Learning and Memory

Some of the earliest descriptions of hippocampal function are a result of observing human behavior following damage to the medial temporal lobe (MTL). Milner (1972) described deficits to both learning and memory following MTL lesions. The MTL is composed of the hippocampus, and the entorhinal, perirhinal, and parahippocampal cortices (reviewed in Squire, 2009). By investigating patients with retrograde amnesia, it became evident that the degree of MTL damage was correlated with how far back in time memory was impaired (reviewed in Nadel and Moscovitch, 1997). Smith and Squire (2009) used functional magnetic resonance imaging to identify brain regions that aligned their activity with temporally graded memories in healthy adults. They observed that as a memory ages, activity indicating recall of that memory shifts from the hippocampus to

the frontal, parietal, and lateral temporal lobes. These findings corroborated the Standard Model of Memory that posits that the hippocampus is important for short term encoding of memory while frontal regions are important for long term storage of memories (reviewed in Nadel and Moscovitch, 1997; Squire, 2009).

Animal models have been a useful tool to further characterize the role of the hippocampus in learning and memory. In 1982, Morris and colleagues trained female rats in a water maze task that assessed spatial learning and memory. When given hippocampal lesions, the rats were unable to complete the task, suggesting a learning deficit. Later work found that lesions given following the acquisition period of the task impaired performance, suggesting a deficit in memory (Frye, 1995). Specific lesions in non-human primates were used to further elucidate the role of the hippocampus in learning and memory. Rhesus monkeys (*Macaca mulatta*) were trained on the delayed non-match-to-sample task (DNMS) before they received hippocampal-specific lesions. The DNMS is a unique task which characterizes hippocampal function as it has both learning (acquisition) and memory (delayed recollection) components. Monkeys that received the hippocampal lesions exhibited deficits in both aspects of the task – they showed impaired acquisition as measured by more errors initially during training and reduced success in the delay conditions which assesses memory ability (Beason-Held et al., 1999). Taken together, lesion studies in humans, rodents, and non-human primates strongly link the hippocampus with aspects of learning and memory performance.

As learning and memory behaviors are closely intertwined, specific tasks have been utilized to directly assess either hippocampal learning or memory performance. Similar to the water maze task, the place learning-set task requires animals to learn the

location of a platform in a water-filled arena. Rats with hippocampal lesions consistently perform poorly on this task while lesions of the parietal cortex do not impair learning performance (Whishaw, 1987). Assessment of hippocampal-dependent memory performance has centered around a large body of work focusing on the distinction between recollection and familiarity in recognition memory (reviewed in Eichenbaum et al., 2007). Following hippocampal lesions, recollection memory, but not familiarity, is lost in an odor recognition task (Fortin et al., 2004). These findings, along with studies in humans (Rugg and Yonelinas, 2003), indicate that the hippocampus is involved in more declarative recognition as opposed to familiar aspects of memory traces (Squire, 2009). Object recognition tasks are used to assess performance on both recognition and spatial memory. Bilateral hippocampal lesions in male rats resulted in deficits in the object location and object-in-place tasks (both spatial memory tasks) as well as the temporal order memory task (Barker and Warburton, 2011). These findings indicate that the role of the hippocampus in both learning and memory is tightly intertwined and appropriate behavioral task development is essential to characterizing the role of the hippocampus in either learning or memory.

More recent technological advances have allowed researchers to pinpoint the finer cellular and molecular mechanisms through which learning and memory are mediated. Using optogenetic control of the dorsal DG, Kheirbek et al. (2013) observed that inhibition of this region, but not the ventral DG, resulted in deficits in a contextual fear learning paradigm. This finding indicates that specific sub-regions of the hippocampus may have different behavioral functions. Place et al. (2016) observed local field potentials within the hippocampus and prefrontal cortex of rats performing a context-

dependent memory task. They found that when animals performed the task accurately theta oscillations between the field potentials in the prefrontal cortex and the hippocampus were perfectly timed, indicating direct connectivity between these two brain regions. Finally, specific cell types within the hippocampus have been identified for encoding memories within specific times and/or places to allow representation of both time and space within the hippocampus (reviewed in Eichenbaum, 2014). Overall, these findings show that memory and learning performance are heavily dependent on the hippocampus and that complex circuitry and cellular systems underlie the specific mechanisms of these behaviors.

Emotional Regulation

In addition to altering some forms of learning and memory, the hippocampus appears to be involved in emotional regulation. Imaging studies in humans have implicated changes in hippocampal volume in patients experiencing emotionally disrupted states. A meta-analysis of studies investigating depressed patients and volume of neural regions found that hippocampal volume was significantly correlated with reported depression. Patients with more depressive symptoms had smaller hippocampi (Campbell et al., 2004). Similarly, patients with chronic post-traumatic stress disorder displayed reduced volumes of left and right hippocampi (Kitayama et al., 2005). More recently, Johnston et al. (2015) observed that patients with treatment-resistant depression failed to deactivate the hippocampus compared to controls during loss events in a win-lose choice task. Overall, these imaging studies in humans suggest that the function and structure of the hippocampus is associated with emotional responsivity.

Like learning and memory, studies using animal models have used lesioning techniques to characterize the role of the hippocampus, and its sub-regions, in emotional regulation. Kim and Fanselow (1992) found that emotional responses to fearful stimuli could be elicited through classical conditioning methods. When rats received hippocampal lesions one day after conditioning, the contextual fear response was eliminated. However, the more time that was allotted between the lesions and the conditioning the more contextual fear remained. These results highlight the interplay between emotional fear and memory and indicate that the hippocampus plays a time-mediated role in this behavioral response. Significant evidence exists suggesting that the ventral hippocampus is primarily involved in emotional regulation. When the most ventral quarter of the hippocampus was lesioned in rats they displayed more time in the open arms of the EPM compared to non-lesioned controls (Kjelstrup et al., 2002). Similarly, lesions of the total ventral hippocampus, including the ventral DG, resulted in reduced freezing behavior in response to foot shock. Despite the extent of these ventral hippocampal lesions (~50% of the total hippocampus), there did not appear to be a deficit in spatial navigation (Bannerman et al., 2003). Kheirbek et al. (2013) used optogenetic control of excitatory or inhibitory activation to assess the DG's regulation of memory and anxiety along the dorsal-ventral axis. Dorsal excitement enhanced overall movement while ventral excitement enhanced time spent on the open arms of the EPM. This suggests that dorsal activity enhances exploration while ventral activity modulates anxiety (Kheirbek et al., 2013).

The extensive connections that the hippocampus has with the amygdala, a region associated with emotion, suggests that the circuits between these two regions may be one

mechanism through which the hippocampus mediates emotional responsivity. Using anterograde and retrograde tracing, Kishi et al. (2006) outlined a topographical map of the connections between the hippocampus and the amygdala. Importantly, the dorsal-ventral axis of the hippocampus maps onto the dorsal-ventral portions of the amygdala suggesting an anatomically- and functionally- specific role for these regions. Finally, like the circuit between the prefrontal cortex and the hippocampus (Place et al., 2016), theta-frequency oscillations fire synchronously in cells in the hippocampus and basolateral amygdala during fear conditioning. The connections between these brain regions is likely very important for emotional enhancement of memory (reviewed in Tully and Bolshakov, 2010). Overall, these findings strongly implicate the hippocampus as a mediator of emotion. Specifically, it appears that the ventral hippocampus is more heavily involved in emotional regulation and that a circuit between the amygdala and the hippocampus allow for hippocampal modulation of emotions and emotional modulation of memories.

Stress Reactivity

Evidence suggests that the hippocampus can impact function of the HPA axis. The HPA axis is a circuit connecting the hypothalamus, pituitary, and the adrenal gland. Its major function is to regulate the release of adrenal hormones into the peripheral nervous system. These hormones include the glucocorticoid family that drives stress responsivity. In a healthy mammal, the HPA axis mediates itself through a negative feedback loop (reviewed in Ulrich-Lai and Herman, 2009). Lesions to the hippocampus disrupt the ability of the HPA axis to turn off, suggesting a role for the hippocampus in HPA modulation (Herman et al., 1995). Lesions to the ventral, but not dorsal, hippocampus exacerbated stress-induced gastric ulcers in rats, indicating a mediator role

of the ventral hippocampus in the physiological stress response (Henke, 1990). Interestingly, hippocampal lesions do not appear to influence HPA activity in response to chronic stress (reviewed in Herman and Mueller, 2006). The effects of these lesions appear to have the most significant effect during recovery from stress-induced glucocorticoid activity, suggesting that the hippocampus' role is in terminating the stress response through modulation of hypothalamic signaling (Herman et al., 1995; reviewed in Ulrich-Lai and Herman, 2009). On the other hand, stimulation of the hippocampus, or the amygdala, in anesthetized rats leads to an increase in plasma corticosterone. When different sub-regions of the hippocampus were investigated it was determined that stimulation of area CA1 induced an increase, while CA3 and the DG induced a decrease in plasma corticosterone (Dunn and Orr, 1984). These findings indicate that the role of the hippocampus in mediating HPA responsivity is fairly complex and, like learning, memory, and emotion, may be sub-region-specific.

This interconnectivity between the HPA axis and the hippocampus allows for stress to modulate hippocampal function. In male rats, 21 days of restraint stress impairs performance on radial arm maze acquisition – a hippocampus-dependent spatial memory task (Luine et al., 1994). Naturally occurring high stress reactivity in mice is accompanied by deficits in both object and spatial memory tasks (Knapman et al., 2010). Similarly, higher levels of corticosterone in humans predict hippocampal atrophy and poor memory performance (Lupien et al., 1998). Diamond et al. (1992) investigated how corticosterone levels differentially affected hippocampal signaling. Importantly, it was observed that the relationship between corticosterone levels and hippocampal firing resembles an inverted U indicating a complex, biphasic effect of stress hormones on

hippocampal function. It is possible that the lower doses of corticosterone may contribute to enhanced survival of the animal through increased vigilance, while higher doses lead to deficits in neural function that contribute to lower survival rates (reviewed in Lupien et al., 2009). These effects are notable when comparing negative and positive stressors. While negative stressors lead to deficits in hippocampal function, positive stressors appear to enhance performance in hippocampal-dependent tasks. Running, considered a positive stressor, enhances memory performance. Specifically, mice housed with a running wheel performed better in the water maze task compared to controls (van Praag et al., 1999a). Similarly, multiple studies have observed that running acts as a rewarding stimulus, by reducing anxiety- and depressive- like behaviors that are, both mediated by the hippocampus (reviewed in Brené et al., 2007). Finally, sexual experience, another positive stressor, reduces anxiety in the novelty suppressed feeding paradigm despite an increase in glucocorticoids (Leuner et al., 2010b). Overall, the evidence clearly indicates that hippocampal function can be modulated by stressors via a hormonal mechanism and that valence of the stressor, positive or negative, and amount of glucocorticoid secretion impact hippocampal function.

Hippocampal functional and structural plasticity

Extensive plasticity is required within the hippocampus to meet the demands of continual input from learning, memory, emotion, and the stress response. While this brain region experiences plasticity of existing cell structure via alterations to dendritic length, branching, and spine density, the hippocampus is unique in that it is one of two brain regions that experiences extensive adult neurogenesis – the birth of new neurons in adulthood (reviewed in Opendak and Gould, 2015). The two regions of the brain that have the capabilities for extensive adult neurogenesis, are the subgranular zone (SGZ) of the DG in the hippocampus and the sub ventricular zone (SVZ) which projects its adult born cells to the olfactory bulb (reviewed in Nowakowski and Hayes, 2008). Adult neurogenesis is composed of a proliferation phase, early survival phase, maturation phase, and late survival phase. During proliferation and the early survival phase, ambient GABA, the primary inhibitory neurotransmitter in the brain, has an excitatory effect on the cells due to the presence of NKCC1 in the immature cells (a NA, K, Cl co-transporter) (Bischofberger and Schinder, 2008; Kempermann et al., 2008; Deng et al., 2010). In the early survival phase (approximately two weeks after birth), adult born cells migrate to the molecular layer of the DG where they begin to put forth dendritic projections. The initiation of this dendritic growth is variable and sensitive to external input (i.e. social condition, stress, and age) but once it begins the process moves rapidly (Zhao et al., 2006). As more glutamatergic projections are made with molecular layer input from the entorhinal cortex and local interneurons, the previously excitatory GABA input switches to its traditional inhibitory role. If a cell survives the first two weeks, it will likely remain integrated into the mature molecular layer (Kempermann et al., 2008).

The postmitotic maturation phase and the late maturation phase are the final two phases of adult neurogenesis. The postmitotic phase is characterized by complete axon elongation of the new DG granule cells to CA3 mossy fibers. About one week after axon elongation, the first dendritic spines appear along the dendritic shafts. The late maturation phase is most notable for having a lowered threshold for LTP (Kempermann, Song, and Gage, 2008). Overall, the entire birth, maturation, and complete functional integration of adult-born granule cells takes about seven weeks. Progenitors divide for 3-5 days followed by three weeks characterized by immature proteins and dendritic elongation. By two weeks, the axon has projected to CA3 and the number of voltage-gated channels has increased to lower the LTP threshold. Finally at seven weeks you have a fully integrated neuron (reviewed in Bischofberger and Schinder, 2008).

The discovery of adult neurogenesis in the hippocampus

Prior to the 1960's, it was commonly believed that new neuron birth did not occur outside of development. It appeared that the structure of the complex brain was stable after birth due to the lack of mitotic elements in cells in the adult brain. Joseph Altman (Altman, 1962) was the first to characterize the birth of news cells in adulthood in the lateral geniculate body, a region in the thalamus, using thymidine autoradiography in response to damage in adult Long Evans rats. These findings were corroborated by Kaplan and colleagues (1984) in a number of brain regions, including the hippocampus, in rats. However, these findings were largely disregarded as artifact until the late 1980's when Nottebohm and colleagues beautifully characterized adult neurogenesis (Alvarez-Buylla and Nottebohm, 1988) and the functional integration of these cells (Paton and Nottebohm, 1984) in the song bird. However, as these findings were in song birds, the

evidence was again largely dismissed as irrelevant for mammals. With the advent of better imaging technology and more complex experimental design, the study of adult neurogenesis in mammals was reinvigorated. In the rat hippocampus, H3-thymidine labeling of adult born cells combined with neuron- and glia- specific markers characterized a trajectory of cell birth and differentiation by comparing dual labeling at multiple time points (Cameron et al., 1993b). Using the thymidine analogue, bromodeoxyuridine (BrdU), hippocampal adult neurogenesis was characterized in an Old World primate (Gould et al., 1999b). By giving BrdU injections to terminally ill patients and then examining their brains postmortem, Eriksson et al. (1998) was able to characterized adult neurogenesis in the human brain. These findings launched a field of investigation into hippocampal adult neurogenesis and its functional role in behavior. Evidence has accrued for adult neurogenesis in other brain regions but the findings and the function of these cells remains controversial (reviewed in Gould, 2007).

Learning, memory, and adult neurogenesis

Learning and memory can influence, and be influenced by, adult hippocampal neurogenesis. LTP plays a known role in facilitating learning and memory (Whitlock et al., 2006; Redondo and Morris, 2011). As the LTP threshold in adult born neurons is low (reviewed in Kempermann et al., 2008), these cells are uniquely suited to respond to aspects of learning and memory. Gould et al. (1999) showed that training on a number of hippocampal-dependent learning tasks enhances adult neurogenesis in male rats. Specifically, BrdU given one week before training on the conditioned eye-blink paradigm using a trace protocol, as opposed to the delay protocol which is not hippocampus-dependent, resulted in nearly double the number of BrdU-labeled cells in the rat

hippocampus. Similar results were observed in the water maze task. Ambrogini et al., (2000) found that spatial learning in the Morris water maze correlated positively with the number of BrdU-labeled cells in the hippocampus. Other evidence has indicated a more complex relationship between adult neurogenesis and learning. When the water maze task is divided into the early and late learning phases, cell survival is dependent on cell birth date. Cells born during the late phase are more likely to survive and mature into functionally integrated neurons whereas cells born in the early phase are more likely to die off. The amount of cell death correlated negatively with water maze performance, suggesting that cells born in the later phases of task learning are more necessary for memory of the task and successful performance (Döbrössy et al., 2003). Interestingly, the relationship between learning and adult neurogenesis depends on the difficulty of the task and how well it was learned (Leuner et al., 2004, 2006). Using trace eye-blink conditioning, Leuner et al. (2004) investigated the impact of learning prior to memory formation on adult neurogenesis. Before memory formation, adult neurogenesis was not impacted during learning the task. However, the number of BrdU-labeled cells did positively predict task performance. Finally, after extensive training on the task, adult neurogenesis was enhanced, suggesting that once a task is learned, neuronal survival is increased.

Like learning, memory influences adult neurogenesis in the DG. Free-ranging black capped chickadees have enhanced seasonal recruitment of adult neurogenesis believed to be important for memory of food caching (Barnea and Nottebohm, 1994). In harsh conditions, where survival depends more heavily on food caches, chickadees have higher levels of adult neurogenesis (Chancellor et al., 2011). On the other hand, red

squirrels, another wild caching species, show no relationship between adult neurogenesis and caching strategy. Instead, memory in this species may be more dependent on the neurogenic reserve theory where cell production early in life is related to later hippocampal function (Johnson et al., 2010). The role of adult neurogenesis in memory has been suggested to be specifically involved in pattern separation – the separate coding of stimuli based on space and time (reviewed in Deng et al., 2010). By using immediate early gene labeling of cell populations, Chawla et al. (2005) observed specific patterns of activity in adult born cells in the DG that preferentially responded to specific environments that had been explored at specific times. Taken together, these findings indicate that memory and learning tasks impact adult neurogenesis in complex ways.

More recently, novel technologies have allowed direct manipulation of adult neurogenesis to observe its impact on learning and memory. When adult hippocampal neurogenesis is inhibited with low-dose x-irradiation, performance on learning and memory tasks are impaired. Irradiated rats performed worse on a non-matching to sample task than controls. This difference is exacerbated when the intervals between the test and the sample trials are long (Winocur et al., 2006). Similarly, irradiated rats showed poor performance on a contextual fear conditioning task, suggesting deficits in learning and memory (Wojtowicz et al., 2008). Low-dose x-irradiation has also been used to characterize the role of adult neurogenesis in pattern separation. Irradiated mice showed deficits in two spatial discrimination tasks, the water maze and spatial mouse touch screen task. Importantly, these deficits were only noticeable when stimuli were presented with small, but not large, spatial separation (Clelland et al., 2016). Treatment with temolozolomide (TMZ), an alkylating agent that damages DNA function, reduces adult

neurogenesis. Rats treated with TMZ showed deficits in the water maze task when they were required to re-learn spatial portions of the task – indicating a role for adult neurogenesis in flexibility of spatial learning and memory (Garthe et al., 2009). Given the above data, it is clear that a relationship exists between adult neurogenesis and learning and memory, however the directionality of that relationship depends on a number of factors.

Emotion and adult neurogenesis

The link between adult neurogenesis and emotional regulation is similarly fraught with complexities. As hippocampal volume correlates with depression (Campbell et al., 2004), it is likely that adult neurogenesis plays a role in depression. Evidence has indicated that treatment with antidepressants increases BrdU labeling in the DG of rats. Using neuron- and glia- specific markers, it is evident that these new cells are more likely to be neurons, indicating that antidepressant treatment increases adult neurogenesis. Only chronic treatment resulted in an increase whereas acute treatment did not impact BrdU expression – a finding which parallels the behavioral effects of antidepressant treatment of depression in humans (Malberg et al., 2000). Airan et al. (2007) used chronic mild stress to induce depressive-like behavior in the forced swim task (FST) in rats. Fluoxetine treatment eliminated this effect while upregulating BrdU-labeling in the DG. Importantly, using voltage-sensitive dye imaging, DG activity was a strong predictor of FST performance (Airan et al., 2009). These findings, overall, suggest that adult neurogenesis plays a role in depression and that it may be necessary for the effectiveness of anti-depressants.

Like the above studies on learning and memory, various techniques have been used to eliminate adult neurogenesis to characterize its role in emotion. X-irradiation prevented the positive effects of the selective serotonin reuptake inhibitor fluoxetine on depressive-like behavior in a serotonin receptor deficient mouse model. These same mice showed deficits in the novelty suppressed feeding paradigm indicating an increase in anxiety-like behavior (Santarelli et al., 2003). Mice expressing herpes simplex virus thymidine kinase (TK), which renders only mitotic cells susceptible to the antiviral drug valganciclovir, showed deficits in proliferation of adult born neurons in the DG after drug treatment. These neurogenesis deficient mice had increased novelty avoidance, a measure of anxiety, increased behavioral despair in the forced swim task, and reduced sucrose preference, a measure of anhedonia (Snyder et al., 2011). Similarly, x-irradiated rats treated with chronic mild stress showed no change in FST performance despite treatment with fluoxetine (Airan et al., 2007). These findings seem to indicate that adult neurogenesis is necessary for antidepressant effectiveness. Deletion of the tropomyosin receptor kinase (Trk) B gene via Cre expression in adult born cells in mice resulted in increased anxiety-like behavior in the open field task up to four weeks later (Bergami et al., 2008). Transgenic mouse models that have eliminated adult neurogenesis through overexpressing BAX in nestin positive cells exhibit enhanced anxiety-like behaviors on a number of behavioral tasks, including the elevated plus maze, light dark box, and predator avoidance (Revest et al., 2009). Models that similarly eliminate adult neurogenesis, but through impeding the cell cycle check point kinase ATR, show deficits in different anxiety-related tasks, such as marble burying and novelty suppressed feeding (Onksen et al., 2011).

These findings suggest a connection between adult neurogenesis, anxiety, and depression and have given rise to the neurogenesis theory of affective disorders (reviewed in Petrik et al., 2012). However, like learning and memory, the exact mechanism and directionality of this relationship remain elusive (reviewed in Krishnan and Nestler, 2008). For example, in the study conducted by Airan et al. (2007) which showed the relationship between chronic mild stress, FST performance, and adult neurogenesis, no effect was observed on anxiety-like behavior in the open field task. Saxe et al., (2006) used x-irradiation to impair contextual fear conditioning but found no effect on elevated plus maze performance suggesting task-specific anxiety-like behavior was not impacted by the loss of adult neurogenesis. Taken together, these findings indicate that adult neurogenesis may play a role in effective treatment of depression and anxiety but the exact relationship is still unclear.

Adult neurogenesis and the stress response

Finally, adult neurogenesis is highly responsive to stress and enrichment. As discussed above, the hippocampus is closely linked with the HPA axis. Adult neurogenesis itself is associated with stress responsivity. The resident intruder paradigm is a task in which a resident animal is presented with an intruder in its home cage – a stressful experience for many species. One hour exposure to this paradigm resulted in decreased cell proliferation in intruder marmosets (Gould et al., 1998). Subordinate tree shews, a species notable for their highly stressful dominance hierarchies, experience rapid stress responses during establishment of the dominance hierarchy resulting in an increase in corticosterone and a suppression of adult neurogenesis (Gould et al., 1997). Exposure to predator odors, a fearful stimulus for rodents, leads to a suppression of cell

proliferation that is blocked with adrenalectomy indicating a adrenal steroid mechanism in male rats (Tanapat et al., 2001). This same effect is not evident in females indicating a sex-specific effect (Falconer and Galea, 2003).

On the other hand, positive stressors appear to increase adult neurogenesis. Mice housed in cages with a running wheel exhibited increased cell proliferation in the DG (van Praag et al., 1999b). This increase in running-induced cell proliferation is prevented with peripheral inhibition of insulin-like growth factor-I, suggesting a mechanism through which physical activity can modulate adult neurogenesis (Glasper et al., 2010). Interestingly, the effects of physical activity on adult neurogenesis are prevented with social isolation (Stranahan et al., 2006). Despite increasing circulating glucocorticoids, acute sexual experience increases cell proliferation in male rats. Increased glucocorticoids are not exhibited with chronic sexual experience yet survival of adult born neurons is increased (Leuner et al., 2010b). Finally, environmental enrichment also has a positive impact on adult neurogenesis. Enhanced adult neurogenesis was first attributed to environmental enrichment in wild chickadees compared to impaired enrichment in captive chickadees (Barnea and Nottebohm, 1994). In laboratory conditions, enrichment that includes social engagement, exploration, and physical activity leads to increased adult neurogenesis in adult (Kempermann et al., 1997) and aged rat populations (Kempermann et al., 1998). Given these findings, it is clear that adult neurogenesis is responsive to input from positive stressors that are potentially beneficial to hippocampal function.

Few studies have been conducted examining the direct relationship of adult neurogenesis and the stress response. The evidence that does exist, suggests that adult

neurogenesis itself can directly impact the stress response. Using a transgenic mouse, Schloesser et al. (2009) completely eliminated adult neurogenesis in the DG then exposed mice to a novel environment for 15 minutes – a mild stressor. Wild-type mice exhibited an increase in corticosterone while knockouts showed no change in corticosterone levels.

Plasticity of existing cell structure within the hippocampus

Like the birth and survival of new neurons, the structure of pre-existing cells within the hippocampus is highly responsive to external input. Dendritic length, branch points, and spine density can all be modified in response to external input. Spines are sites for potential excitatory synaptic connections that are primarily along the dendritic shaft but are also present on the axon and cell body – suggesting possible differences in function. However, dendritic spines exhibit the largest experience-dependent fluctuations (reviewed in Holtmaat and Svoboda, 2009). Globus et al. (1973) first showed that dendritic spines change in the cortex in response to environmental input – spine density increases in rats housed in enriched conditions. Subsequent studies have shown considerable data on how important spine plasticity, and stability, is for the changing brain – particularly in the hippocampus. Harris and Stevens (1989) and Harris et al. (1992) used electron microscopy to characterize spines and their role in development and LTP in area CA1 of the hippocampus. They observed increased density in spines in the hippocampus from development through maturation in rats and that larger spines were more likely to be LTP-responsive. These findings indicated that spine density within the hippocampus was altered with age and that spines were likely responsive to behavioral input.

Indeed, extensive evidence implicates spines in learning and memory. Spatial learning in the water maze task (Moser et al., 1994) and learning an olfactory discrimination task (Knafo et al., 2004) increase spine density along CA1 dendrites in rats. Similarly, using trace eye-blink conditioning, a task that requires the hippocampus for acquisition, Leuner et al. (2003) showed that spine density was enhanced in area CA1 – an enhancement that was prevented with an NMDA antagonist. These studies provide examples of learning and memory tasks that show a positive relationship with spine density in the hippocampus. Similarly, the dendritic tree is responsive to learning and memory tasks. Tronel et al. (2010) showed that spatial learning in the water maze task enhanced development of dendritic arbors of adult born neurons in the DG in rats. These enhancements persisted for months after learning suggesting that they also played a role in memory of the learned task.

Like adult neurogenesis, a detrimental relationship exists between negative stress and existing cell structure. Repeated restraint stress leads to atrophy of dendrites in area CA3 of the hippocampus in rats (Watanabe et al., 1992; Magarinos and McEwen, 1995). Spine density is impoverished within minutes of the onset of a stressor in the hippocampus – an effect that is dependent on signaling through corticotrophin-releasing hormone (Chen et al., 2008). Interestingly, exposure to an acute stressor (brief restraint) enhances hippocampal spine density in males but impairs it in female rats (Shors et al., 2001). On the other hand, positive stressors can beneficially impact spine density. One week of sexual experience increased spine density along dendrites of granule cells within the DG of male rats (Glasper et al., 2015). Two month exposure to enriched housing (i.e. toys, running wheels) rescued spine density and memory performance in CA1 NMDA

receptor knockout mice (Rampon et al., 2000). Taken together, these findings of positive and negative stressors parallel the observed effects of stress on adult neurogenesis.

Spine density in the hippocampus is also impacted by emotional state. Depressive-like behavior exhibited through learned helplessness is accompanied by spine density deficits in areas CA1, CA3, and the DG of the hippocampus (Hajszan et al., 2009). Rats that naturally display a more anxious phenotype show depressed spine density in DG granule cells compared to less anxious rats (Adamec et al., 2012). Chronic corticosterone treatment induced depressive- and anxiety- like phenotypes in mice after 35 days. Spine density in the hippocampus was concomitantly suppressed with behavioral displays of these affective disorders. Treatment with the anti-depressant fluoxetine reversed both behavioral and structural deficits (Wang et al., 2013). Taken together, these findings indicate that dendritic structure and spine density are responsive to external input, be it stress or enrichment.

Mechanisms underlying dendritic spine plasticity

Using multiple techniques to isolate individual synapses, cellular and molecular mechanisms have been identified which elucidate how the above experiences can impact spine changes. Engert and Bonhoeffer (1999) identified undeveloped spines on hippocampal CA1 pyramidal neurons and investigated the mechanisms underlying spine growth. Development of spines appeared activity-, NMDAr-, and LTP- dependent (Engert and Bonhoeffer, 1999). Neural activity, NMDAr, and LTP all play a role in experience-dependent dendritic plasticity (reviewed in Holtmaat and Svoboda, 2009). Furthering these findings, Matsuzaki et al. (2004) applied glutamate to synapses within area CA1 of the hippocampus. Excitatory postsynaptic current stimulation leads to a

massive enhancement in spine size that was evident within 10 seconds. Interestingly, small spine enhancement lasted longer than enhancement in large spines. The enlargement appeared NMDA-, calmodulin-, and actin polymerization- dependent. The magnitude of the enlargement correlated with whether the change was permanent or long-lasting (Matsuzaki et al., 2004). Combined, these data suggest that LTP can induce spine growth and that the stages of LTP, the size of the spine changes, and the persistence of these changes reflect a cellular and molecular mechanism for experience-dependent plasticity of dendritic spines.

The above description of adult neurogenesis and alterations of existing cell structure paint a fairly thorough picture of the range of hippocampal plasticity. On a larger scale, these changes to new and existing cell structure occur to facilitate learning (Moser et al., 1994; Gould et al., 1999a), memory (Leuner et al., 2003; Winocur et al., 2006), enrichment (Kempermann et al., 1997), emotion (Malberg et al., 2000; Hajszan et al., 2009; Revest et al., 2009), and the stress response (Magarinos and McEwen, 1995; Tanapat et al., 2001). Regulating this wide-range of behavioral functions requires the capacity for extensive plasticity. As many of these behaviors are directly or indirectly related to successful offspring rearing, understanding how hippocampal plasticity is altered in the postpartum period is important for characterizing the mechanisms underlying successful parenting behavior. Importantly, this may help to inform us about the development of negative affect in the postpartum period that can be detrimental for both parent and child.

Hormones and hippocampal plasticity

There are a number of cellular and molecular signaling mechanisms through which factors such as the local environment, social experience, and internal motivation can directly influence plasticity of hippocampal function and structure (Kempermann et al., 2008; Lie and Gotz, 2008; Nowakowski and Hayes, 2008). The hippocampus, in general, is rich in hormone receptors, providing a site of action directly on the existing neural architecture for neuroendocrine signaling (McEwen, 1999). In the subgranular zone (SGZ) of the DG, precursor cells which generate adult born neurons have extensive neurotransmitter receptors, as well as end feet on nearby vasculature, that allow these cells to directly receive afferent neurotransmitter and neuromodulator input (Filippov et al., 2003). These connections, both vasculature and receptor, allow for chemical and signal input to the hippocampus, thus allowing for experience-dependent alterations of adult neurogenesis and existing cell structure (Kempermann et al., 2008; Tully and Bolshakov, 2010). Given this, it is no surprise that hormones can modify both functional and structural plasticity of the hippocampus. Below, I outline the ways in which hormones associated with parenting can impact hippocampal plasticity.

Hormones, learning, and memory

Previous work has investigated the role of hormones in hippocampal-dependent learning and memory performance. Gonadectomized male rats show deficits in radial arm maze acquisition (Kritzer et al., 2001; Daniel et al., 2003). This deficit is restored with peripheral testosterone treatment but not estradiol treatment (Kritzer et al., 2001). Castration does not impair performance on the water maze task (Sandstrom et al., 2006; Spritzer et al., 2008). However, when assessed on a delayed-matching-to-place version,

which altered the learned location of the platform, castrated males performed more poorly than controls. This deficit was reversed with testosterone treatment (Sandstrom et al., 2006). Interestingly, the benefits of testosterone on the water maze may be towards perseverance as opposed to memory retention as higher testosterone levels correlated with longer swim path length (Spritzer et al., 2008). Estradiol also appears to affect learning and memory in males. Following a one or four hour delay prior to the test portion of the radial arm maze, male Sprague-Dawley rats treated with chronic estradiol performed better on the test portion compared to control males, females, and estradiol treated females (Luine and Rodriguez, 1994). Chronic estradiol treatment (seven days) also improves radial arm maze performance in intact male and female mice (Heikkinen et al., 2002). An intrahippocampal injection of estradiol immediately post-training on the radial arm maze improved memory retention in male rats whereas injections following a one hour delay did not (Packard et al., 1996). Overall, evidence from these studies indicates that gonadal steroids, both testosterone and estrogen, can influence learning and memory in males.

Like males, learning and memory performance in females improves with increased steroid hormones. Compared to females in lower progestin states (estrus, diestrus, non-pregnant, nulliparous), female rats in proestrus (high progesterone, high estradiol) perform better on the water maze task (Frye, 1995) as well as the object recognition and object in place task (Paris and Frye, 2008). Priming with estradiol 48 or 72 hours, or with progesterone 8 hours, prior to testing on the water maze improves memory performance in female rats – evident by increased retention intervals of the location of the platform (Sandstrom and Williams, 2001). Two days of acute estradiol

treatment can maintain these effects for 4 days while after treatment for 10 consecutive days the enhancement in memory retention persists (Sandstrom and Williams, 2004). A single dose of physiologically relevant estradiol (1.5, 2, or 3µg/kg) enhances learning and memory on a social recognition and object recognition in female ovariectomized mice within 40 minutes (Phan et al., 2012). Using the aromatase inhibitor, letrozole, Tuscher et al., (2016a) impaired local estradiol synthesis within the dorsal hippocampus of female mice. Blockage of estradiol synthesis in this brain region resulted in a deficit in object recognition. Taken together, these findings indicate that both endogenous and exogenous estradiol have a positive impact on hippocampal-dependent learning and memory performance in females.

Vasopressin and OT appear to play a sex-specific role in social memory – AVP appears to regulate social recognition in males while OT regulates this behavior in females (for review see Gabor et al., 2012). Peripheral injections of AVP increased social recognition in both male and female rats. However, treatment with the AVP antagonist dPTyr(Me)AVP impaired this behavior in males alone, suggesting that AVP binding to its receptor is sufficient but not necessary for social recognition in female rats (Bluthe´ and Dantzer, 1990). Central administration of anti-AVP serum to the dorsal and ventral hippocampus, but not the nucleus olfactorious or the septal region, impairs social recognition behavior in male rats (van Wimersma Greidanus and Maigret, 1996). Male mutant mice that are lacking the gene for the AVP 1b receptor (V1bR) show deficits in social recognition memory but no differences in learning an olfactory discrimination task compared to wild type controls (Wersinger et al., 2004). Further investigation into the hippocampal function of V1bR deficient mutants indicated that, while retaining

familiarity of objects, they lack the ability to distinguish temporal order, suggesting a deficit in temporal memory performance (DeVito et al., 2009). While peripheral OT did not alter social recognition in intact female rats, treatment with OT receptor antagonists prevented the females' ability to distinguish a novel juvenile from a familiar juvenile (Engelmann et al., 1998). Interestingly, administration of an anti-OT serum directly to the ventral hippocampus also decreased time spent with a novel animal, indicating that in male rats OT can have some effect on social recognition memory (van Wimersma Greidanus and Maigret, 1996). These findings indicate that both AVP and OT can impact social recognition memory and that their effects are somewhat sex-specific.

Finally, glucocorticoids can have a significant impact on learning and memory performance. In a contextual fear conditioning task, male rats treated with metyrapone, a glucocorticoid synthesis inhibitor, 90 minutes before training showed attenuated fear conditioning indicating that acquisition (learning) was impaired (Cordero et al., 2002). Interestingly, the effects of glucocorticoids on fear conditioning are dose-dependent. Rats treated with a high dose of corticosterone for 21 days before fear conditioning showed a fear response to both the conditioned tone and the contextual environment during the retrieval phase. Rats treated with a low dose only showed a response to the tone presentation (Marks et al., 2015). These findings indicate that corticosterone's effects on learning and memory in this task are dependent on time and dose of treatment in relation to training. Infusions of a glucocorticoid receptor antagonist (RU 28362) directly to the hippocampus 24 hours after training on the water maze impaired retention performance in male Sprague-Dawley rats (Roozendaal et al., 2003). Unlike male rats, females are more susceptible to deficits in water maze performance as a result of elevated baseline

corticosterone levels – a difference that is eliminated following removal of the adrenal gland (Beiko et al., 2004). Overall, glucocorticoids appear to enhance memory and learning during emotionally arousing experiences but impair memory retrieval during test situations (for review see de Quervain et al., 2009).

Hormones and emotional responsivity

Like learning and memory, hormones can influence hippocampal-dependent emotional responses. Males show reduced anxiety-like behavior with estradiol treatment as gonadectomized male rats that receive systemic estradiol show reduced anxiety-like behavior in the open field task (Filova et al., 2015). Testosterone treatment is also anxiolytic in male rats in the open field task but this effect is dependent on the conversion of testosterone to estradiol via aromatase within the DG of the hippocampus (Carrier et al., 2015). Similarly, female rats treated with estradiol show reduced anxiety-like behavior in the open field task (Bowman et al., 2002). In pro-estrus, when estradiol is highest and sexual receptivity is displayed, female mice show reduced anxiety-like behavior on the elevated plus maze (Walf et al., 2009). Systemic administration of estradiol reduced anxiety-like behavior in a number of anxiety assessment tasks including the elevated plus maze (Walf and Frye, 2005). The anxiolytic effect of estradiol appears to be dependent on signaling through the beta estrogen receptor (Erβ). Treatment with the selective estrogen receptor modulator (SERM) diarylpropionitrile (DPN), which acts as an agonist on Erβ, is anxiolytic while activation of the alpha receptor (Erα) has no effect on anxiety-like behavior. DPN's effects were inhibited by concomitant treatment with the SERM tamoxifen (TMX), an Erβ antagonist with some agonistic proclivity for Erα (Watanabe et al., 1997). Erβ knockout mice show no attenuation in anxiety-like behavior

on the elevated plus maze (Walf and Frye, 2006). Overall these data show that estradiol's effect on anxiety, like its effects on learning and memory, appear beneficial.

While OT and AVP can both influence anxiety (for review see Neumann & Landgraf 2012), they traditionally have opposing effects – OT is anxiolytic while AVP is anxiogenic. When male rats are treated with OT, but not AVP, directly to prelimbic regions, anxiety-like behavior is reduced (Sabihi et al., 2014). Females treated with a regimen of estrogen and progesterone to mimic the end stages of pregnancy show decreased anxiety-like behavior on the EPM and activity of the immediate early gene c-fos in the ventral hippocampus. Accompanying these changes is an increase in OT receptor ligand binding – the inhibition of which disrupts the attenuation in anxiety, suggesting an OT dependent mechanism (Windle et al., 2006). In contrast to OT, AVP appears anxiogenic in maternal rodents. Intracerebroventricular infusions of AVP increase, while OT infusions decrease, anxiety-like behavior in lactating rat dams (Bosch and Neumann, 2008). Taken together, these data indicate that the hormonal milieu in postpartum females provides a number of mechanisms that may drive anxiolytic behavior in mothers. Importantly, it also suggests that these hormones may act together to mediate these changes.

Glucocorticoids tend to have an anxiogenic and depressive effect. Chronic, low-dose treatment with corticosterone increases anxiety-like behavior in the light-dark box, and increases depressive-like behavior in the forced swim task in male mice (Murray et al., 2008). Chronic (21 day) treatment with corticosterone increases depressive-like behavior in the forced swim task in male Long Evans rats. This effect is escalated with increasing doses (Johnson et al., 2006). Anxiety-like behavior on the elevated plus maze

and the tail suspension test is elevated in male mice that received chronic corticosterone treatment. This effect is reversed with fluoxetine treatment, an anti-depressant (Wang et al., 2013). Higher physiological doses of corticosterone that mimic stress-induced elevations of glucocorticoids increase anxiety in male rats on the elevated plus maze even when given in an acute dose (Mitra and Sapolsky, 2008). As low, acute doses of corticosterone do not appear to initiate depressive- or anxiety- like behavior (Johnson et al., 2006; Murray et al., 2008) it is evident that glucocorticoids' impact on emotional responsivity are only evident after higher doses corresponding to more stressful experiences.

While PRL is traditionally associated with milk production in females and its impact on learning and memory is relatively unstudied (however, see Torner et al., 2013), it appears to have a significant impact on emotional responsivity in both males and females. Chronic infusions of PRL into the ventral hippocampus have an anxiolytic effect in virgin male rats on the elevated plus maze. This effect is dose-dependent and is attenuated with the down-regulation of PRL receptor (PRLr) expression (Torner et al., 2001). Ovariectomized females treated with intracerebral infusions of PRL directly to the ventral hippocampus show reduced anxiety-like behavior on the elevated plus maze and an attenuated rise in glucocorticoids following restraint stress (Donner et al., 2007). Similarly, PRL treatment directly to the ventral hippocampus increases swimming time in the forced swim task and enhances novel object-directed exploration, suggesting anti-depressive and anti-anxiety effects (Alvarez and Banzan, 1994). These findings indicate that central PRL can act directly on the ventral hippocampus to influence emotional responsivity.

Hormones and structural plasticity

Along with the changes observed in functional plasticity, hormones can alter both new and existing structural plasticity of the hippocampus. Most of the work investigating estradiol's influence on adult neurogenesis has focused on females. However, the limited evidence from males indicates that estradiol can enhance cell survival but not cell proliferation. Male meadow voles treated with peripheral estradiol one week post-BrdU injection, at the time of axon extension, show enhanced survival of adult born neurons without exhibiting alterations in cell proliferation (Ormerod et al., 2004). Within the SVZ male rats treated with testosterone or estradiol show increased BrdU-labeling indicating that testosterone and estradiol can increase adult neurogenesis in this brain region (Farinetti et al., 2015). In females, both endogenous and exogenous estradiol can alter structural plasticity. In virgin, cycling female rats, cell proliferation in the DG peaks during pro-estrus when estradiol levels are highest (Tanapat et al., 1999). Ovariectomized female rats that receive a moderate, acute dose of estradiol experience an increase in cell proliferation in the DG. Interestingly, low and high doses, as well as chronic estradiol treatment, do not alter cell proliferation (Tanapat et al., 2005). When estradiol is administered to female rats four hours prior to BrdU injection an increase in cell proliferation is observed. However, if given 48 hours before the BrdU-labeling, cell proliferation decreases (Ormerod et al., 2003). Agonists for both Erα and Erβ increase cell proliferation in female rats when given four hours before BrdU injection (Mazzucco et al., 2006). Taken together, these findings indicate that estradiol alters adult neurogenesis. In females, estradiol's effects on cell proliferation in the DG are time- and dose- dependent.

Gonadal steroids can also impact dendritic plasticity of existing neurons in males and females. Gonadectomy of male rats resulted in an increase in electrical transmission along mossy fibers of CA3 neurons as well as increased LTP and dendritic sprouting. These changes were induced by an increase in brain derived neurotrophic factor (BDNF) signaling through Trk receptors. Using multiple androgen inhibitors, the lack of testosterone was determined to be the neuroendocrine alteration responsible for the changes (Skucas et al., 2013). Bath application of estradiol to hippocampal slices from male rats induced LTP in area CA1, CA3, and the DG. Erα-dependent spinogenesis was observed in area CA1 as only the Erα agonist, propyl-pyrazole-triol (PPT), induced spine growth while the Erβ agonist, DPN, did not (Mukai et al., 2007). Ovariectomized female rats lacking gonadal steroids show significantly reduced dendritic spine density in the CA1 region of the hippocampus. Spine density in this region is returned to baseline after treatment with estradiol or progesterone (Gould et al., 1990). As estradiol can reverse spine density deficits within 30 minutes of administration (MacLusky et al., 2005; Phan et al., 2012), it is likely that hippocampal spine density fluctuates rapidly with endogenous hormone levels in females (Gould et al., 1990; MacLusky et al., 2005). Intact female mice show enhanced spine density following estradiol infusions directly to the dorsal hippocampus in the CA1 region. This increase is prevented with administration of either extracellular signal-regulated kinase (ERK) or mammalian target of rapamycin (mTOR) inhibitors suggesting that these signaling pathways are necessary for estradiol-induced increases in spine density (Tuscher et al., 2016b).

The rapid effects of estradiol on hippocampal plasticity are dependent on estrogen receptor and glutamate receptor signaling. Compared to estradiol treatment alone, Erβ

and Erα agonists cause similar improvements in memory performance (Boulware et al., 2013) and Erβ agonists induce changes in anxiety-like behavior (Walf and Frye, 2005). Signaling through these receptors appears dependent on activation of metabotropic glutamate receptor 1a (mGluR1a), as treatment with the mGluR1a antagonist LY367385 to the dorsal hippocampus inhibits the memory enhancing effects of Er agonists (Boulware et al., 2013). The differing effects of Erβ and Erα are a result of receptor differences. While the two receptor subtypes have similar DNA- and ligand- binding domains, they have differing N-terminal regions (Tremblay et al., 1997). This discrepancy results in activation of separate metabotropic glutamate receptors. Signaling through Erα activates mGluR1 which in turn drives cAMP response element-binding protein (CREB) phosphorylation. Erβ activation triggers mGluR2/3 signaling resulting in a downregulation of calcium mediated CREB phosphorylation (Boulware et al., 2005). These pathways can allow for the rapid, non-genomic effects of estradiol on hippocampal plasticity (for review see Walf and Frye, 2006).

While no studies have reported OT- or AVP- driven changes in existing cell structure of the hippocampus, evidence suggests that OT may drive new cell growth within the hippocampal. Central and peripheral OT treatment, but not AVP, has been shown to increase cell proliferation primarily in the ventral DG of male rats, even under stressful conditions. Repeated administration of OT, but not AVP, actually enhances the number of new neurons in this brain region suggesting that OT may play a neuroprotective role (Leuner et al., 2012). Daily treatments with OT for two weeks increased cell proliferation in male rats. Additionally, the dendritic maturation of these adult born cells was enhanced (Sánchez-Vidaña et al., 2016). Administration of OT to

nulliparous women increases functional connectivity within the hippocampus during presentation of infant-related cues (Riem et al., 2012), suggesting that OT may contribute to altered neuroplasticity of the hippocampus in mothers. The mechanism(s) by which OT may drive adult hippocampal neurogenesis remains unclear. The hippocampus does have receptors for both OT and AVP – suggesting a site for direct activation. However, it is also possible that OT positive neurons from other regions that connect directly to the hippocampus, such as from the amygdala, may contribute to OT-induced hippocampal plasticity (reviewed in Gimpl et al., 2001). Finally, as estrogen can also modulate social recognition memory, it is possible that OT and AVP mechanisms may be acting on hippocampal plasticity via estradiol (reviewed in Gabor et al., 2012).

Glucocorticoids tend to have a negative impact on hippocampal structural plasticity. In humans, hippocampal volume is reduced with long-term cortisol elevations. This reduction was strongly correlated with the degree of elevation as well as the baseline levels at the time of testing (Lupien et al., 1998). Similar patterns are observed in rodents. Basal levels of adrenal steroids negatively correlated with adult neurogenesis in the DG of rats (Sapolsky and Meaney, 1986). Treatment with acute corticosterone decreases H3-thymidine labeling density, while removal of endogenous adrenal steroids via adrenalectomy increases neuron-specific cell proliferation in the DG of male rats (Cameron and Gould, 1994). Low-dose corticosterone reduces adult neurogenesis and overall hippocampal volume in male CD1 mice (Murray et al., 2008). Interestingly, suppression of cell proliferation in the DG of male rats by two weeks of daily injections of corticosterone is prevented with co-administration of OT (Sánchez-Vidaña et al., 2016). Repeated administration of corticosterone for 35 days induces a depressive

phenotype in male C57BL6 mice. Spine density within the CA1 region of the hippocampus is suppressed concomitant with the behavioral phenotype. Both the depressive-like behavior and the spine density deficit are reversed following 25 days of treatment with the anti-depressant fluoxetine (Wang et al., 2013).

Receptors for glucocorticoids and mineralocorticoids are present throughout the hippocampus providing a direct site of action for stress-induced glucocorticoids to act on this brain region (Han et al., 2005). In cell culture, two week incubation of hippocampal neurons with corticosterone reduces dendritic spines. This impairment is a result of glucocorticoid-induced reduction of caldesmon, an actin-linked protein which stabilizes actin filaments for spine growth (Tanokashira et al., 2012). Precursor cells within the SGZ have few, if any, glucocorticoid or mineralcorticoid receptors preventing direct action on the proliferative population by adrenal steroids (Cameron et al., 1993a). The effect of glucocorticoids on adult neurogenesis appears to be through indirect modulation of n-methyl-D-aspartate (NMDA) receptors. Treatment with NMDA antagonists increased cell birth in the DG of adult male rats (Cameron et al., 1995). Thus, it is possible that adrenal steroids are interacting with NMDA signaling to modify adult neurogenesis (for review see Gould and Tanapat, 1999). Diamond et al. (1992) investigated how corticosterone levels differentially affected hippocampal signaling. Importantly, it was observed that the relationship between corticosterone levels and hippocampal firing resembles an inverted U indicating a complex, biphasic effect of stress hormones on hippocampal function. It is possible that the lower doses of corticosterone may contribute to enhanced survival of the animal through increased

vigilance and neuroplasticity, while the higher doses lead to deficits in neural function that contribute to lower survival rates (reviewed in Lupien et al., 2009).

Data on PRL and hippocampal structural plasticity is somewhat limited. While no data has been reported on PRL's influence on hippocampal spine density, PRL appears to influence adult neurogenesis. PRL has been shown to mediate adult neurogenesis in C57BL6 mouse fathers. While males of this species are not naturally biparental, they will exhibit some paternal care behaviors if left with their offspring. Fathers that remained in contact with their pups had increases adult neurogenesis in the DG and the olfactory bulb. These newborn cells co-labeled with PRLr-ir while PRLr knockout fathers had reduced adult neurogenesis (Mak and Weiss, 2010). PRLr expression has been observed in the DG (Bakowska and Morrell, 1997; Shingo et al., 2003; Nogami et al., 2007) suggesting a site of action for PRL to modulate DG plasticity. Despite these findings the mechanism and the extent to which PRL mediates hippocampal plasticity remains unknown.

The evidence provided above indicates that estradiol, OT, AVP, glucocorticoids, and PRL can impact functional and structural plasticity of the hippocampus. As these hormones are modified in the postpartum period of fathers, they may act as a neuroendocrine mechanism driving paternal experience-dependent hippocampal plasticity. Furthermore, it is evident that many of these hormones can work together to alter neuroplasticity. These findings, combined with the data presented in the previous sections of this chapter, highlight the complex physiological state that accompanies the postpartum period in males of biparental species. As the neuroendocrine changes associated with parenting can have a significant impact on a brain region that mediates

important aspects of behavioral and emotional function, it is evident that a thorough investigation of these interactions should be undertaken in males.

Conclusion

The evidence provided above describes the literature on hippocampal plasticity and how it is modified in the postpartum period. Additionally, it is evident that the hormones associated with parenting have an impact on neuroplasticity of this region (Ormerod et al., 2004; Walf and Frye, 2005). While the neural circuitry underlying maternal care behaviors has been elucidated and its regulation via hormonal fluctuations during the postpartum period are known, parallel work in fathers is limited. Research that has investigated these mechanisms in fathers has utilized appropriate models of paternal care such as the California mouse (i.e. Glasper et al., 2011). The California mouse is an excellent model of paternal care as males of this species exhibit all aspects of paternal behavior (Bester-Meredith et al., 1999) and previous studies have laid the groundwork for investigating hormones (Trainor and Marler, 2002) and hippocampal plasticity in the postpartum period (Glasper et al., 2011; Lambert et al., 2011) in this species. Despite the work that has been done, there remain gaps in the knowledge of how the hippocampus is modified in the paternal postpartum period and how hormones may contribute to any changes.

In the early-postpartum period (Chauke et al., 2012) and at weaning (Glasper et al., 2011) anxiety-like behavior is not altered in California mouse fathers. However, no work has investigated this aspect of hippocampal function in the mid-postpartum period in males of a biparental species. Anxiety-like behavior is attenuated by offspring contact in the maternal postpartum period (Lonstein, 2005). In California mouse fathers, the mid-postpartum period is characterized by a unique shift from passive to active father-offspring interaction (Bester-Meredith et al., 1999) to accommodate more

mobile pups (Vieira and Brown, 2002). Thus, it is possible that anxiety-like behavior may be differentially altered as a result of specific father-offspring interaction across the postpartum period. Hippocampal structural plasticity, specifically suppressed adult neurogenesis, is altered at weaning in California mouse fathers (Glasper et al., 2011). However, this aspect of structural hippocampal plasticity has yet to be investigated in fathers prior to this time-point. Additionally, to date no work has investigated other aspects of hippocampal structural plasticity, such as spine density and dendritic structure, in males of any biparental species. As hippocampal structure is also impacted by offspring contact in mothers (Leuner et al., 2007), it is possible that fathers may experience differential alterations in structural plasticity of this region at different times across the postpartum period.

Increased circulating estradiol after only brief exposure to pups promotes paternal care in California mouse fathers (Trainor and Marler, 2002). However, the level of circulating estradiol in the mid- and late- postpartum periods remains unknown. Thus, it is unclear if circulating estradiol is involved in both promoting and maintaining paternal care behaviors. While evidence suggests that estradiol can impact hippocampal plasticity through activation of its receptors (Walf and Frye, 2005), no work to date has investigated to what extent estradiol receptor expression in this region may be altered in the paternal postpartum period. As circulating estradiol is elevated in the early postpartum period in fathers, it is possible that this elevation may initiate increased receptor expression (Barton and Schapiro, 1988) in the hippocampus, providing a site of action for this hormone to modify hippocampal plasticity in the postpartum period. Taken together, the paternal postpartum period in California mice is characterized by changes in

estradiol and hippocampal plasticity. However, the extent of these changes, their temporal nature, and to what extent estradiol may be acting as a neuroendocrine mechanism underlying hippocampal plasticity remain unclear.

Chapter 2: Fatherhood contributes to increased hippocampal spine density and anxiety regulation in California mice

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Abstract

Introduction: Parenting alters the hippocampus, an area of the brain that undergoes significant experience-induced plasticity and contributes to emotional regulation. While the relationship between maternal care and hippocampal neuroplasticity has been characterized, the extent to which fatherhood alters the structure and function of the hippocampus is far less understood.

Methods: Here, we investigated to what extent fatherhood altered anxiety regulation and dendritic morphology of the hippocampus using the highly paternal California mouse (Peromyscus californicus).

Results: Fathers spent significantly more time on the open arms of the elevated plus maze, compared to non-fathers. Total distance traveled in the EPM was not changed by paternal experience, which suggests that the increased time spent on the open arms of the maze indicates decreased anxiety-like behavior. Fatherhood also increased dendritic spine density of granule cells in the dentate gyrus and basal dendrites of pyramidal cells in area CA1 of the hippocampus.

Conclusions: These findings parallel those observed in maternal rodents, suggesting that the hippocampus of fathers and mothers respond similarly to offspring.

Introduction

Fatherhood-induced changes to the brain are not well understood, in large part due to the small number of paternally behaving mammals. The California mouse (*Peromyscus californicus*) is an excellent rodent model in which to study parenting, as males and females of this biparental species care for offspring similarly (Dudley, 1974). This species provides a rare opportunity to study the effects of paternal care on brain plasticity. While few studies exist, available evidence suggests that males and females of biparental species experience similar changes to the structure of the brain – especially the hippocampus. The hippocampus has received much attention because of continued structural modifications (i.e., adult neurogenesis; spinogenesis) throughout adulthood and because of the role it plays in emotional regulation, cognition, and stress reactivity – all of which are altered during the postpartum period of maternal rodents. The paternal brain may undergo similar changes, however, it has been far less studied.

As previously mentioned, hippocampal structural morphology is significantly altered during the postpartum period in mothers (reviewed in Leuner and Gould, 2010a). Rodent mothers experience reduced adult neurogenesis (Leuner et al., 2007; Pawluski and Galea, 2007; Glasper et al., 2011) and increased dendritic spine density in the hippocampus (Kinsley et al., 2006; Pawluski and Galea, 2006; Leuner and Gould, 2010a). Given that both adult neurogenesis (Jessberger et al., 2009; Clelland et al., 2016) and changes in dendritic spines (Leuner and Shors, 2013) may underlie hippocampal function, it is not surprising that emotional regulation is also altered during the post-partum period in mothers. Specifically, maternal rodents exhibit reduced anxiety-like behavior on the elevated plus maze (EPM) during the postpartum period (Lonstein, 2005)

– an effect that is dependent on offspring interaction. Interestingly, some fatherhood-induced alterations to the hippocampus have also been observed. Fatherhood decreases new neuron survival in monogamous voles (Lieberwirth et al., 2013) as well as in California mouse fathers (Glasper et al., 2011), however, this change in adult neurogenesis is not accompanied by altered hippocampal function 1 month following the birth of pups (Glasper et al., 2011). To date, the extent to which fatherhood alters dendritic morphology of the hippocampus, and whether this change is associated with anxiety regulation, has yet to be investigated.

Methods

Gonadally intact male, gonadally intact female, and tubally ligated female California mice (60–90 days old) were obtained from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC) or bred in our colony. Mice were provided ad libitum access to food and water, and were maintained on a reversed 16:8 light/dark cycle (lights off at 11:00 h). Two groups were used: non- fathers (n = 8) and fathers (n = 12). Non-fathers were gonadally intact virgin males that were pair housed with tubally ligated females, while fathers were gonadally intact virgin males that were pair housed with gonadally intact virgin females. Paired mice cohabitated on average 51.1 days before an average of 1.6 pups were born. Four fathers had a second litter present at the time of euthanization. Two litters were born on the same day of euthanization, 1 litter was born 24 h prior to euthanization, and 1 litter was present for nearly 1 week before euthanization. A 5-min EPM task was used to assess anxiety-like behavior, as previously described in the California mouse (Chauke et al., 2012). Testing occurred on PND19,

during a time of peak pup retrieval in California mouse fathers (Bester-Meredith et al., 1999). Behavior was observed 2 h after lights out, under red light illumination, and monitored by EthoVision® XT behavioral tracking software (Noldus, Leesburg, VA). Percent time spent in the open arms, total distance traveled, and number of arm entries was calculated. In the event that a mouse fell off of the EPM (n = 3), they were quickly placed into the center of the maze and allowed to explore the maze until a total of 5 min had passed. Heat maps were generated to represent the average location on the maze for each group. Warmer colors indicated more, while cooler colors represented less, time spent in a location of the maze. Three non- fathers and 1 father were excluded from analysis due to >40% immobility during testing (Chauke et al., 2012). These excluded mice remained completely immobile for extended periods of time on the open or center arms of the maze and therefore were eliminated from the study. At the conclusion of EPM testing, mice were returned to their home cages and remained undisturbed until weaning on PND35.

On PND35, mice were deeply anesthetized, cervically dislocated, and brains were quickly harvested and processed for Golgi impregnation using a Rapid Golgi Staining Kit (FD Neurotechnologies, Columbia, MD), as previously described (Haim et al., 2016).

Dendritic remodeling analyses were performed, as previously described (Glasper et al., 2010), using a Zeiss AxioImager microscope with a stage controller and neuroimaging software (Neurolucida, Williston, VT). Five neurons per brain and five dendrites per neuron were used to assess spine density, dendritic length, and dendritic branching from randomly selected Golgi-impregnated cells throughout the entire rostral-caudal extent of the dentate gyrus (DG) and area CA1 of the hippocampus.

Data were analyzed using Prism 6.0 Software for MacOSX (GraphPad Software Inc., San Diego, CA). Unpaired Student's t-tests were performed to assess the effects of fatherhood on anxiety-like behavior and hippocampal structural morphology. Pearson correlations were per- formed, where appropriate. Mean differences were statistically different when $P \leq 0.05$.

Results

Fatherhood significantly decreased anxiety-like behavior on the EPM. Increased percent time spent exploring the open arms of the EPM was observed among fathers, com- pared to non-fathers ($t(14) = 2.53$, $P \leq 0.05$; Fig. 1A). No differences in the total distance traveled within the EPM were observed ($P > 0.05$; Fig. 1B). Open and closed arm entries did not differ between groups ($P > 0.05$; Fig. 1C). Due to increased freezing behavior, three non- fathers were excluded from the analyses. This likely increased the variance in open arm entries that may have prevented non-fathers and fathers from being statistically different from each other.

Fatherhood increased dendritic spine density. Increased dendritic spine density on secondary and tertiary dendrites was observed on DG granule cells ($t(18) = 2.099$, $P \leq 0.05$; Fig. 2A), while dendritic length and the number of branch points were not altered by paternal experience ($P > 0.05$). Basal dendritic spine density of pyramidal cells within area CA1 of the hippocampus was increased by fatherhood ($t(18) = 2.831$, $P \leq 0.05$; Fig. 2B), how- ever, no change in dendritic spine density was observed on pyramidal cell apical dendrites within area CA1 ($P > 0.05$). CA1 pyramidal cell basal dendritic tree lengths (non-father: 697.5 ± 50.80; father: 639.0 ± 68.36) and number of branch points

(non-father: 7.20 ± 0.638; father: 5.567 ± 0.669) were not different (P > 0.05). However, fathers had significantly shorter CA1 pyramidal cell apical dendritic tree lengths (t (18) = 2.615, P ≤ 0.05; non-father: 845.9 ± 48.8; father: 638.2 ± 55.86) and fewer branch points (t (18) = 2.752, P ≤ 0.05; non-father: 8.175 ± 0.284; father: 5.817 ± 0.668).

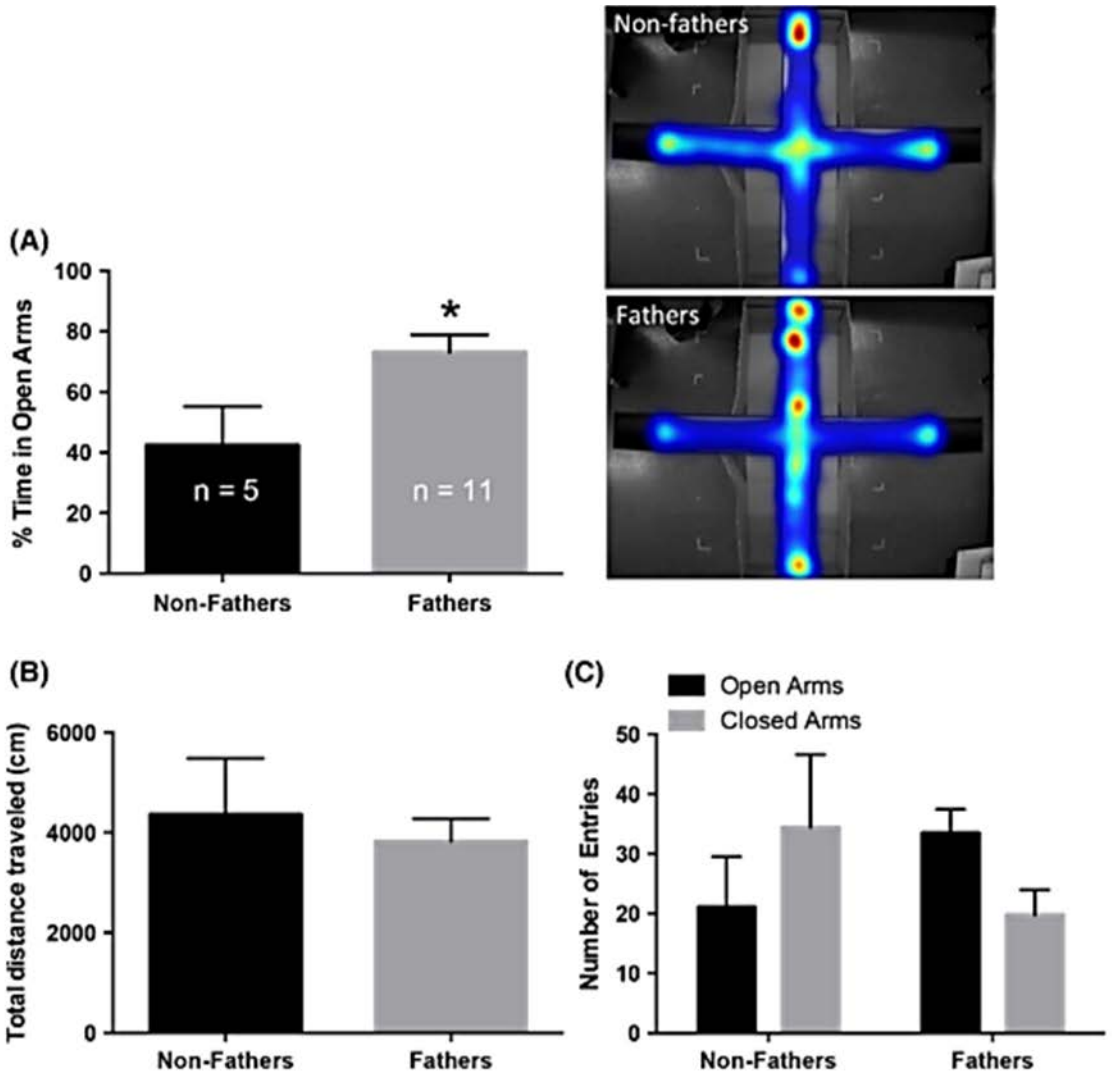

Figure 1. Fatherhood decreases anxiety- like behavior in California mice. (A) Fatherhood increases the percent time spent in the open arms of the elevated plus maze (EPM), compared to non-fathers. Heat maps indicate the average location of mice. Warmer colors represent more, while cooler colors represent less, time spent in a location on the EPM. Greater heat is observed on the open arm of the elevated plus maze in fathers. (B) No difference in the total distance traveled within the EPM was observed. (C) The number of entries into the open or closed arms did not differ between groups. Bars represent mean+SEM. *P ≤ 0.05.

Performance on the elevated plus maze did not correlate with spine density in the DG or area CA1 of the hippocampus ($P > 0.05$).

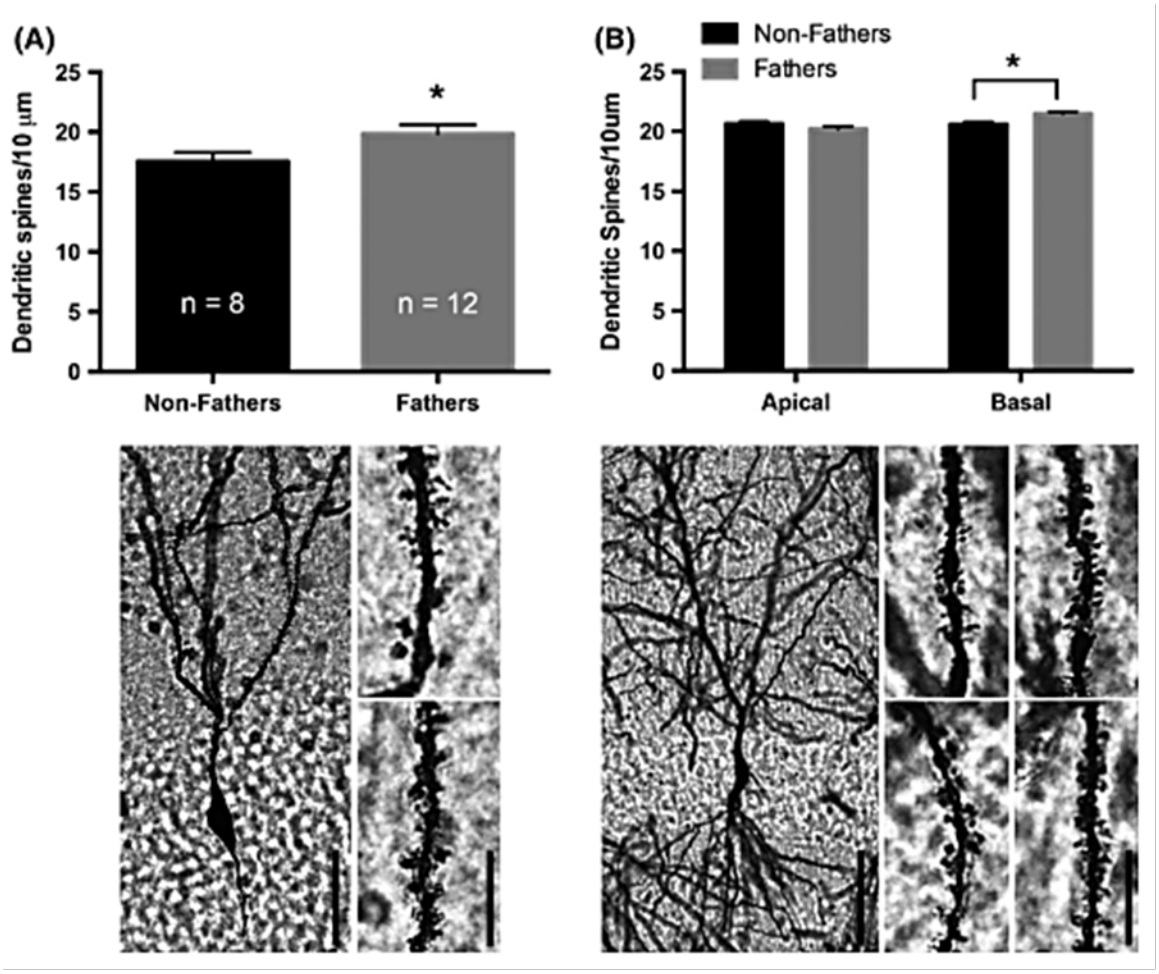

Figure 2. Fatherhood increases hippocampal dendritic spine density. (A, top) Fatherhood increases dendritic spine density of granule cell neurons in the dentate gyrus (DG). (A, bottom) The photomicrograph on the left depicts a representative DG granule cell from a male California mouse. Dendritic segments on the right are representative of non-fathers (top) and fathers (bottom). (B, top) Dendritic spine density on basal, but not apical, dendrites of CA1 pyramidal cells is increased with fatherhood. (B, bottom) The photomicrograph on the left depicts a representative CA1 pyramidal cell from a male California mouse. Dendritic segments represent non-fathers (left), fathers (right), apical (top), and basal (bottom). Bars represent mean+SEM. Scale bars: cells = 40 µm, segments = 10 µm. *$P \leq 0.05$

Discussion

This study examined the effects of fatherhood on hippocampal plasticity in California mice, a biparental species that forms strong bonds with both their mate and offspring

(Gubernick and Nordby, 1993). Our results suggest that fatherhood alters the structure and function of the hippocampus – a brain region that undergoes significant experience-induced plasticity. The hippocampus plays an important role in the regulation of anxiety (Kheirbek et al., 2013). Parenting-induced enhancements in anxiety regulation have been shown in maternal rodents (Lonstein, 2005). We demonstrate that fatherhood decreases anxiety-like behavior during a period of time when pup retrieval is elevated in fathers of this species (Bester-Meredith et al., 1999). This offspring-induced decrease in anxiety-like behavior, among fathers, is similar to that observed in maternal rodents – an effect that is independent of suckling (Lonstein, 2005). This suggests that pup contact, and not a mechanism related to lactation, may indeed drive these observed effects in fathers. However, not all biparental male rodents demonstrate enhanced anxiety regulation with paternal experience. Male prairie voles (*Microtus ochrogaster*) exhibit increased anxiety-like behavior on the EPM a few days following the birth of offspring (Lieberwirth et al., 2013). Timing of behavioral testing may be the key to understanding these discordant observations. Starting at birth, California mouse fathers interact with their offspring by demonstrating huddling and licking behaviors that are followed by a surge in pup retrievals that occurs between PND15 and PND21 (Bester-Meredith et al., 1999). We may have observed a different anxiety profile had we measured performance on the EPM earlier during the postpartum period. Interestingly, new California mouse fathers and paired virgins exhibit decreased anxiety-like behavior on PND 3–4 when compared to isolated virgins and expectant fathers (Chauke et al., 2012). An analysis of the development of emotional regulation in fathers of this species should be carefully investigated.

Here, we demonstrate for the first time that fatherhood increases dendritic spine density of DG granule cells and basal CA1 pyramidal cells – an observation previously seen in maternal rodents (Kinsley et al., 2006; Pawluski and Galea, 2006; Leuner and Gould, 2010a; Salmaso et al., 2011). Collectively, these data suggest that the effects of offspring on parenting-induced hippocampal plasticity are similar between sexes. It is important to note that enhanced dendritic plasticity in fathers has been observed in the prefrontal cortex of the biparental marmoset (*Callithrix jacchus*; Kozorovitskiy et al., 2006) and in the medial precentral cortex of California mice (Kozorovitskiy, 2007). The hippocampus was not assessed in these previous studies. Our study also observed dendritic atrophy of apical dendrites of CA1 pyramidal cells in fathers, compared to non-fathers. Chronic stress has been reported to reduce dendritic complexity of CA1 neurons in rats (Donohue et al., 2006; Pawluski and Galea, 2006). We do not know whether California mouse males find fatherhood stressful (i.e., activation of the HPA axis). The degree to which fatherhood alters the stress response and its differential effects on region-specific hippocampal structural plasticity is unknown.

Available evidence from fathers of biparental species suggests that paternal care is influenced through direct father–offspring contact (Dixson and George, 1982; Bredy et al., 2004). Increased offspring contact improves memory (Aguggia et al., 2013), decreases anxiety (Maniam and Morris, 2010), and decreases depression (Boccia et al., 2007) in maternal rodents, however, the extent to which altered offspring contact (i.e., separation from offspring) changes hippocampal function in fathers is unknown. It is likely that offspring contact may be neuroprotective in species where strong pair bonds exist between parents and offspring during the postpartum period. Direct comparisons

between species that demonstrate different social structures could shed an interesting light on the role of bonding in parenting-induced neuroplasticity.

The mechanisms responsible for fatherhood-induced changes in neuroplasticity are unknown, however, many of the observed changes in neuroplasticity in maternal rodents are due to alterations in circulating hormones (Lucas et al., 1998; Darnaudéry et al., 2007; Brusco et al., 2008). Although paternal rodents do not undergo pregnancy, parturition, or lactation, interaction with offspring has been shown to alter hormone concentrations (for a comprehensive review, see Saltzman and Ziegler, 2014). One such hormone that is altered with offspring interaction is prolactin (PRL). Higher PRL concentrations are observed in California mouse fathers 2d postpartum, compared to virgin males or expectant fathers (Gubernick and Nelson, 1989) and PRL concentrations in marmoset fathers are higher than in males without offspring (Dixson and George, 1982). To date, the direct effects of PRL on hippocampal dendritic spine density, in fathers of any species, is unknown. However, given PRL's role in mediating offspring contact, and its likely role in other forms of offspring-induced structural plasticity (i.e., adult neurogenesis; (Mak and Weiss, 2010; Lévy et al., 2011), it is possible that PRL plays a role in our observed findings. Another potential contributor to our fatherhood-induced changes in hippocampal neuroplasticity is oxytocin (OT). By PND3, California mouse fathers have higher OT concentrations than fathers separated from their offspring on PND0 (Gubernick et al., 1995), suggesting that bond formation may contribute to alterations in OT concentrations in this species. While direct manipulation of OT and its effects on hippocampal neuroplasticity in males is unknown, administration of OT to nulliparous women increases functional connectivity within the hippocampus during

presentation of infant-related cues (Riem et al., 2012), suggesting that OT may contribute to altered neuroplasticity of the hippocampus in parents. Finally, given that vasopressin (AVP) has been associated with offspring-induced alterations to structural plasticity in marmoset fathers (Kozorovitskiy et al., 2006), it is likely a candidate here. AVP correlates with paternal care in California mouse fathers (Bester-Meredith and Marler, 2003) and AVP gene expression is elevated in prairie vole fathers (Wang et al., 2000).

In conclusion, these results demonstrate that the post-partum period in fathers is a time of significant plasticity within the hippocampus. DG structural morphology and anxiety regulation are enhanced during a time of peak pup interaction in fathers. These data suggest that interaction with offspring may influence mood and structural changes within the brain of fathers as it does in maternal rodents. However, whether the changes in hippocampal structure underlie the observed behavioral change is still unknown and should be explored. Additionally, future studies should investigate whether maternal California mice also exhibit similar modifications to DG neuroplasticity. Taken together, these novel data increase our knowledge of paternal experience-induced plasticity and raise interesting questions about the mechanisms driving these observed effects.

Chapter 3: Offspring exposure reduces passive stress coping behaviors during forced swim and differentially alters hippocampal dendritic morphology in California mouse fathers

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Abstract

Individuals within monogamous species form bonds that appear to buffer against the negative effects of stress on physiology and behavior. In some species, involuntary termination of the mother/offspring bond results in increased symptoms of negative affect in the mother, suggesting that the parent/offspring bond may be equally as important as the pair bond. To our knowledge, the extent to which affect in paternal rodents is altered by involuntary termination of the father/offspring bond is currently unknown. Here, we investigated whether separation from offspring alters passive stress coping behaviors and dendritic morphology in hippocampal subfields of California mouse (*Peromyscus californicus*) fathers. Irrespective of paternal experience, separated mice displayed longer durations of immobility, compared to control (non-separated) mice. However, fathers separated from their mate and offspring showed shorter latencies to the initial bout of immobility and more bouts of immobility compared to control fathers. Separation from mate alone did not significantly alter latency to immobility or the bouts of immobility. In the dentate gyrus (DG) and area CA1 of the hippocampus, fatherhood was accompanied by increased dendritic spine density – an effect that was eliminated in the DG following separation from offspring. Non-separated fathers displayed reduced apical dendritic length and numbers of apical and basal branch points in area CA1, compared to separated fathers. Regardless of offspring presence, fatherhood was associated with reduced dendritic spine density in area CA3 of the hippocampus. Our data are the first to suggest that offspring exposure reduces passive stress coping behavior, while also contributing region-specific changes in hippocampal dendritic morphology in fathers of a biparental species.

Introduction

Bond formation is one of the strongest relationships observed between two individuals. In monogamous species, these bonds are typically formed after individuals spend significant periods of time in physical contact with one another (Lim and Young, 2006; McGraw and Young, 2010). Increased physical contact may serve as a social buffer (Kikusui et al., 2006), one that includes providing protection from the deleterious effects of stress on physiology and behavior (DeVries et al., 2003). For example, involuntary termination of strong social bonds can result in significant alterations to health, brain, and behavior (Norcross and Newman, 1999; Grippo et al., 2008; Lieberwirth et al., 2012; Sun et al., 2014). For many species, the parent/offspring bond is the first experience that has long-lasting effects on offspring development (Rilling and Young, 2014). Likewise, it is evident that interactions with offspring can alter both neuronal plasticity and brain function of parents (Jenkins et al., 2016). Much of this evidence results from observations of mother and offspring interactions, while the effects of father/offspring interactions on neuroplasticity within the paternal brain have received far less attention.

Prevailing evidence suggests that interactions with offspring can affect neuronal structure of the maternal brain in many regions, including the hippocampus and the prefrontal cortex. Dendritic spine density is increased in the dentate gyrus (DG; 12)and area CA1 of the hippocampus of maternal rodents. Similar enhancements in dendritic spine density are observed in the prefrontal cortex (Leuner and Gould, 2010a). However, maternal experience does not result in global enhancements in dendritic plasticity, as dendritic atrophy of area CA1 and CA3 of the hippocampus is observed following

parturition in rats (Pawluski and Galea, 2006). These effects of offspring on structural plasticity of the hippocampus may be driven by offspring contact alone, as virgin female rats exposed to pups, acutely or chronically, have enhanced neuroplasticity within the DG and subventricular zone (i.e., cell proliferation, cell survival; Furuta and Bridges, 2009), however, to our knowledge no studies have investigated whether pup exposure alters dendritic morphology in virgin females.

Similar to alterations in brain plasticity, emotionality in maternal rodents during the postpartum period can be impacted by interactions with offspring. In humans, mothers with increased positive feelings toward their baby had greater gray matter volume in areas of the brain associated with maternal motivation and behaviors (Kim et al., 2010). Breastfeeding mothers report lower anxiety (Groër, 2005), while disrupted maternal care is linked to increased risk for postpartum depression and anxiety (Machado et al., 2014; Murray et al., 2014). These studies suggest that mother/infant contact may prevent development of negative affect during the postpartum period and studies in rodents appear to support this hypothesis. Repeated separation of rat mothers from offspring leads to increased depressive-like (Boccia et al., 2007) and anxiety-like (Maniam and Morris, 2010) behaviors. Reduced anxiety-like behavior in maternal rats is dependent on recent offspring contact (Lonstein, 2005). Like alterations in structural morphology, exposure to pups alone can affect in virgin females. Nulliparous female rats exposed to pups for 21 days show reduced depressive-like behavior on the forced swim task (Pawluski et al., 2009). Taken together, the maternal literature suggests that the relationship between the mother and offspring is sufficient to permeate both the mother's behavior and neuronal morphology.

Due to the lack of mammalian models of paternal care, far less is known about how interactions with offspring alter the brain and behavior of fathers. Human fathers show enhanced brain activity in regions associated with parenting, like the ventral prefrontal cortex and amygdala, following exposure to infant cues (Swain, 2011). Additionally, striatal volume is negatively correlated with symptoms of depression in fathers 12-16 weeks postpartum (Kim et al., 2014). These findings in fathers, while few, suggest that brain regions associated with parenting undergo plasticity during the postpartum period and may be sensitive to disruptions in normal parental care - an effect that is similar to observations in mothers. Affect and structural plasticity of the brain in rodent and non-human primate models of paternal care mimic what has been seen in rodent mothers. In the California mouse father, a reduction in anxiety-like behavior and a maintenance of adult-born neurons is observed on postnatal day (PND) 16 (Hyer et al., 2016) - effects that may be driven by increased pup contact at this time (Bester-Meredith et al., 1999). Pup contact alone increases adult neurogenesis in the DG of male prairie voles *(Michrotus ochragaster;* Ruscio et al., 2008). Together, these data suggest that interaction with offspring influences both function and structure of the paternal brain.

Our goal was to enhance our knowledge of fatherhood-related neuroplasticity by determining to what extent early separation from offspring alters hippocampal dendritic morphology and affective behaviors during the forced swim test. We examined passive stress coping strategies, during the forced swim test, followed by Golgi-Cox analysis of dendritic morphology in hippocampal subfields (DG, CA1, and CA3) of California mouse males that were separated from their offspring on PND 1, compared to males that remained with their offspring until weaning. Given that California mouse fathers play key

roles in the physical and psychological development of their offspring (Gubernick et al., 1993; Bredy et al., 2007) and fatherhood alters emotional responsivity in this species (Lieberwirth et al., 2013; Glasper et al., 2016; Hyer et al., 2016), we hypothesized that separation from offspring would negatively alter the father's passive stress coping strategies during the forced swim test, as well as decrease dendritic plasticity across the hippocampus. Our data suggest that in California mouse fathers, pup exposure decreases passive coping strategies during the forced swim test and induces differential structural remodeling of the hippocampus. Taken together, these findings suggest that the father/offspring bond may be important for improving both affective behavior and structural plasticity in fathers of a biparental species.

Materials and Methods

Animals

Virgin male, gonadally intact virgin female, and tubally ligated virgin female California mice (60-90 days of age) were obtained from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC) or were descendants of these mice bred in our colony. Mice were provided ad libitum access to food and water and were housed on a 16:8 reversed light/dark cycle (lights off at 10:00h). Males were pair housed (non-fathers, 37.33 ± 3.21 days; fathers, 58.95 ± 32.37 days) with gonadally intact or tubally ligated females, allowed to mate, and remained undisturbed throughout the duration of the pregnancy until PND1 (where applicable). An average of 1.64 ± 0.58 offspring were in each litter. On PND1, half of the males were removed from their home cages and individually housed, resulting in two groups [separated non-father (n=10);

separated father (n = 11)]. The remaining males were not disturbed until behavioral testing, resulting in two additional groups [control non-father (n=8); control father (n=12)]. All experiments were approved by the University of Maryland Institutional Animal Care and Use Committee and conformed to the guidelines provided by the National Institutes of Health for the care and use of animals.

A small subset of the data from these experiments, DG and CA1 dendritic morphology from control non-fathers and fathers, have been presented in a smaller study (Glasper et al., 2016) that examined fatherhood-induced effects on dendritic morphology and anxiety-like behavior. Those data have been included in this article for adequate comparison to the separated groups. This is explicitly stated and referenced when these data are presented.

Forced Swim Task

On PND21 (time matched for non-fathers), all male mice were tested on the forced swim task, a common behavioral test used to assess active and passive stress coping behavioral strategies (de Kloet et al., 2016). The version of the task used here was modified to consist of a single session that is commonly used in mice, compared to 2 test sessions often used in rats (Petit-Demouliere et al., 2005; de Kloet et al., 2016). Testing began ~2hr after lights out and was performed under red light illumination. Males were placed in holding cages and transported to the behavioral room for testing. The forced swim test consisted of placing mice, for 5 minutes each, in a Plexiglas cylinder (30cm diameter, 43cm deep) that was filled ¾ of the way with 23-25°C tap water. Behavior was digitally recorded from a side view of the cylinder at 30 frames per second (Bogdanova et al., 2013), to better distinguish between swimming and immobility behaviors. Behavior

during the task was analyzed with EthoVision®XT 11 behavioral tracking software (Noldus, Leesburg, VA). The first 2 minutes of the task were used for habituation, while the final 3 minutes constituted the test portion of the task (Porsolt et al., 1977; Can et al., 2012). Thus, the following behaviors were assessed from the latter 3 minutes of the task and used to assess passive stress coping behavior: latency to the first bout of immobility, duration of immobility, and frequency of immobility bouts. Immobility was defined as remaining parallel to the surface of the water with only slight motions to remain afloat, while swimming was defined as continuous motion of paws and head. Use of automated detection systems typically reduces observer-related error. However, due to individual differences in behavioral patterns and minor changes in camera placement over the course of the experiment, slight modifications to the analysis parameters were made (Bogdanova et al., 2013). This resulted in different mobility settings for immobility when using EthoVision to analyze the behaviors. Floating behavior was set between 4% and 8% of pixel variation per 3 frames. The number of mice scored at each percentage of pixel variation are as follows: 4%, n = 1; 5%, n = 23, 7%, n = 1, 8%, n = 12. Flipping behavior during the forced swim task greatly increases the chance of drowning in California mice (unpublished observations); therefore, any mice that exhibited flipping behavior during the forced swim test were quickly removed and were not included in any analyses (n = 3). Following testing, mice were returned to their home cage and remained undisturbed until weaning of offspring on PND35. Total duration outside of the home cage was <15 minutes.

Golgi-impregnation

On PND35, mice were euthanized via cervical dislocation and brains were harvested, rinsed with dH_2O, and processed for Golgi impregnation per manufacturer's recommendations (Rapid Golgi Staining Kit, FD Neurotechnologies, Columbia, MD). Briefly, brains were submerged into equal parts of solutions A and B and stored in the dark at room temperature. Solution was refreshed 24hrs later. Fourteen days later, brains were transferred to solution C and stored in the dark at -4°C for 24hrs. Solution C was refreshed and brains were maintained under these conditions for 10 days. Tissue, of 100µm thickness, was sectioned in solution C using a vibrating microtome (Leica Microsystems, Chicago, IL). Sections were immediately mounted onto gelatinized slides and allowed to dry overnight. Slides were then placed into mailers and rinsed with dH_2O for 8min. Tissue was exposed to equal parts of solutions D and E for 10min, rinsed, and then dehydrated in increasing concentrations of ethanol (50%, 75%, 95%, and 100%), cleared in xylene, and coverslipped under Permount (Fisher Scientific, Fair Lawn, NJ).

Dendritic Remodeling Analysis

Dendritic remodeling analyses were performed, as previously described (Glasper et al., 2016). Granule cells within the DG, as well as pyramidal cells in areas CA1 and CA3 were analyzed for spine density (100x under oil immersion), and dendritic length and number of branch points (40x under oil immersion). Analyses were conducted using a Zeiss AxioImager microscope with a stage controller and neuroimaging software (Neurolucida, Williston, VT) by trained individuals with no knowledge of groups. Dendritic spine density measurements were taken from dendritic sections, with a mean of 10.24µm. These sections were on dendritic branches that were 2.9 ± 0.83 branches from the soma. For all analyses, dendrites were fully stained, relatively isolated, and

predominately in one focal plane. Five neurons per brain and five dendrites per neuron were analyzed. Neurons were sampled from the entire rostral-caudal extent of the hippocampus.

Statistics

Data were analyzed using GraphPad Prism version 6.0f for Mac OS X, (GraphPad Software, La Jolla California USA, www.graphpad.com). All data were checked for equality of group variances and were transformed, if necessary, to meet normality assumptions. All behavioral data were log transformed [Y = Log (Y)]. Two-way analysis of variance (ANOVA) was used to assess the effects of separation and paternal experience on behavioral and neuronal endpoints, followed by Sidak's multiple comparison tests, when appropriate. All mean differences were considered statistically different when the P-value was equal to or less than 0.05. For post hoc analyses, the multiplicity-adjusted P-value was reported for each comparison. Effect sizes were calculated for all neural plasticity analyses using Cohen's *d* and eta squared for ANOVA. Statistical outliers were determined using the ROUT method that identifies outliers from a non-linear regression, which resulted in the exclusion of a separated control and a separated father from all analyses. Given all exclusions, the final n sizes are as follows: control non-father, n=8; control father, n=12, separated non-father, n=6; separated father n=8.

Results

Behavior

Separation increased passive stress coping behavior during the forced swim task. A two-way ANOVA revealed a main effect of separation ($F_{(1, 30)} = 9.019$; $p = 0.005$), but no main effect of paternal experience ($p = 0.772$), or a significant interaction between separation and paternal experience ($p = 0.095$) on latency to immobility. Post-hoc analysis revealed that latency to immobility was significantly shorter in separated fathers, compared to control fathers ($p = 0.002$; Fig 1A). Control and separated non-fathers did not significantly differ in their latency to immobility ($p = 0.651$). A two-way ANOVA revealed a significant main effect of separation ($F_{(1,30)} = 6.435$; $p = 0.017$), but no main effect of paternal experience ($p = 0.620$) or an interaction between separation and paternal experience ($p = 0.919$) on the duration of immobility during the forced swim task . Regardless of paternal experience, separation resulted in a longer duration of immobility during the forced swim task (Fig 1B). A two-way ANOVA also revealed a main effect of separation ($F_{(1,30)} = 0.683$; $p = 0.006$), but no main effect of paternal experience ($p = 0.922$) or an interaction between separation and paternal experience ($p = 0.186$) on the bouts of immobility. Post-hoc analysis revealed that separated fathers performed more bouts of immobility, compared to control fathers ($p = 0.0128$; Fig 1C).

Dendritic Morphology

Dendritic Spine Density

Paternal experience and separation altered hippocampal dendritic spine density in a region-specific manner. A two-way ANOVA revealed a significant main effect of separation ($F_{(1, 30)} = 7.429$; $p = 0.011$; $d = 0.979$, $\eta^2 = 0.248$), but not a significant main effect of paternal experience ($p = 0.074$), or an interaction between paternal experience and housing ($p = 0.219$) on DG dendritic spine density. Post-hoc analysis revealed that

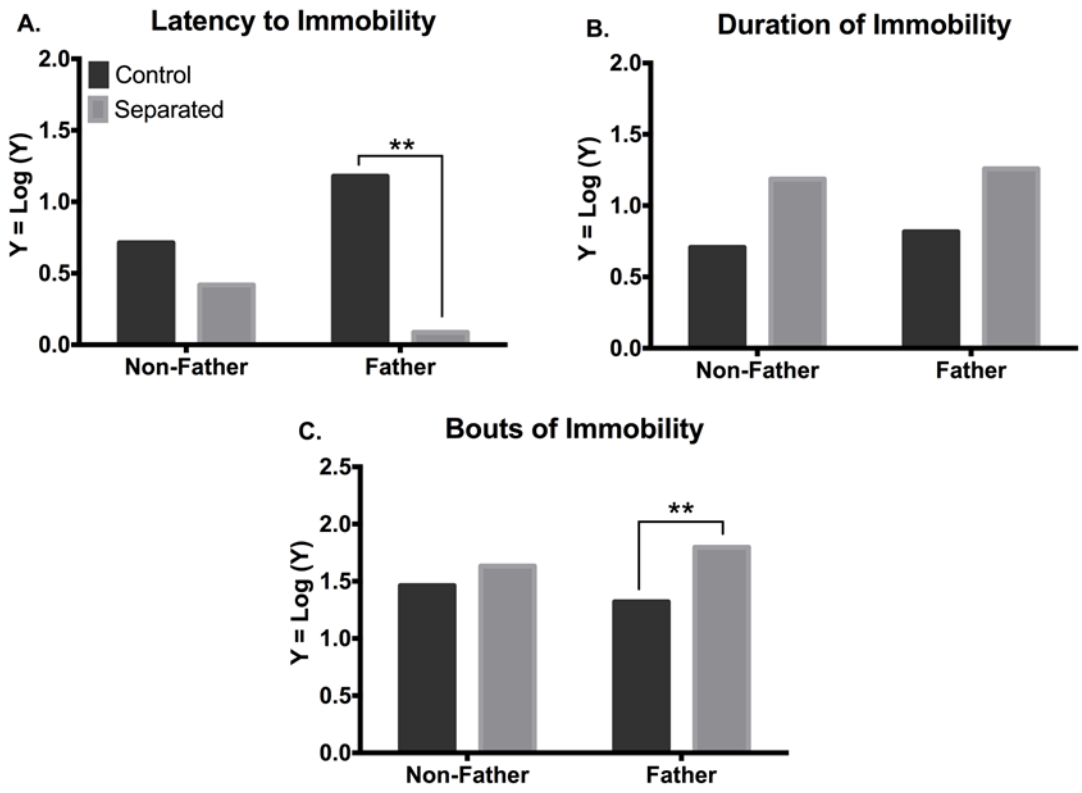

Fig 1. Separation increases passive stress coping behavior during the forced swim task in male California mice. (A) Twenty days of separation from mate and offspring significantly shortens latency to the initial bout of immobility during the forced swim task, compared to control fathers. (B) Regardless of paternal experience, 20 days of isolation housing increases the duration of immobility during the forced swim task. (C) Twenty days of separation from mate and offspring increases the number of immobility bouts during the forced swim task, compared to control fathers. **$p \leq 0.01$. Data presented as Mean ± SEM.

separation significantly reduced DG dendritic spine density in fathers, compared to control fathers ($p = 0.009$; Fig 2A; see Glasper et al., 2016). No main effect of separation ($p = 0.351$), paternal experience ($p = 0.322$), or an interaction between separation and paternal experience ($p = 0.236$) was observed on dendritic spine density of CA1 apical dendrites (Fig 2B; see Glasper et al., 2016). A two-way ANOVA revealed a significant main effect of paternal experience ($F_{(1, 30)} = 36.43$; $p < 0.0001$; $d = 2.168$, $\eta^2 = 1.215$), but no main effect of separation ($p = 0.506$) and no interaction between paternal experience and separation ($p = 0.328$) on CA1 basal dendritic spine density. Post-hoc analysis revealed that paternal experience increased dendritic spine density on CA1 basal

pyramidal cells among control ($p = 0.001$) and separated ($p = 0.0001$) groups (Fig 2C; see Glasper et al., 2016). A two-way ANOVA revealed a main effect of paternal experience ($F_{(1, 30)} = 4.204$; $p < 0.05$; $d = 9.69$, $\eta^2 = 0.140$), but no main effect of separation ($p = 0.905$) or an interaction between paternal experience and separation ($p = 0.622$) on dendritic spine density on CA3 apical pyramidal cells dendrites of area CA3 of the hippocampus. Paternal experience decreased dendritic spine density on CA3 apical pyramidal cells regardless of separation (Fig 2D). No main effect of separation ($p = 0.294$), paternal experience ($p = 0.190$), or an interaction between separation and paternal experience ($p = 0.183$) was observed on spine density along CA3 basal pyramidal cell dendrites (Fig 2E).

Dendritic Tree Length and Branching

Separation and paternal experience alter CA1, but not DG or CA3, dendritic tree complexity (Table 1, Fig 3). *DG*: Two-way ANOVA did not reveal a main effect of separation ($p = 0.474$), paternal experience ($p = 0.778$), or an interaction between separation and paternal experience ($p = 0.197$) on the length of DG granule cell dendritic trees. Additionally, two-way ANOVA did not reveal a main effect of separation ($p = 0.575$), paternal experience ($p = 0.434$), or an interaction between separation and paternal experience ($p > 0.999$) on DG granule cell branching. *CA1*: Two way ANOVA revealed a main effect of separation ($F_{(1, 30)} = 11.93$; $p = 0.002$; $d = 1.241$, $\eta^2 = 0.398$), a main effect of paternal experience ($F_{(1, 30)} = 5.544$; $p = 0.025$; $d = 0.846$, $\eta^2 = 0.185$) (Glasper et al., 2016), but no interaction between separation and paternal experience ($p = 0.394$) on the length of the apical dendritic tree of CA1 pyramidal cells. Post-hoc analysis revealed that separated fathers had significantly longer apical dendritic trees than control fathers

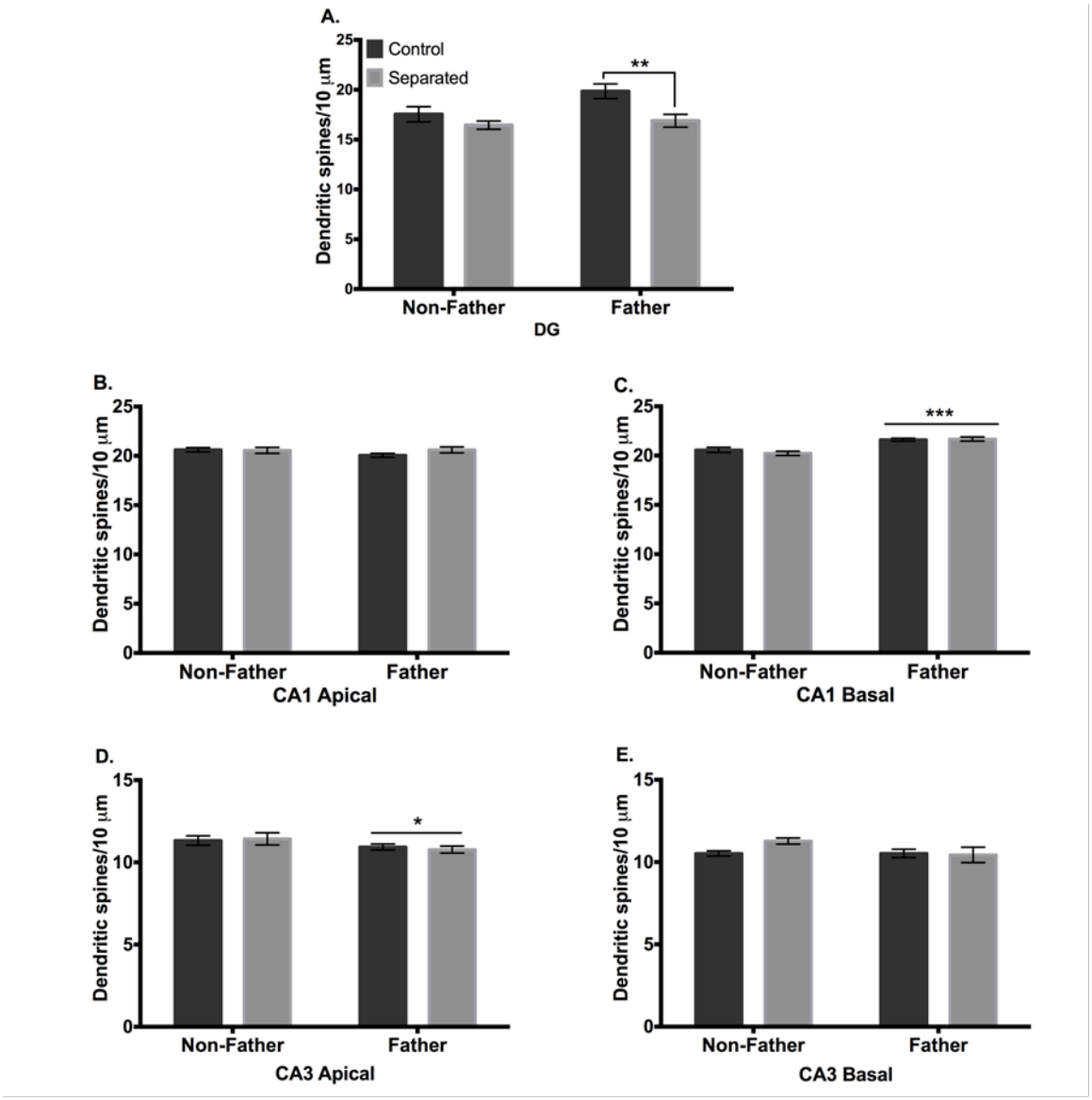

Fig 2. Paternal experience and separation differentially alter dendritic spine density in hippocampal subfields of California mouse fathers. (A) Separation from mate and offspring on postnatal day 1 decreases dendritic spine density of dentate gyrus (DG) granule cells, compared to control fathers. Separation from mate alone does not alter DG dendritic spine density. (B) Neither paternal experience nor separation alters dendritic spine density of CA1 apical pyramidal cells. (C) Paternal experience increases dendritic spine density on CA1 basal, but decreases CA3 apical pyramidal cells (D). (E) Neither paternal experience nor separation alters dendritic spine density of CA3 basal pyramidal cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data presented as Mean ± SEM.

($p=0.004$). Likewise, two-way ANOVA revealed a main effect of separation ($F_{(1, 30)} = 16.64$; $p = 0.000$; $d = 1.465$, $\eta^2 = 0.555$), a main effect of paternal experience ($F_{(1, 30)} = 6.992$; $p = 0.013$; $d = 0.95$, $\eta^2 = 0.233$) (Glasper et al., 2016), but no interaction between

separation and paternal experience (p = 0.393) on the number of branch points on the apical dendritic tree of CA1 pyramidal cells. Post-hoc analysis revealed that separated fathers had significantly more branch points along apical dendrites than control fathers (p=0.001). Two-way ANOVA revealed a main effect of separation ($F_{(1, 30)} = 6.914$, p = 0.013, d = 1.881, $\eta^2 = 0.230$), but not paternal experience (p = 0.722) or an interaction between separation and paternal experience (p = 0.982) on the length of the basal dendritic tree of CA1 pyramidal cells. Regardless of fatherhood, separation resulted in a longer dendritic tree of basal dendrites in area CA1. Two-way ANOVA revealed a significant main effect of separation ($F_{(1, 30)} = 7.876$; p = 0.009; d = 1.008, $\eta^2 = 0.262$), but no main effect of paternal experience (p = 0.292), or an interaction between separation and paternal experience (p = 0.337) on the number of branch points on the basal dendritic tree of CA1 pyramidal cells. Post-hoc analysis indicated that separated fathers had more branch points along the basal dendritic tree compared to non-separated fathers (p = 0.013). *CA3:* Two-way ANOVA did not reveal a main effect of separation (p = 0.646), paternal experience (p = 0.689), or an interaction between separation and paternal experience (p = 0.556) on the length of the apical dendritic tree of CA3 pyramidal cells. Additionally, two-way ANOVA did not reveal a main effect of separation (p = 0.328), paternal experience (p = 0.941), or an interaction between separation and paternal experience (p = 0.791) on the number of branch points on the apical dendritic tree of CA3 pyramidal cells. Two-way ANOVA did not reveal a main effect of separation (p = 0.238), paternal experience (p = 0.385), or an interaction between separation and paternal experience (p = 0.685) on the length of the basal dendritic tree of CA3 pyramidal cells. Additionally, two-way ANOVA did not reveal a significant main effect of separation (p =

0.098), paternal experience (p = 0.194), or an interaction between separation and paternal experience (p = 0.573) on the number of branch points on the basal dendritic tree of CA3 pyramidal cells.

Table 1. Separation and paternal experience alter dendritic tree complexity in CA1, but not DG or CA3, of the hippocampus in California mice.

Neural Measures		**Non-Fathers**		**Fathers**	
		Control	**Separated**	**Control**	**Separated**
Length (µm)	DG	802.7 ± 65.79	771.10 ± 50.93	747.64 ± 32.37	856.41 ± 62.73
	CA1 Apical	841.3 ± 51.08	1004.89 ± 48.94	637.98 ± 55.78	910.85 ± 76.62
	CA1 Basal	697.54 ± 50.80	892.8 ± 65.03	672.82 ± 72.33	864.68 ± 77.72
	CA3 Apical	815.68 ± 65.60	755.06 ± 30.29	758.45 ± 52.65	765.96 ± 52.09
	CA3 Basal	939.26 ± 94.52	870.56 ± 65.94	1050.86 ± 72.22	911.38 ± 92.28
Branch Points	DG	5.50 ± 0.41	5.33 ± 0.34	5.27 ± 0.19	5.10 ± 0.25
	CA1 Apical	8.18 ± 0.28	10.13 ± 0.51	6.03 ± 0.66	9.05 ± 0.60
	CA1 Basal	7.20 ± 0.64	8.47 ± 0.81	5.78 ± 0.66	8.40 ± 0.50
	CA3 Apical	6.63 ± 0.62	7.33 ± 0.45	6.82 ± 0.44	7.22 ± 0.64
	CA3 Basal	7.75 ± 0.75	6.10 ± 0.68	8.30 ± 0.53	7.48 ± 0.88

CA1 apical length was significantly shorter in control fathers, compared to separated fathers. Separation, regardless of paternal experience, resulted in increased basal dendritic tree length in CA1. Control fathers had significantly fewer branch points on

both apical and basal dendritic trees, compared to separated fathers. Values represent Mean ± SEM. Bold text indicates main effects, while shaded cells represent significant group differences.

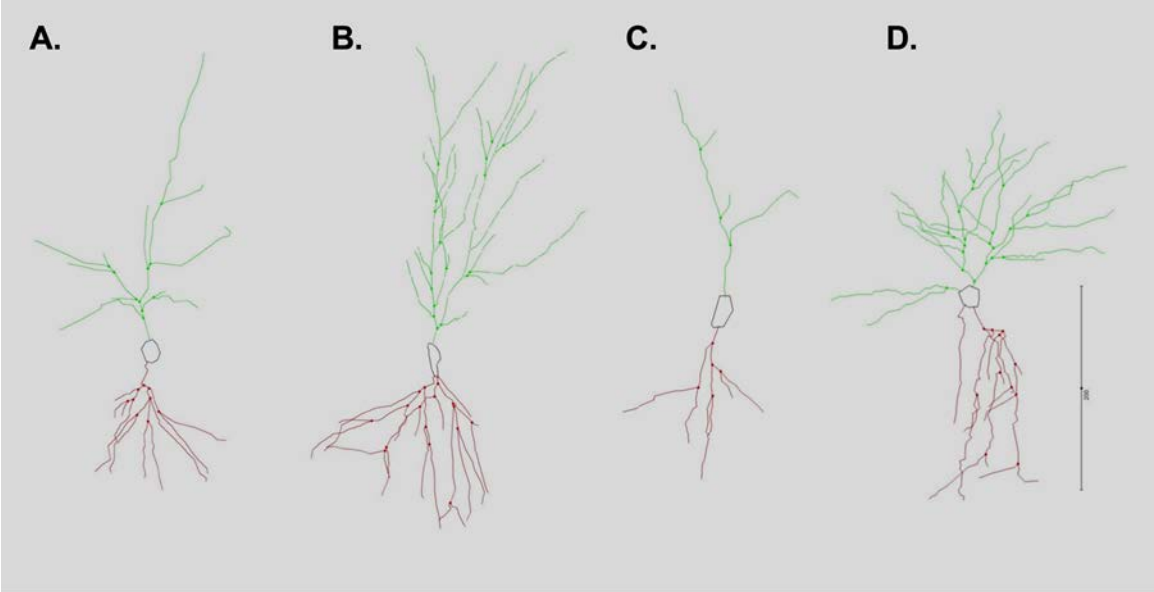

Fig 3. Microlucida tracings of Golgi-impregnated CA1 pyramidal cells. Representative tracings of a representative (A) control non-father, (B) control father, (C) separated non-father, and (D) separated father. Scale bar = 200 μm.

Discussion

The biparental care strategy in the California mouse is necessary for offspring survival and development. In the wild, when the California mouse father is removed from the nest and mothers are forced to forage under challenging environmental conditions, offspring survival sharply declines, compared to offspring that are reared with their fathers under similar conditions (Gubernick et al., 1993). Offspring raised with their father have higher body temperatures (Dudley, 1974) and are more social with siblings (Vieira and Brown, 2003), compared to offspring reared without their fathers. Thus, the presence of the father significantly improves offspring survival, physiology, and behavior. Given this significant parental role, it is reasonable to assume that a strong relationship exists between the father and offspring and that perturbations to this relationship may results in

alterations to neural structures and brain function, specifically in areas of the brain that are modified by paternal experience.

While quality and quantity of paternal care in biparental species significantly impacts offspring behavior and structural plasticity (Bredy et al., 2004, 2007; Jia et al., 2009; Pinkernelle et al., 2009), to our knowledge, this is the first study to demonstrate that preventing paternal males from caring for their offspring can negatively impact the father's brain and behavior. We report that 20 days of isolation housing increases passive stress-coping behaviors in the forced swim task in male California mice. Separation from offspring exacerbates this effect, as latency to the first bout of immobility, a marker for passive stress coping, was significantly shorter among separated California mouse fathers, while separation from a mate did not yield a significant difference in non-fathers. Additionally, bouts of immobility were increased following separation, with paternal experience exaggerating this effect. Overall, separation effects were observed in the duration of immobility among California mouse males. These behavioral data suggest that separation has profound, long-lasting effects on California mouse males and the combination of separation and paternal experience can exacerbate passive stress coping behaviors above what is observed following mate separation alone.

In addition to increased passive stress coping following separation, we observed region-specific changes to the hippocampus as a result of parental experience and separation. In the DG, fathers show an increase in spine density along granule cell dendrites at weaning (PND35). If fathers were removed from offspring, and isolate housed for 34 days, the effects of paternal experience on DG dendritic spine density are no longer observed. In the CA1 region, fatherhood alone enhanced spine density along

basal dendrites of pyramidal cells, regardless of housing condition. We also observed an effect of fatherhood on CA3 pyramidal neurons. Specifically, fatherhood decreased dendritic spine density on apical, but not basal, dendrites of pyramidal cells in area CA3 in both separated and control fathers. Along with these changes in spine density, we observed that separation and paternal experience influenced dendritic tree complexity in area CA1, but not in the DG or area CA3. Fathers that remained with their offspring had shorter apical dendritic trees and fewer branch points along both apical and basal trees of CA1 pyramidal cells. Isolation housing increased the length of the basal dendritic tree along pyramidal cells in area CA1, regardless of paternal experience. Taken together, these changes in dendritic morphology indicate that fatherhood alone can differentially alter hippocampal structural morphology while also adding to the body of literature on isolation and/or separation-induced effects on neuromorphology.

Unlike other biparental species that require pregnancy to form pair bonds (Williams et al., 1992; Resendez et al., 2012), California mice form pair bonds following sexual intercourse alone (Ribble and Salvioni, 1990; Gubernick and Nordby, 1993). Given this, in order to assess the effects of offspring separation on affective behavior and dendritic morphology, we compared males that were removed from their offspring and mate to males that were separated from their mate alone. Any differences observed between these two groups could then be attributed to paternal experience and not simply isolation, as isolation has been shown to enhance depressive-like behavior (Grippo et al., 2008), impair immune function (Glasper and DeVries, 2005; Martin et al., 2006), and decrease dendritic spine density (Silva-Gómez et al., 2003). We show that isolation resulted in fathers floating sooner and more frequently during the forced swim task,

compared to fathers that remained with their offspring and mate. It is important to note that separation, whether from mate or mate and offspring, increased floating duration in the forced swim task in California mouse males. These data are the first to make two important observations in this species. First, limiting interactions with offspring results in increased passive stress coping behaviors in fathers. This is similar to observations in maternal rodents, as disruption to mother/offspring interactions leads to emotional dysregulation and negative affect (Liu et al., 1997; Maniam and Morris, 2010). While not assessed in this study, it is possible that offspring contact may moderate hypothalamic-pituitary-adrenal axis activity. In virgin male prairie voles, licking and grooming of pups is associated with reductions in corticosterone concentrations following swim stress, suggesting that interactions with offspring may reduce the stress response (Bales et al., 2006). Secondly, we observe that separation from a mate results in an impaired coping response during the forced swim test. There is a rich body of literature highlighting the relationship between bond disruption and passive stress coping behavior (Grippo et al., 2008; Bosch et al., 2009; Lieberwirth et al., 2012). For example, in the socially monogamous prairie vole (*Microtus ochragaster*), both short and long separations from a bonded mate result in extended durations of immobility during the forced swim task in both males (5 days; 51) and females (6 weeks; 7). Additional effects of separation are observed in other highly social, monogamous species. Male, Siberian dwarf hamsters (*Phodopus sungorus*) are more inactive, have increased body, seminal vesicle, and testicular mass, and display heightened cortisol concentrations following four weeks of separation from their bonded female (Castro and Matt, 1997). Following 4-weeks of bond separation, both male and female prairie voles show reduced sucrose intake (a measure of

negative affect) (Grippo et al., 2007) and females show increased immobility in the forced swim task, decreased time spent on the open arms of the elevated plus maze, and increased pup-directed attack behavior (Grippo et al., 2008).

The hippocampus is sensitive to parental experience (reviewed in 54) and is structurally modified by motherhood in region-specific ways. Specifically, both the DG (Leuner and Gould, 2010a) and area CA1 (apical and basal; 54, 56) show enhanced dendritic spine density during the postpartum period in rat dams. Not surprisingly, in a species that exhibits paternal care, we observe similar findings in our paternal males, where increased dendritic spine density was observed in the DG and along CA1 basal pyramidal cell dendrites (Glasper et al., 2016). While much attention has not been given to paternal experience-induced modifications to dendritic architecture, we acknowledge that this effect is not limited to subregions of the hippocampus. First-time and experienced marmoset fathers have enhanced dendritic spine density in prefrontal cortex pyramidal neurons (Kozorovitskiy et al., 2006) - an effect that is also observed in maternal rats (Leuner and Gould, 2010a).

We demonstrate, for the first time, that separation from offspring reduces dendritic spine density in the DG of fathers, compared to fathers that remain with their offspring. This suggests that siring a litter is not sufficient for enhanced DG spine density and that persistent offspring exposure may be necessary for maintaining enhanced DG dendritic spine density that accompanies fatherhood in the California mouse. This effect of separation on dendritic spine density in the hippocampus is region-specific, as both control and separated fathers exhibited increased dendritic spine density along basal CA1 dendrites. This finding suggests that separation alone is not sufficient to eliminate a

fatherhood-induced increase in dendritic spine density in area CA1 of the hippocampus and reveals the possible long-lasting consequence of, even minimal, paternal experience on specific hippocampal excitatory synapses. To our knowledge, no studies have investigated the effects of maternal separation on hippocampal spine density in rat dams, however, periodic separation from offspring decreases cell proliferation in the DG of rat dams (Sung et al., 2010), suggesting that hippocampal structural plasticity is also sensitive to disruption of dam/offspring interactions. Future studies should investigate whether early weaning of offspring impairs dendritic architecture within the hippocampus of maternal rodents.

Alterations to CA1 and CA3 dendritic architecture are observed during the postpartum period of maternal rodents, however, to date, changes in dendritic length and branching within the DG have not been reported. Primiparous maternal rats show dendritic atrophy on both apical and basal CA1 and CA3 pyramidal neurons 24-31 days postpartum (Pawluski and Galea, 2006). We observed a similar effect of parenthood-induced atrophy to CA1 apical length and apical and basal branch points in control California mouse fathers, compared to control non-fathers (Glasper et al., 2016). Interestingly, in the current study, separated fathers exhibited CA1 lengths and numbers of branch points similar to separated non-fathers, suggesting that siring offspring, without maintaining offspring interaction, is not sufficient to prevent separation-induced dendritic atrophy in the CA1 region of the hippocampus.

Despite a lack of evidence for maternal-induced alterations to dendritic morphology in the DG, other aspects of DG plasticity in this region have been shown to be temporally altered in both mothers and fathers. Adult neurogenesis is impaired in

female rats in the early and mid-postpartum period (Leuner et al., 2007; Pawluski and Galea, 2007) yet returns to baseline at weaning (11). In California mouse fathers, adult neurogenesis is maintained from PND 9-16 (Hyer et al., 2016), however, at weaning, both California mouse fathers and mothers show a suppression of adult neurogenesis (Glasper et al., 2011). Our current findings suggest that dendritic morphology in the DG is not altered at weaning. However, evidence from the literature suggests that parental-induced changes to DG plasticity may not be long-lasting and thus a more thorough examination of dendritic morphology in this region over the entire postpartum period may be necessary.

The CA3 region of the hippocampus is highly susceptible to stress (Watanabe et al., 1992; Magarinos and McEwen, 1995; McEwen et al., 1997), and chronic stress, or repeated injections of exogenous stress hormones, decreases dendritic complexity within this region in male rats (Gould et al., 1990; Woolley et al., 1990a; Watanabe et al., 1992; Magarinos and McEwen, 1995; Galea et al., 1997). We observed reduced dendritic spine density on CA3 apical dendrites, but no changes in CA3 dendritic complexity, irrespective of separation. Rat dams demonstrate atrophy of CA3 apical and basal dendritic trees during the postpartum period, but dendritic spine density is unaltered (Pawluski and Galea, 2006). On the other hand, unlike males, treating postpartum female rats with corticosterone results in increased dendritic complexity in the CA3 region – specifically, increased density of mushroom spines (Workman et al., 2013). Sex differences in CA3 dendritic plasticity may be a direct result of sex-specific steroid hormones (Galea et al., 1997). Currently, the mechanisms underlying sex differences in parenting-induced CA3 dendritic plasticity are unknown. It is possible that alterations in

stress responsivity following offspring interaction may be sex-specific. Taken together, our findings in the CA3 region, combined with evidence from the literature, suggest that this region is highly responsive to external input and that the directionality of these effects can be mixed.

Conclusion

The formation and maintenance of bonds are essential for highly social species. Our data suggest that forced dissolution of the pair bond induces passive stress coping behaviors and contributes to region-specific alterations in hippocampal structure in California mouse males. Permanent disruption of the interactions between California mouse fathers and their offspring may further contribute to these changes that accompany pair bond dissolution. Furthermore, our data suggest that, while some changes to hippocampal structural plasticity are sensitive to fatherhood, minimal interactions with offspring may not be sufficient to maintain these changes that persist with continuous offspring exposure. To our knowledge this is the first study to investigate to what extent limiting father/offspring care, in a biparental species, alters behavioral and neural endpoints in the father. Offspring interaction is important for emotional health during the postpartum period of males, a finding that has also been observed in postpartum females (Ramchandani and Psychogiou, 2009). The expression of postpartum depression and anxiety in fathers can negatively impact the father/child relationship, suggesting that father/offspring interactions are important for the health of the father and can have significant effects on development of the child (Bögels et al., 2008; Möller et al., 2016). It will be important to identifying mechanisms underlying both the neural and behavioral alterations observed during the postpartum period in males and how they may be similar

or different than postpartum females. This will ultimately contribute to our understanding of parenting-induced neuroplasticity.

Chapter 4: Neurogenesis and anxiety-like behavior in male California mice during the mate's postpartum period

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Running title: Anxiety and adult neurogenesis are altered by fatherhood

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Abstract

Our understanding of postpartum anxiety (PPA) in fathers is limited, despite the negative consequences of anxiety on the father and child. Offspring contact reduces PPA in mothers; however, parallel investigations in fathers has gone unaddressed. Adult neurogenesis in the dentate gyrus (DG) contributes to anxiety regulation and is altered during the postpartum period, yet the effects of fatherhood on the production, or survival, of newborn cells in the DG, and the role of adult neurogenesis in PPA regulation, have not been examined. Using the biparental California mouse (*Peromyscus californicus*), we examined the relationships among postnatal day, anxiety-like behavior and adult neurogenesis in fathers. We hypothesized that attenuated anxiety-like behavior and enhanced adult neurogenesis would be observed when father–offspring contact was increased. We observed a reduction in anxiety-like behavior on the elevated plus-maze, but only at PND 16, a time of peak pup retrieval. Fatherhood reduced 1-week survival of newborn cells; however, surviving cells were maintained until 2 weeks postpartum. In contrast, non-fathers experienced a significant reduction in the survival of newborn cells between 1 and 2 weeks postpartum. Fatherhood also increased the numbers of newborn cells that expressed a neuronal phenotype. Collectively, these findings suggest that offspring interaction contributes to reductions in anxiety-like behavior and the maintenance of newborn neurons in the DG of fathers. These data contribute to our knowledge of the postpartum affective state in fathers, findings that may contribute to improved health of both the father and offspring.

Introduction

The maternal postpartum period is accompanied by increased emotional resilience (for review, see Macbeth and Luine, 2010), yet postpartum anxiety (PPA) occurs frequently (5–12%; reviewed in Lonstein, 2007; Barrett and Fleming, 2011) and is influenced by a number of factors (reviewed in Agrati and Lonstein, 2015), including the bond between mother and offspring (Maniam and Morris, 2010; Sung et al., 2010). In rodents, females exhibit reduced anxiety-like behavior throughout the postpartum period (reviewed in Macbeth and Luine, 2010) that is dependent on recent offspring contact but is not linked to lactation (Lonstein, 2005; Maniam and Morris, 2010).

While motherhood-induced anxiety has been afforded attention, little is known about anxiety regulation in fathers, likely due to the limited research on paternal human males (reviewed in Ramchandani and Psychogiou, 2009) and the low occurrence of paternal care in other mammals (~ 6%; Kleiman and Malcolm, 1971). While detrimental to fathers, PPA can have negative impacts on offspring as well (reviewed in Ramchandani and Psychogiou, 2009), making it necessary to identify underlying mechanisms contributing to postpartum emotional states in fathers. Pioneering research suggests that anxiety-like behavior is increased (Lieberwirth et al., 2013) or unaltered (Glasper et al., 2011; Chauke et al., 2012) in fathers. Given differences in species used and times at which behavior was assessed, no consensus can be drawn about fatherhood-induced anxiety regulation at present.

The California mouse (*Peromyscus californicus*) exhibits extensive paternal care (Gubernick and Alberts, 1987) that has been well-characterized (Bester-Meredith et al., 1999), providing a model with which to explore fatherhood-induced changes in emotional

regulation. We recently observed a decrease in PPA-like behavior on postnatal day (PND) 19 in California mouse males (Glasper et al., 2016), a time of reported peak retrievals by fathers of straying pups (Bester-Meredith et al., 1999). However, the extent to which altered anxiety-like behavior is specific to this time during the postpartum period, and to what extent anxiety-like behavior is associated with structural changes within the brain, remains unknown.

Anxiety-like behavior is mediated by the dentate gyrus (DG) of the hippocampus (Kheirbek et al., 2013), a brain region that exhibits extensive adult neurogenesis (reviewed in Leuner and Gould, 2010b) and is altered during the postpartum period of both males and females(Leuner et al., 2007; Pawluski and Galea, 2007; Glasper et al., 2011; Lieberwirth et al., 2013). Previous work suggests that increased adult neurogenesis may contribute to decreased anxiety-like behavior (Revest et al., 2009); however, the extent to which adult neurogenesis is associated with anxiety-like behavior during the postpartum period is unknown. Prior research in paternal rodents has also examined cell survival in the amygdala (Lieberwirth et al., 2013), given the role that the basolateral amygdala plays in anxiety-like behavior (Tye et al., 2011). Given our lack of knowledge surrounding the relationship between anxiety-like behavior and adult neurogenesis in fathers, we hypothesized that reduced anxiety-like behavior would be associated with an increase in adult neurogenesis in the hippocampus, but not in the basolateral amygdala, of paternal males, specifically during a time of peak pup retrieval.

Materials and methods

Animals

Gonadally intact virgin male and female California mice, bred in our colony, were descendants of mice obtained from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Tubally ligated female California mice were obtained from the Peromyscus Genetic Stock Center. All mice were 60–90 days of age at the start of experimentation. Mice were provided with ad libitum access to food and water and were housed on a 16 : 8 reversed light : dark cycle (lights off at 10:00 h). All experiments were approved by the University of Maryland Institutional Animal Care and Use Committee and conformed to the guidelines provided by the National Institutes of Health for the care and use of animals.

Experimental design

Gonadally intact males were paired with either tubally ligated females or gonadally intact females to form two experimental groups: non-fathers and fathers. Non-fathers cohabited for an average of 42.6 days while fathers cohabitated for an average of 48.8 days, which resulted in an average of 2.09 births. Non-fathers (time-matched) and fathers received intraperitoneal injections of the DNA synthesis marker bromodeoxyuridine (BrdU; 200 mg/kg) on PND 2 to determine the extent to which adult neurogenesis was altered throughout the postpartum period. One-week cell survival was assessed on PND 9 (non-fathers, n = 5; fathers, n = 7) and 2-week cell survival was assessed on PND 16 (non-fathers, n = 4; fathers, n = 6). Previous work has shown that maternal rats experience suppressed cell proliferation shortly postpartum (Leuner et al., 2007). Using a separate cohort of mice, we investigated the extent to which cell

proliferation was altered during the postpartum period in fathers. Non-fathers (time-matched; n = 5) and fathers were injected with BrdU on PND 2 (n = 6), PND 9 (n = 4) or PND 16 (n = 5) and perfused 2 h later.

Elevated plus-maze (EPM) task

Assessment of anxiety-like behavior was performed in the same mice used to assess cell survival (see above) on PND 2, PND 9 or PND 16. Mice were removed from their home cages and placed in a holding cage for transportation to an adjacent behavioral room. After a 10 min acclimation, mice were tested on the EPM. The maze stood 75 cm above the floor with arms measuring 11.5 x 55 x 45 cm tall. Mice were placed in the center of the maze, facing an open arm, and observed for 5 min. Behavior was digitally recorded and analyzed with EthoVision XT 11 behavioral tracking software (Noldus, Leesburg, VA, USA). Recordings were taken from a top-down view at a rate of 30 frames per second. Latency to enter the arms, duration of time spent in the arms, and number of arm entries were assessed (Walf and Frye, 2007; Chauke et al., 2012). Duration of time spent in the open arm was calculated as a percentage of time spent in the open arm divided by the total time spent in both arms, excluding the center, multiplied by 100. Mice were returned to their holding cages immediately following the conclusion of testing where they remained for 60 min. Mice that fell off of the maze (14/31) were quickly returned to the center of the maze and allowed to continue exploring the maze until a total duration of 5 min had elapsed. Mice that froze for > 40% of the time (Chauke et al., 2012), or fell off of the maze more than twice, were excluded from the study. This resulted in the exclusion of one PND 16 non-father, one PND 2 father and three PND 16 fathers. Final N sizes were as follows: non-fathers (collapsed across all PNDs; see

Results), n = 14; PND 2 fathers, n = 4; PND 9 fathers, n = 7; and PND 16 fathers, n = 6. Mice were perfused 60 min following the completion of behavioral testing.

Immunohistochemistry

Mice were anesthetized using a ketamine–xylazine cocktail and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0. Brains were dissected from the skull and post-fixed for at least 48 h at 4 °C. Coronal sections (40 lm) were cut throughout the entire rostrocaudal extent of the DG on a vibrating microtome (Leica Microsystems, Chicago, IL, USA) into a bath of 0.1 M phosphate-buffered saline (PBS), pH 7.5. Sections containing the DG and basolateral amygdala were identified using the mouse brain atlas (Franklin and Paxinos, 2007).

Immunoperoxidase staining for BrdU

For BrdU peroxidase staining, a 1 : 8 series of sections were mounted onto glass Super Frost Plus slides (Fisher Scientific, Pittsburgh, PA, USA), dried, and pretreated by heating in 0.1 M citric acid, pH 6.0. Tissue was rinsed with PBS, incubated in trypsin for 10 min, denatured in 2 M HCL : PBS for 30 min, rinsed with PBS, incubated overnight in purified mouse anti-BrdU (1 : 200; BD Biosciences, San Jose, CA, USA; cat. no. 347580), incubated in biotinylated horse anti-mouse (1 : 200; Vector, Burlingame, CA, USA; cat. no. BA-2000) for 60 min, rinsed in PBS, incubated with avidin–biotin complex (Vector), rinsed with PBS, and then reacted in 0.01% diaminobenzadine with 0.003% H2O2. All slides were counterstained with cresyl violet, dehydrated, cleared with Citrisolv (Fisher Scientific), and coverslipped under Permount (Fisher Scientific). BrdU labeling was not apparent in one PND 2 father, one PND 9 father and one PND 16 father, resulting in the exclusion of these mice from all analyses.

BrdU and neuron-specific class III beta-tubulin (TuJ-1) immunofluorescence

Double labeling with immunofluorescence for BrdU and TuJ-1 was conducted to determine the percentage of cells that expressed a neuronal phenotype. TuJ-1 labels immature neurons but not glial cells (Biolegend, San Diego, CA, USA; Jepsen et al., 2000). Immunofluorescence protocols were adapted from Glasper et al. (2011). Freefloating sections were rinsed in 0.1 M tris-buffered saline (TBS), pH 7.5, denatured in 2 M HCl : TBS for 30 min, rinsed and incubated in rat anti-BrdU (1 : 200; Accurate Chemical, Westbury, NY, USA; cat. no. OBT0030G) and Alexa Fluor 488 mouse anti-tubulin b 3 (1 : 500; BioLegend; cat. no. A488-435L), overnight, at 4 °C. The next day, sections were rinsed with TBS, incubated with biotinylated goat anti-rat (1 : 250; EMD Millipore, Billerica, MA, USA; cat. no. AP183B) for 90 min, rinsed again, and incubated for 30 min in the dark with streptavidin-conjugated Alexa 568 (1 : 1000; Molecular Probes, Eugene, OR, USA; cat. no. S11226). Finally, sections were rinsed, mounted onto glass slides, dried, and coverslipped using glycerol in TBS (3 : 1).

Data analysis

Quantitative analysis was conducted on coded slides. The number of BrdU-labeled cells on every eighth unilateral section throughout the entire rostrocaudal extent of the DG (granule layer, subgranular zone and hilus) and basolateral amygdala were counted at 100 9 magnification under oil immersion on a Zeiss Primo Star light microscope (Zeiss, Thornwood, NY, USA) by using a modified version of the optical fractionator method (West et al., 1991; Ngwenya et al., 2005). The simplified formula for the estimated total number of labeled cells was: N Σ Q 9 (1/ssf), which is the total

number of labeled cells counted (N Σ Q) multiplied by the reciprocal of the section sampling fraction (1/ssf or 1/8) (Leuner et al., 2009).

Brightfield photomicrographs were taken with a Zeiss AxioImager microscope with a stage controller attached to a computer with Microlucida Imaging Software (Williston, VT, USA). Images were cropped and optimized by adjusting brightness and color balance in Microsoft PowerPoint 2010.

Immunofluorescence analysis was conducted at 40 9 oil immersion using a Zeiss AxioImager microscope with a stage controller and neuroimaging software (Neurolucida, Williston, VT, USA). To determine cell fate, the percentage of BrdU+ cells that were also TuJ-1+ were counted in 25 randomly selected cells within the granule cell layer and subgranular zone of the DG. To verify double labeling throughout their extent, images of these cells were captured using epifluorescent filters for red (BrdU) and green (TuJ-1), pseudo-colored, and then merged to identify double-labeled cells (yellow in the merged images).

Statistics

Data were analyzed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). One-way or two-way ANOVA was used to assess the effects of fatherhood and PND on behavioral and neuronal endpoints, followed by Holmes–Sidak multiple comparisons test when appropriate. Pearson's correlation was used to determine the relationship between EPM performance and adult neurogenesis. All comparisons used a two-tailed test and mean differences were considered statistically

different when the P-value was ≤ 0.05. For post hoc analyses, the multiplicity- adjusted P-value was reported for each comparison.

Results

Enhanced anxiety regulation was observed 16 days into the postpartum period of fathers

At PND 16, fathers showed significant reductions in anxiety-like behavior. No significant difference was observed in the percent time spent on the open arms of the EPM among non-fathers at any time-matched PND (P > 0.05); therefore, non-fathers were collapsed into one group for this analysis. One-way ANOVA revealed a significant main effect in the percent time spent in the open arms of the EPM ($F_{3,27}$ = 3.82; P = 0.02). Post hoc analysis revealed that, on PND 16, fathers spent significantly more time on the open arms of the EPM than did non-fathers (P = 0.01; Fig. 1). PND 2 and PND 9 fathers did not significantly differ from non-fathers or PND 16 fathers (P > 0.05 for each comparison). Neither the number of arm entries nor the latency to enter either arm of the EPM was different among non-fathers and fathers at the three PNDs (P > 0.05).

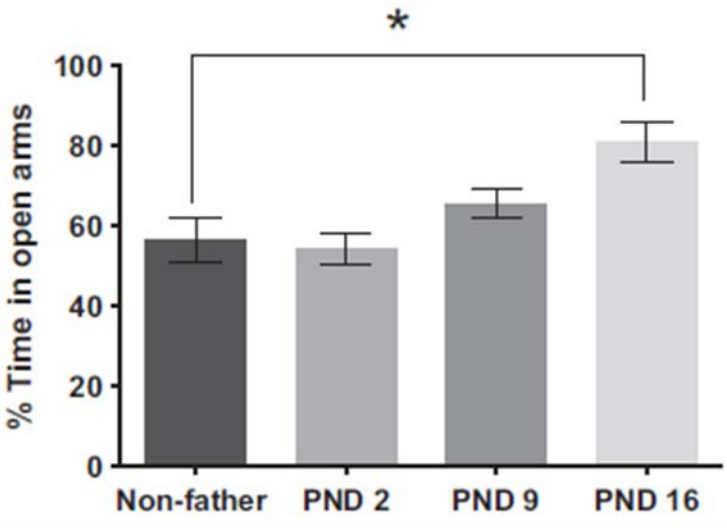

Fig. 1. Fatherhood reduced anxiety-like behavior but only during a period of increased pup retrieval. Percent time spent on the open arms of the EPM only differed from non-fathers at PND 16. No other PNDs were significantly different from each other. *P ≤ 0.05. Bars represent mean ± SEM.

Fatherhood increased survival, but not proliferation, of newborn cells in the DG

The number of BrdU-labeled cells remained constant in fathers from PND 9 to PND 16, whereas in non-fathers there was a significant decline in cell survival. Two-way ANOVA revealed a significant main effect of PND ($F_{2,25} = 21.90$; $P = 0.00$) and a significant interaction between paternal experience and PND ($F_{2,25} = 5.16$; $P = 0.01$) in the survival of BrdU-labeled cells within the DG of the hippocampus. Post hoc analysis revealed significantly fewer BrdU-labeled cells in the DG of non-fathers at PND 16 compared to non-fathers at PND 9 ($P = 0.00$; Figs 2A and B). Post hoc analysis also demonstrated that non-fathers had significantly more BrdU-labeled cells than fathers at PND 9 ($P = 0.04$; Figs 2A and B). The number of BrdU-labeled cells in the DG was not different between PND 9 fathers and PND 16 fathers ($P > 0.05$). Fatherhood did not alter cell proliferation. One-way ANOVA revealed no significant differences between non-fathers and fathers in the 2-h survival of BrdU-labeled cells in the DG at PND 2, PND 9 or PND 16 ($P > 0.05$; Fig. 2C).

Fatherhood did not alter proliferation or survival of new cells in the basolateral amygdala

Fathers showed no alterations in cell proliferation or survival in the basolateral amygdala. Two-way ANOVA did not reveal a main effect of PND or paternal experience, or an interaction in 1-week (PND 9) or 2-week (PND 16) survival of newborn cells in the basolateral amygdala ($P > 0.05$ for all comparisons; Supporting Information Figs S1A and B). One-way ANOVA revealed no main effect of PND on the proliferation of new

cells in the basolateral amygdala of nonfathers or fathers (P > 0.05; Supporting Information Fig. S1C).

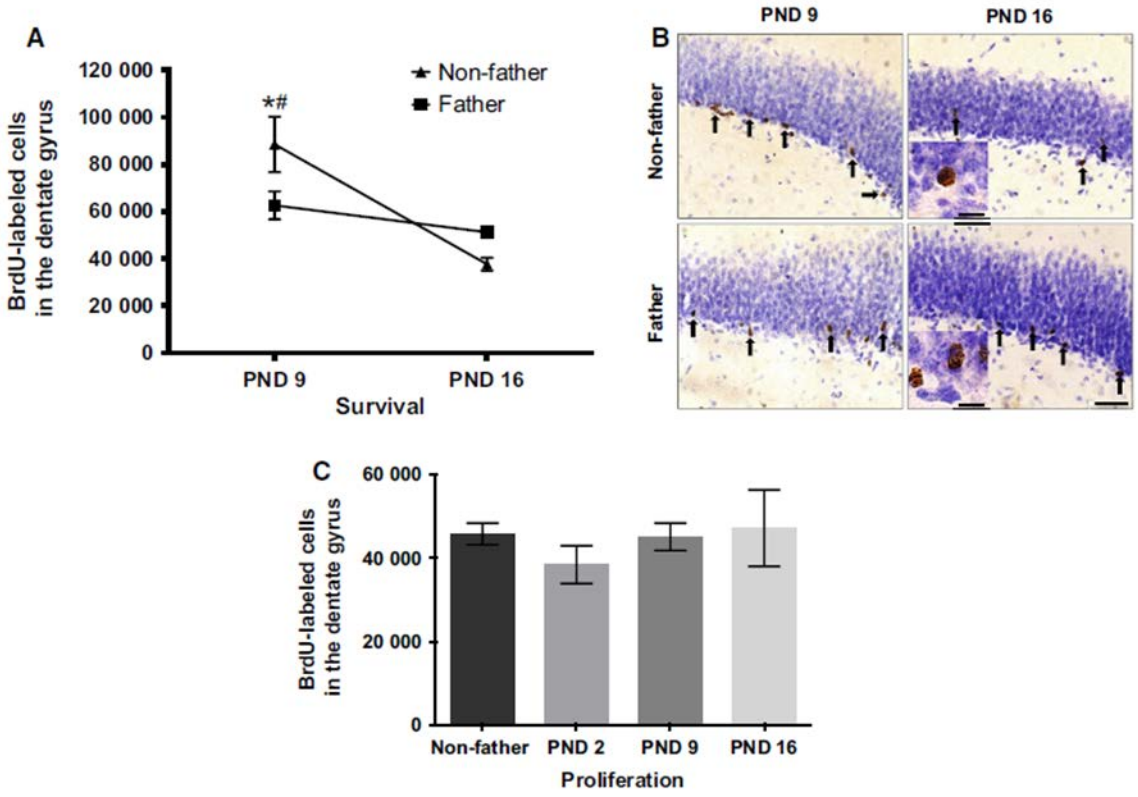

Fig. 2. Fatherhood increased the survival of newborn neurons in the dentate gyrus (DG) of the hippocampus. (A) Fathers had significantly fewer BrdU-labeled cells at PND 9; however, these surviving cells were maintained at PND 16. Non-fathers experience a significant decline in cell survival from PND 9 to PND 16. *P ≤ 0.05 for non-fathers at PND 9 vs. PND 16. #P ≤ 0.05 for non-fathers vs. fathers at PND 9. Bars represent mean ± SEM. (B) Photomicrographs (20x) of representative BrdU-labeled cells in the DG of California mouse non-fathers and fathers at PND 9 and PND 16. Arrows point to BrdU-labeled cells. Insets depict clusters of BrdU-labeled cells (x100 oil). (C) Neither fatherhood nor PND altered cell proliferation in the DG. Bars represent mean SEM. Scale bars, 40 µm (B), 5 µm (insets).

Fatherhood increased the number of BrdU-labeled cells that expressed the neuronal marker TuJ-1

Fathers had significantly more BrdU-labeled cells that co-expressed the neuronal marker TuJ-1, regardless of PND. Two-way ANOVA revealed a significant main effect of

fatherhood ($F_{1,18}$ = 7.03, P = 0.02; Fig. 3) but not PND (P > 0.05) on the number of BrdU-labeled cells that co-labeled with the neuronal marker TuJ-1.

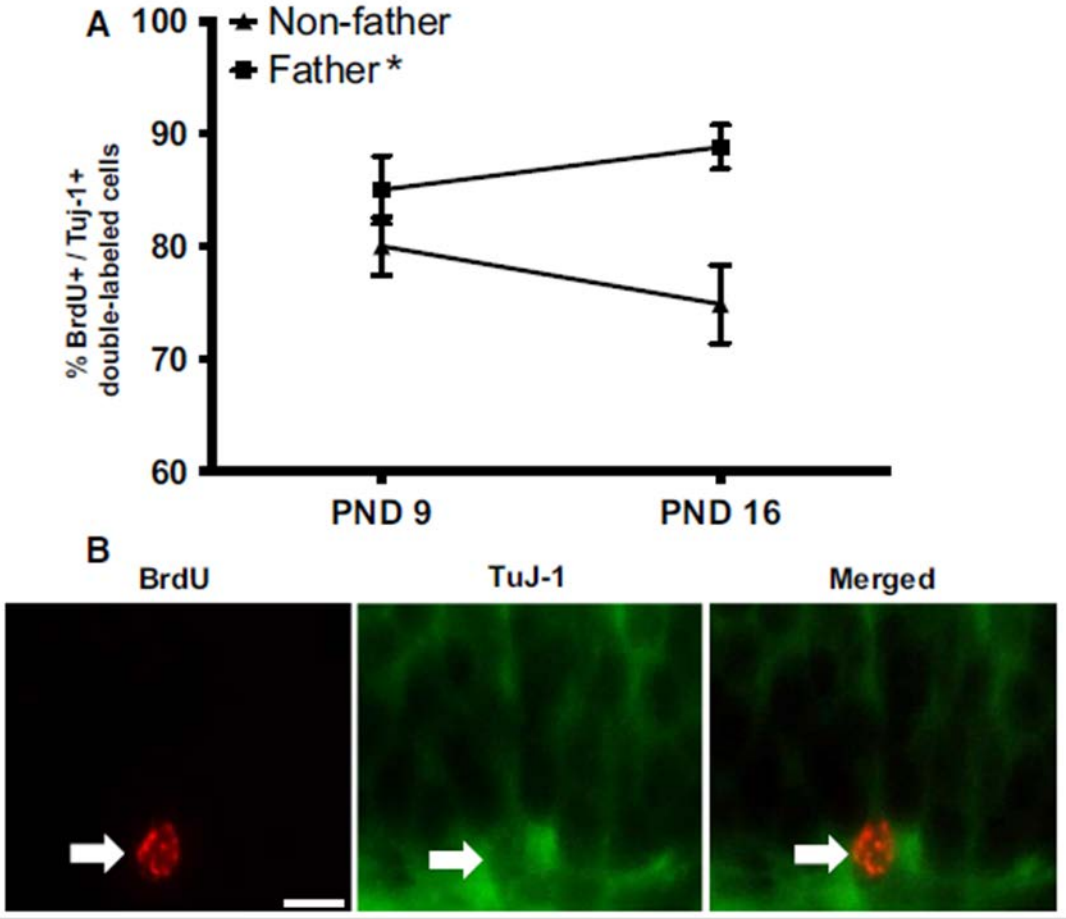

Fig. 3. Fatherhood increased neuronal differentiation. (A) Fatherhood increased the number of BrdU-labeled cells that were TuJ-1-positive. *P ≤ 0.05. Bars represent mean ± SEM. (B) Photomicrograph (x40 oil) of a representative BrdU-labeled cell that co-label with the immature neuronal marker TuJ-1. Arrows indicate cell location. Scale bar, 40 µm.

No relationship existed between cell survival and anxiety-like behavior

Pearson's correlations did not reveal a relationship between cell survival in the DG or the basolateral amygdala at PND 16 and the percentage of time spent on the open arm of the EPM (P > 0.05 for each comparison; data not shown).

Discussion

Our data show that anxiety-like behavior is reduced during the postpartum period in fathers, but only at a time when adult neurogenesis is maintained in the DG of the hippocampus. While the number of adult-born cells diminishes between 1 and 2 weeks post-BrdU injection in non-fathers, no changes in BrdU-labeled cells were observed during this time period in California mouse fathers despite an initial reduction in the 1-week survival of cells. These data indicate that, within the period of peak pup retrieval in California mouse fathers, anxiety-like behavior is decreased and adult neurogenesis is maintained. Our findings suggest that the type of father–offspring interaction may drive changes in emotional resilience and associated structural plasticity in fathers.

Parenting alters emotional regulation

Offspring contact is anxiolytic to the postpartum mother (Lonstein, 2005; Maniam and Morris, 2010). Lonstein (2005) observed that rat dams spent more time on the open arms of the EPM during the first week of lactation. This reduction in anxiety-like behavior was eliminated if dams were separated from their offspring for > 4 h prior to testing. Maniam and Morris (2010) observed similar offspring contact-induced effects in maternal rats. Dams that were separated from their offspring for 180 min a day exhibited increased anxiety-like behavior on the EPM from 4 to 17 weeks postpartum. The elimination of suckling, the removal of hormones associated with parturition, and the presentation of only distal pup cues does not alter anxiety-like behavior in rat dams. This confirms that the reduction in anxiety-like behavior in rats dams is dependent on other forms of offspring contact, such as grooming and huddling (Lonstein, 2005). More recent findings suggest that offspring contact-induced reductions in anxiety-like behavior may also be

the case in fathers. We have recently observed a decrease in anxiety-like behavior at PND 19 in California mouse fathers (Glasper et al., 2016). Given that the anxiolytic profile, in our present study and prior work, does not develop until ~ 2 weeks post-birth, it is possible that increased pup retrieval observed at this time point in the California mouse may be directly contributing to our observed effects on emotional regulation.

This hypothesis is supported by previous studies, in California mice and in prairie voles, which did not observe decreased anxiety-like behavior in paternal males shortly after the birth of pups. No changes in anxiety-like behavior were observed in California mouse fathers that were tested on PND 3–5 (Chauke et al., 2012). Interestingly, paternal prairie voles demonstrated increased anxiety-like behavior when tested at PND 6 and 7 (Lieberwirth et al., 2013). Within the first week of life, pup retrieval is low in these two paternal species. Therefore, if retrieval of pups is driving our effects on anxiety-like behavior, it is not surprising that we only observed this effect when pup retrieval was high (PND 16; Bester-Meredith et al., 1999).

It should be noted that while pup retrieval is not enhanced until ~ 2 weeks following the birth of offspring in the California mouse, fathers do engage in many other parental behaviors early in the postpartum period that result in high levels of offspring contact, such as huddling and grooming (Bester-Meredith et al., 1999). Despite these initial interactions with offspring, anxiety-like behavior is not altered until pup retrieval is at its peak. It is possible that once offspring are more mobile it is useful for fathers to be less fearful in entering novel or exposed areas to retrieve their pups. This type of exploratory behavior, resulting in increased father–offspring contact, may be important for driving attenuation of anxiety in fathers, a finding that would be similar to that

observed in mothers. Rat dams require recent physical contact with offspring to express reduced anxiety (Lonstein, 2005). Like maternal rats, our findings suggest that fathers may require a specific type of offspring contact to drive the reduction in anxiety-like behavior; specifically, more active father– offspring interaction via pup retrievals.

Parental experience alters adult neurogenesis in the hippocampus

Both the maternal and paternal brain undergo significant structural plasticity during the postpartum period (for a comprehensive review, see Leuner and Gould, 2010b). Both adult neurogenesis and cell proliferation are impaired during the postpartum period of maternal rats (Leuner et al., 2007; Pawluski and Galea, 2007) and mice (Glasper et al., 2011). Paternal California mice (Glasper et al., 2011) and prairie voles (Lieberwirth et al., 2013) exhibit reduced cell survival during the late postpartum period. On the other hand, C57BL6 male mice, which do not typically exhibit paternal care in the wild, have increased adult neurogenesis in the DG and the olfactory bulb (Mak and Weiss, 2010), and prairie vole fathers exhibit no changes in cell proliferation in the DG (Lieberwirth et al., 2013). Until now, cell survival has only been assessed at single time points following the birth of offspring in fathers. By examining the trajectory of new cell survival in the postpartum brain, we observed that the survival of new cells in the DG is initially reduced in California mouse fathers. Of the cells that do survive to 1 week postpartum, these cells are maintained until at least 2 weeks postpartum, at a time when anxiety-like behavior is also reduced.

We observed a reduction in 1-week survival of adult-born neurons in fathers compared to non-fathers. These findings are consistent with suppressed short-term

survival in maternal rats (Pawluski and Galea, 2007). However, in mothers this reduction is a direct result of a lactation-induced increase in glucocorticoids (Leuner et al., 2007). As fathers do not lactate, another mechanism is probably responsible for the reduction in short-term cell survival observed here. A likely candidate mechanism, known for its ability to reduce cell survival, is glucocorticoids; however, previous work in the California mouse suggests that fatherhood does not alter glucocorticoid levels in this species (Harris et al., 2012). As California mouse fathers are actively engaged in parenting early in the postpartum period, it is likely that this suppression in cell survival is a result of energy expenditure being shunted away from supporting structural plasticity of new cells, which are not fully functional, and moved toward functions that will aid in the care of offspring. This hypothesis, however, has yet to be directly tested.

Fatherhood increased the percentage of cells that co-expressed TuJ-1, a marker of immature neurons, between 1 and 2 weeks post-BrdU injection. Motherhood, however, does not alter the percentage of cells that express a neuronal phenotype (Leuner et al., 2007; Pawluski and Galea, 2007). Our findings suggest that fatherhood not only maintains the survival of newborn cells during the postpartum period but also increases the likelihood that these cells will be neurons. While cell survival is altered in paternal males, cell proliferation is not altered by fatherhood (present data; (Lieberwirth et al., 2013). This is in contrast to decreased cell proliferation observed in maternal rodents (Leuner et al., 2007; Pawluski and Galea, 2007). Reduced cell proliferation in maternal rodents is a result of lactation- induced increases in glucocorticoids (Leuner et al., 2007). Given that male rodents do not lactate, the lack of an effect on cell proliferation in fathers is not surprising here.

Finally, we did not observe any change in cell proliferation or adult neurogenesis in the basolateral amygdala, even though prairie vole fathers display a decrease in cell survival in this brain region (Lieberwirth et al., 2013). While the amygdala is involved in anxiety (Tye et al., 2011), the role of adult neurogenesis in this region is less clear (for review, see (Gould, 2007). On the other hand, adult neurogenesis in the DG has been implicated in anxiety-like behavior (Revest et al., 2009). In prairie voles, fatherhood decreases cell survival in the DG and is anxiogenic (Lieberwirth et al., 2013), yet the current study suggests that fatherhood has the opposite effect in California mice. Thus, it is possible that these functional and structural changes may be driven by fatherhood, yet the directionality is species specific.

A possible hormonal mechanism mediating fatherhood-induced neuroplasticity

Given that a correlation did not exist between anxiety-like behavior and adult neurogenesis in the DG of the hippocampus, it is likely that changes in new cell survival may not completely underlie anxiety regulation in fathers. This direct hypothesis, however, has yet to be tested. However, what is known is that prolactin is consistently elevated in rodent, non-human primate, and human fathers (Gubernick and Nelson, 1989; Reburn and Wynne-Edwards, 1999; Delahunty et al., 2007). This elevation in prolactin output is positively associated with father–offspring contact (Dixson and George, 1982; Roberts et al., 2001; Fleming et al., 2002; Ziegler et al., 2009). This relationship probably drives the motivation for paternal care (for review, see Storey and Ziegler, 2015). Offspring interaction results in higher prolactin receptor mRNA and increased adult neurogenesis in the DG of C57BL6 mouse fathers (Mak and Weiss, 2010). Prolactin

treatment also reduces anxiety-like behavior in non-paternal male and maternal female rats (Torner et al., 2001). Taken together, offspring contact may attenuate anxiety and enhance adult neurogenesis via a prolactin-related mechanism. To date, no one has explored the relationships among prolactin, anxiety and adult neurogenesis in males of a biparental species.

Conclusions

Our data suggest that increased offspring contact, during the postpartum period, contributes to reductions in anxiety-like behavior and the maintenance of newborn neurons in the DG of the hippocampus in fathers. It is likely that enhanced anxiety regulation and hippocampal structural plasticity leads to better parenting behavior (for reviews, see Leuner et al., 2010b; Agrati and Lonstein, 2015). Offspring health (for reviews, see Brand and Brennan, 2009; Ramchandani and Psychogiou, 2009), development (Glasheen et al., 2010) and neuroplasticity (Bredy et al., 2007; Lippmann et al., 2007) are all responsive to parental care and data suggest that the father's mental health is as important as the mother's mental health (for review, see Ramchandani and Psychogiou, 2009). Therefore, increased understanding of mechanisms underlying the postpartum affective state in males is of great importance to the father and, possibly, to the child.

Chapter 5: Inhibition of estrogen receptor beta impairs adult neurogenesis in California mouse fathers

Introduction

The postpartum period is characterized by a number of alterations to functional and structural hippocampal plasticity in both mothers and males that exhibit paternal care (Leuner et al., 2007; Leuner and Gould, 2010a; Macbeth and Luine, 2010; Glasper et al., 2011, 2016; Hyer et al., 2016). Effective regulation of hippocampus-mediated behaviors, like anxiety and stress, are essential for rearing healthy offspring. In mothers, hippocampal plasticity is often driven by hormonal mechanisms that are a result of pregnancy, parturition, and lactation (Pawluski and Galea, 2006; Leuner et al., 2007; Agrati et al., 2008; Galea et al., 2014). As males do not undergo the same physiological alterations that accompany reproduction in females, the neuroendocrine mechanisms underlying paternal experience-related hippocampal plasticity are less clear. The search for these neuroendocrine mechanisms in fathers is even more elusive due to significant species variation in the hormonal regulation of paternal care in biparental mammals (for reviews, see Wynne-Edwards & Timonin 2007; Saltzman & Ziegler 2014). Thus, isolating a target mechanism that may underlie fatherhood-induced hippocampal plasticity is a complex undertaking.

The California mouse (*Peromyscus californicus*) is a biparental, monogamous species, where males exhibit all aspects of paternal care aside from nursing (Gubernick and Alberts, 1987). Previous work has indicated that California mouse fathers experience hippocampal plasticity throughout the postpartum period. Fathers show reduced anxiety-like behavior and maintained survival of adult born neurons in the dentate gyrus (DG)

region of the hippocampus during the middle of the postpartum period (Glasper et al., 2016; Hyer et al., 2016). Changes to hippocampal plasticity can persist until weaning as California mouse fathers show reduced adult neurogenesis in the late postpartum period (Glasper et al., 2011). Altered hippocampal structural and functional plasticity appears around postnatal day (PND) 15 when offspring development causes a shift in parental care strategy (Bester-Meredith et al., 1999). Pups begin to show considerably more locomotion as well as rearing behavior near this age (Vieira and Brown, 2002). Concomitant with this change in locomotion, pups develop the ability to thermoregulate (Rosenfeld et al., 2013). These developmental changes allow the pups to venture outside of the nest. To accommodate their more mobile offspring, California mouse fathers and mothers shift their parental care from passive (i.e., huddling and grooming) to more active care behaviors (i.e., retrievals) (Bester-Meredith et al., 1999; Frazier et al., 2006). As this shift in paternal care strategy occurs at the same time when altered hippocampal function and structure is evident, it is likely that specific types of father-offspring interaction contribute to paternal experience-induced hippocampal plasticity.

Maternal rats experience reduced anxiety-like behavior during pregnancy (Macbeth et al., 2008a) and in the postpartum period (Lonstein, 2005), similar to what has recently been observed in California mouse fathers (Glasper et al., 2016; Hyer et al., 2016). Evidence suggests that the steroid hormone estradiol, and its receptors, may contribute to this anxiolytic effect in pregnant and lactating mothers. Pregnant female rats have increased levels of circulating estradiol and reduced anxiety-like behavior on the elevated plus maze (EPM) after only 9 days of gestation, compared to non-pregnant virgins (Macbeth et al., 2008a). Compared to virgin females, lactating primiparous

female rats show reduced fear behaviors in the light-dark box six days postpartum (Agrati et al., 2008). Similar to the maternal condition, cycling female rodents in proestrus have reduced anxiety-like behavior on a number of tasks, including the EPM - at a time when estradiol peaks and sexual receptivity is displayed (Walf and Frye, 2005; Walf et al., 2009). Anxiety-like behavior on the EPM is increased in ovariectomized female rats that do not receive hormone replacement, while subcutaneous estradiol replacement is anxiolytic in these females (Frye and Walf, 2004). These findings indicate that elevated levels of estradiol in females modulate anxiety-like behavior.

The hippocampus, which mediates anxiety-like behavior (Bannerman et al., 2003; Kheirbek et al., 2013), is a site of action for estradiol in the brain (for reviews, see Walf & Frye 2006; Galea et al. 2006). When administered directly to the hippocampus of ovariectomized rats via cannula infusions, estradiol attenuates anxiety-like behavior similar to systemic treatment (Walf and Frye, 2005). Interestingly, this estradiol-induced decrease in anxiety-like behavior appears dependent on activation of the β estrogen receptor (Erβ; Walf & Frye 2005; Walf et al. 2009). By administering selective estrogen receptor modulators (SERMs) for estradiol receptor subtypes Erα (propyl-pyrazole-triol) and Erβ (diarylpropionitrile), Walf & Frye (2005) found that activation of Erβ, but not Erα, 48 hours prior to testing reduced anxiety-like behavior on the EPM and during the open field task in ovariectomized female rats. When co-administered with the SERM tamoxifen (TMX), an Erβ antagonist with slight agonisitic properties for Erα (Watanabe et al., 1997), the estradiol-induced attenuation of anxiety, via either 17β-estradiol or Erβ agonists, was inhibited. Erβ knockout male mice show no change in anxiety-like behavior following estradiol treatment suggesting this receptor is necessary for these anxiolytic

effects (Walf and Frye, 2006). Taken together, these findings indicate that, in females, elevated estradiol is anxiolytic and that this reduction in anxiety-like behavior is likely driven by activation of Erβ in the hippocampus.

Both natural fluctuations and manipulated levels of circulating estradiol can influence adult neurogenesis. In non-lactating female rats, the number of newborn cells in the DG fluctuates throughout the estrus cycle. In proestrus, when estradiol levels are highest, cell proliferation is increased in female rats compared to the estrus and diestrus stages (Tanapat et al., 1999). However, the effects of estradiol on cell proliferation in female rats are time and dose dependent (Ormerod et al., 2003; Tanapat et al., 2005). An acute, moderate dose of estradiol administered to recently ovariectomized female rats will increase cell proliferation, however cell proliferation is not altered following acute treatment with low or high doses or with chronic treatment (Tanapat et al., 2005). Estradiol given 4 hours prior to administration of bromodeoxyuridine (BrdU), a synthetic thymine analogue that labels proliferating cells, increases the number of newly labeled cells in the DG of female rats. However, if given 48 hours prior to BrdU injection, cell proliferation decreases in female rats. The estradiol-dependent decrease in cell proliferation after 48 hours appears dependent on adrenal activity as adrenalectomized females do not display this decrease (Ormerod et al., 2003).

The role of estradiol in male hippocampal plasticity mirrors many of the effects observed in females. Gonadectomized male rats that receive estradiol, but not those that receive testosterone, show anxiolytic behavior in the open field task within five minutes of administration. This indicates a fast-acting, non-genomic pathway for the effects of estradiol on anxiety (Filova et al., 2015). Carrier et al. (2015) observed that a ten day,

slow release treatment with either estradiol or testosterone reduced anxiety-like behavior in the open field task. Testosterone's anxiolytic effect was eliminated with administration of fadrozole, an aromatase inhibitor, to the DG, indicating that the aromatization of testosterone into estradiol is driving the reduction in anxiety. While administration of testosterone metabolites to male rats, wildtype mice, and female rats results in similar reductions in anxiety-like behavior, Erβ knockout male mice show no alterations in anxiety-like behavior following treatment with steroid hormones (Frye et al., 2008). These findings suggest that Erβ is necessary for estradiol's effect on anxiety-like behavior in males as it is in females.

Despite the limited research on males, estradiol appears to influence adult neurogenesis in the DG of males and females similarly. In male meadow voles, intraperitoneal injections of estradiol increase cell survival if administered concomitant with axon extension that occurs when cells are 1 week old (Ormerod et al., 2004). The mechanism by which estradiol alters adult neurogenesis is still unclear. However, distinct localization of specific estradiol receptors suggests that different receptor subtypes may have differential effects on cell proliferation and adult neurogenesis. Greater immunoreactivity for Erβ is evident in the granule cell layer of the DG, while Erα is primarily localized to the subgranular zone where cells are proliferating (Blurton-Jones et al., 2004). The effects of estradiol on adult neurogenesis in meadow voles appear to target survival as opposed to proliferation as estradiol's enhancement of cell survival is independent from any impact on proliferation (Ormerod et al., 2004). These findings suggest that the mechanism by which estradiol impacts survival of new neurons in the DG may be through activation of Erβ (for review, see Galea et al., 2006). Overall, it is

evident that activation of Erβ in both males and females has similar repercussions on anxiety and adult neurogenesis. Given this, it is possible that alterations to anxiety-like behavior (Glasper et al., 2016; Hyer et al., 2016) and hippocampal adult neurogenesis (Hyer et al., 2016) in California mouse fathers may be driven by activation of Erβ.

We performed a series of investigations to determine the role of estradiol in hippocampal functional and structural plasticity across the postpartum period in fathers. First, we determined the relative expression of *Erβ* mRNA in the hippocampus using quantitative polymerase chain reaction (qPCR) to assess *Erβ* mRNA expression in the hippocampus among virgin males and early-, mid-, or late- postpartum fathers (PND 2, 16, and 30, respectively). Additionally, expression of other hormones associated with parenting and hippocampal plasticity (e.g., prolactin, vasopressin, oxytocin) was determined. Second, we characterized circulating estradiol concentrations in virgin males and fathers at these same time-points. Last, we determined to what extent anxiety-like behavior and adult hippocampal neurogenesis were altered through Erβ activation in California mouse fathers by treating virgins and fathers with TMX for eight days from PND 9 to PND 16 and then assessing EPM performance and adult neurogenesis. Specifically, it was expected that *Erβ* mRNA expression and estradiol would be increased at PND 16 concomitant with the previously observed reduction in anxiety-like behavior and the maintenance of adult neurogenesis in California mouse fathers (Glasper et al., 2016; Hyer et al., 2016). If Erβ mediates the observed reduction in anxiety-like behavior and the maintained survival of adult born neurons in fathers, TMX treatment was expected to attenuate these affects. By characterizing mRNA expression of hormone receptors that have been linked with changes in anxiety and adult neurogenesis, we may

be able to elucidate how hormone receptors in the hippocampus are altered across the postpartum period in fathers and how this receptor expression may contribute to paternal experience-induced hippocampal plasticity in the California mouse.

Methods

Animals

Adult male and female California mice, descendants of mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC), were born in our colony, weaned on postnatal day (PND) 30, and housed in same-sex dyads until 60-90 days of age before being utilized for experimental procedures. All mice were provided *ad libitum* access to food and water and were maintained on a 16:8 reversed light:dark cycle (lights off at 11:00 hours). All experiments were approved by the University of Maryland Institutional Animal Care and Use Committee and conformed to the guidelines provided by the National Institutes of Health for the care and use of animals.

Experiment 1

Experimental Design

Gonadally intact male and female California mice were paired in either male/male (virgin) or male/female dyads (father). Male/female pairs were housed undisturbed, aside from routine cage changes, allowed to mate, and give birth to an average of 1.67 ± 0.12 offspring after 45.73 ± 2.04 days of pairing. Male/male pairs were housed undisturbed aside from routine cage changes for 42.33 ± 1.84 days. Following birth (time-matched for

virgin controls), experimental mice were sacked and brain tissue and blood samples were collected on PND 2, PND 16, or PND 30.

Tissue collection

To determine fatherhood-induced changes in hormone receptor expression in the hippocampus across the postpartum period, whole hippocampi were dissected from the brains of experimental males on PND 2, 16, or 30 (time-matched for virgin males). Briefly, mice were cervicaly dislocated, brains were rapidly extracted from the skulls, hemispheres were separated, and hippocampi were removed. Hippocampi were individually placed into an RNAse-free centrifuge tube and flash frozen in liquid nitrogen. Total time from cervical dislocation to liquid nitrogen was on average 370.42 ± 8.88 seconds. Tissue was then stored at -80°C until processing.

Quantification of mRNA expression

mRNA expression was quantified using a qPCR protocol adapted from Venezia and colleagues (2016). Tissue samples were homogenized in Trizol (Life Technologies Carlsbad, CA, cat. no. 15596026) using a glass Dounce homogenizer. The following protocol follows manufacturer's instructions. Briefly, RNA was isolated by centrifuging each sample with chloroform at 12000 rpg for 12 minutes at 4°C. The top layer was carefully extracted and mixed with isopropyl alcohol. Samples were again centrifuged at 12000 rpg for 8 minutes at 4°C forming a pellet. All liquid was removed from the sample tube and the pellet was reconstituted with 20µl of 1X TE Buffer (Integrated DNA Technologies, Coralville, IO, cat. no. 11-05-01). Following reconstitution, samples were diluted with 1X TE Buffer to a concentration of 1:50. RNA was quantified using a DU800 Spectrophotometer (Beckman Coulter Brea, CA). One µg of total RNA was

reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, cat. no. 4368814). Real-time qPCR was used to assess mRNA expression of *Erβ, PRLr, V1a, OTr*, *ActB*, and *TBP*. Primer:probe assays were purchased pre-made (*ActB*, *TBP*) or designed (*Erβ, V1ar, OTr, PRLr*) using Integrated DNA Technologies' PrimeTime qPCR Assay designer (Table 1). Efficiency for each primer:probe assay was determined prior to use using serial dilutions. qPCR data was normalized to the geometric mean of *ActB* and *TBP* using the -ΔΔCt method and expressed as fold induction ($2^{-\Delta\Delta Ct}$) of mRNA expression compared to the control group (1.0-fold induction).

Table 1. Designed primer and probe sequences.

Gene	Forward Primer	Reverse Primer	Probe
Estrogen receptor β	GCTGATGTGGCGC TCGAT	CCCTCATCCCTGT CCAGAAC	ACCACCCTGGCAA GCTCATCTTT
Prolactin receptor	CGACATTTGTGGA TCTCAGGTT	CTGCCCTTGCTTT CATCCTA	AGGTGGTATTGTC CATTCAGAAGACC
Vasopressin 1a receptor	CGCCTCTTGGGTG CTGAGT	CGATTTCGATCAT AGAGAAGATGAA GT	CTACTGAGCACAC CGCA
Oxytocin receptor	TTCCTTGGGCGCA TTGAC	GTGCTGGACGCCT TTCTTC	CGTGCAGATGTGG AGCGTCTGG

Plasma Collection and Estradiol ELISA

A subset of the above mice (n=5) and a second cohort of mice (n=27) was used to determine circulating estradiol levels in virgins and fathers at PND 2, 16, and 30. Following rapid decapitation, trunk blood was collected, placed on ice, and then centrifuged at 9000 rpg for 30 minutes at 4°C. Plasma was isolated into a fresh tube and then stored at -80°C until processing. Total time from death till plasma extraction was 105.78 ± 6.63 seconds. Plasma estradiol levels were determined using enzyme-linked immunoabsorbant assay (ELISA). Samples were prepared per manufacturer's instructions

(Calbiotech, Spring Valley, CA, cat no ES180S-100). Assay sensitivity was < 3 pg/ml and the intra-assay coefficient of variation for this assay was 8.8%.

Experiment 2

Experimental Design

Gonadally intact male and female California mice were paired in either male/male (virgin) or male/female (father) dyads. Male/female pairs were housed undisturbed, aside from routine cage changes, for an average of 49.45 ± 5.68 days before they gave birth to an average of 1.57 ± 0.10 offspring. Virgin male pairs were time-matched to the male/female pairs. Mice were weighed on the day of pairing, on the day of BrdU injection (PND 2), and on the first day of treatment (PND 9). A subset of experimental mice were additionally weighed on the day of EPM testing and perfusion (PND16). All weight measurements were taken prior to injections or behavioral testing.

Drug Treatments

All males received intraperitoneal injections of BrdU (200 mg/kg; Sigma cat. no. B5002) on PND 2 to label proliferating cells. To determine the role of Erβ in the previously observed alterations to anxiety-like behavior and adult neurogenesis (Hyer et al., 2016), virgins and fathers were injected subcutaneously with TMX (100µg/g; Sigma cat. no. T5648) or a vehicle control (SAL; 10% ethanol, 90% saline) once daily from PND 9 to PND 16. To control for injection effects, a separated cohort of mice were handled, but did not receive injections, beginning on PND 9 through PND 16. All treatments were administered between 1100 and 1200 hours. Paternal behavior was quantified in fathers on PND 15. On PND 16, all male mice were placed on the EPM, to assess anxiety-like behavior, and then perfused via transcardial perfusion (see below).

Paternal Behavior

To determine if inhibition of Erβ alters paternal care, fathers were observed interacting with their offspring for 20 minutes between 1300 and 1400 hours on PND 15. Briefly, fathers and mothers were removed from the home cage and placed into a holding cage while pups were displaced from the nest. The father was then placed back into the home cage, opposite the pups, and his behavior was recorded using a Canon Vixia HFM500 camera (Canon, Arlington, VA, USA). Recordings were imported into EthoVision®XT11 (Noldus Leesburg, VA, USA) and paternal behaviors were recorded by a trained observed that was not aware of experimental treatments. The following behaviors were quantified: huddling, retrieving, grooming, and nest building. Operational definitions of paternal care behaviors were adapted from Bester-Meredith and colleagues (1999). Huddling was defined as the father arching his back over top of at least one pup and retrieving was defined as when the father grasped the pup with his mouth until he released the pup. Grooming consisted of the father licking at least one pup and nest building was defined as when the father was manipulating bedding material around the pup(s) or nest site. Latency to first, frequency, and duration of each behavior was observed.

Elevated plus maze task

Anxiety-like behavior was assessed using the EPM on PND 16 for all males between 1300 and 1600 hours. Mice were removed from their home cages and placed in a holding cage for transportation to an adjacent behavioral room. Mice habituated to the behavioral room for ten minutes prior to the beginning of the EPM task. Following this, mice were placed into the center of the maze, which stood 75 cm above the floor with

arms measuring 11.5 x 55 x 45 cm, facing an open arm, and behavior was digitally recorded for 5 minutes. Performance on the EPM was analyzed with EthoVision®XT 11 behavioral tracking software. Recordings were taken from a top-down view at a rate of 30 frames per second. Latency to enter arms, duration of time spent in the arms, and number of arm entries were assessed (Glasper et al., 2016; Hyer et al., 2016). Duration of time spent in the open arm was calculated as a percentage of time spent in the open arm divided by the total time spent in both arms, excluding the center, multiplied by 100. Mice that fell off of the maze were quickly returned to the center of the maze and allowed to continue exploring the maze until a total duration of 5 minutes had elapsed. Mice that fell off of the maze more than twice or froze for > 40% of the time (Chauke et al., 2012; Glasper et al., 2016; Hyer et al., 2016) were excluded from all analyses. Mice were perfused following the completion of behavioral testing.

Immunohistochemistry

Mice were anesthetized using a ketamine-xylazine cocktail and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.5. Brains were dissected from the skull and postfixed for 24 hours at 4°C before being moved to phosphate-buffered saline (PBS), pH 7.5. Coronal sections (40 µm) were cut throughout the entire rostrocaudal extent of the DG on a vibrating microtome (Leica Microsystems, Chicago, IL, USA) into a bath of 0.1M PBS, pH 7.5. Sections containing the DG were identified using the Peromyscus californicus brain atlas (Mikula et al., 2007).

Immunoperoxidase staining for BrdU

For BrdU peroxidase staining, a 1:8 series of sections were mounted onto glass Super Frost Plus slides (Fisher Scientific, Pittsburgh, PA, USA), dried, and pretreated by

heating in 0.1M citric acid, pH 6.0. Tissue was rinsed with PBS, incubated in trypsin for 10 min, denatured in 2M HCl:PBS for 30 min, rinsed with PBS, incubated overnight in purified mouse anti-BrdU (1:200; BD Biosciences, San Jose, CA, USA; insert cat. no.), incubated in biotinylated horse anti-mouse (1:200; Vector, Burlingame, CA, USA; insert cat. no.) for 60 min, rinsed in PBS, incubated with avidin-biotin complex (Vector), rinsed with PBS, and then reacted in 0.01% diaminobenzadine with 0.003% H_2O_2. All slides were counterstained with cresyl violet, dehydrated, and cleared with Citrisolv (Fisher Scientific) and coverslipped under Permount (Fisher Scientific).

BrdU and neuron-specific class III beta-tubulin (TuJ-1) immunofluorescence

Double labeling with immunofluorescence for BrdU and TuJ-1 was conducted to determine the percentage of BrdU-labeled cells that expressed a neuronal phenotype. TuJ-1 labels immature neurons but not glial cells (Biolegend, San Diego, CA, USA; (Jepsen et al., 2000). Immunofluorescence protocols were adapted from Glasper et al., (2011). Free-floating sections were rinsed in 0.1 M tris-buffered saline (TBS), pH 7.5, denatured in 2 M HCl : TBS for 30 min, rinsed and incubated in rat anti-BrdU (1 : 200; Accurate Chemical, Westbury, NY, USA; cat. no. OBT0030G) and Alexa Fluor 488 mouse anti-tubulin β 3 (1 : 500; BioLegend; cat. no. A488-435L), overnight, at 4°C. The next day, sections were rinsed with TBS, incubated with biotinylated goat anti-rat (1 :250; EMD Millipore, Billerica, MA, USA; cat. no. AP183B) for 90 min, rinsed again, and incubated for 30 min in the dark with streptavidin-conjugated Alexa 568 (1 : 1000); Molecular Probes, Eugene, OR, USA; cat. no. S11226). Finally, sections were rinsed, mounted onto glass slides, dried, and coverslipped using glycerol in TBS (3 : 1).

Data Analysis

Quantitative analysis of peroxidase-stained BrdU-labeled cells was conducted on coded slides. The number of BrdU-labeled cells on every eighth unilateral section throughout the entire rostrocaudal extent of the DG (granule cell layer, subgranular zone and hilus) were counted at 100 x magnification under oil immersion on a Zeiss Primo Star light microscope (Zeiss, Thornwood, NY) using the optical fractionator method (West et al., 1991; Ngwenya et al., 2005). The simplified formula for the estimated total number of labeled cells was: $N \sum Q$ x (1/ssf), which is the total number of labeled cells counted ($N \sum Q$) multiplied by the reciprocal of the section sampling fraction (1/ssf or 1/8) (Leuner et al., 2009). Brightfield photomicrographs were taken with a Zeiss AxioImager microscope with a stage controller attached to a computer with Microlucida Imaging Software (Williston, VT, USA). Images were cropped and optimized, by adjusting brightness and color balance, in Microsoft PowerPoint 2010. Immunofluorescence analysis of Tuj-1-labeled cells in the DG was conducted at 40 x oil immersion using a Zeiss AxioImager microscope with a stage controller and neuroimaging software (Neurolucida, Williston, VT, USA). To determine cell fate, the percentage of BrdU+ cells that were also TuJ-1+ were counted in 25 randomly selected cells within the granule cell layer and subgranular zone of the DG. To verify double labeling throughout their extent, images of these cells were captured using epifluorescent filters for red (BrdU) and green (TuJ-1), pseudo-colored, and then merged to identify double-labeled cells (yellow in the merged images).

Statistics

All data were analyzed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). One-way analysis of variance (ANOVA) was

used to assess the effects of fatherhood on mRNA expression in the hippocampus and plasma estradiol levels across the postpartum period, as well as the effects of treatment on paternal behavior, followed by appropriate multiple comparisons tests. Two-way ANOVA, followed by appropriate multiple comparisons test, was used to determine differences in EPM performance and BrdU labeling. All data were checked for equality of group variances and, if necessary, transformed using log transformation [Y = Log (Y)] to reduce heterogeneity of variance. Given that the estradiol data did not meet assumptions of normality, means were normalized so that the lowest value in the data set was 0% and the highest value in the data set was 100%. All comparisons used a two-tailed test and mean differences were considered statistically different when the P-value was ≤ 0.05.

For Experiment 1 some hippocampal samples were not used as they showed no amplification during the qPCR analyses. As a result, sample sizes varied across analyses (for final n sizes, see Figure 1). The following exclusions were required for Experiment 2: Behavioral exclusions due to > 40% freezing behavior (n=4; 1 handled virgin, 1 SAL virgin, 1 TMX virgin, 1 TMX father) or falling off of the EPM two times or more (n=2; 1 SAL virgin, 1 TMX father) and/or missed BrdU injections (n=12; 1 handled virgin, 2 SAL virgins, 1 TMX virgin, 1 handed father, 2 SAL fathers, 5 TMX fathers).

Results

Experiment 1

qPCR. No significant differences were observed between time-matched virgin groups for any measures, thus all virgin data were combined for analyses. One-way

ANOVA revealed a significant effect of postnatal day on *Erβ* mRNA expression ($F_{3,13}$ = 3.72; p = 0.04; Figure 1A). Post-hoc analysis revealed that at PND 16, fathers had greater *Erβ* mRNA expression than PND 2 (p = 0.01) and PND 30 fathers (p = 0.02). While not significant, comparisons between virgins and PND 16 fathers were marginally significant (p = 0.07), with more *Erβ* mRNA expression compared to virgin males. One-way ANOVA indicated that virgin males had significantly more *PRLr* mRNA expression ($F_{3,12}$ = 4.30; p = 0.03; Figure 1B) than fathers at all three time points. Post hoc analyses

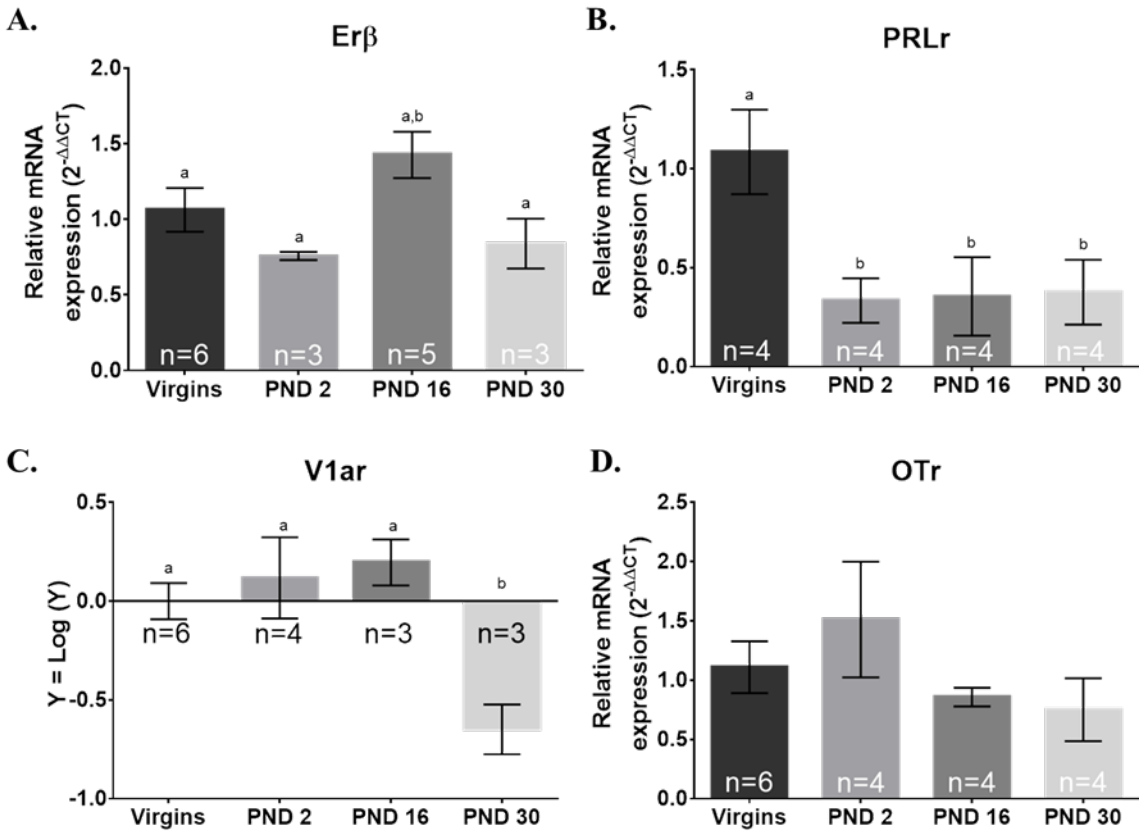

Fig. 1. Variations in hormone receptor mRNA expression in the hippocampus across the postpartum period of male California mice. A) *Erβ* mRNA expression was higher in PND 16 fathers compared to fathers at PND 2 and PND 30. B) Fatherhood reduced *PRLr* mRNA expression throughout the postpartum period compared to virgins. C) *V1ar* mRNA expression is reduced at weaning (PND 30), but no differences are observed among virgins, PND 2, and PND 16 fathers. D) Fatherhood does not alter *OTr* mRNA expression in the hippocampus. Bars reflect mean ± SEM. b, significantly different from PND 2 and PND 30. ($p < 0.05$).

indicated that virgins had more PRLr mRNA than fathers at PND 2 ($p = 0.012$), PND 16 ($p = 0.013$), and PND 30 ($p = 0.015$). One-way ANOVA revealed a significant effect of postnatal day on *V1ar* mRNA expression ($F_{3,14} = 7.54$; $p = 0.003$; Figure 1C). Post-hoc analysis indicated that at PND 30 fathers had less *V1ar* mRNA expression compared to virgins ($p = 0.003$), PND 2 fathers ($p = 0.001$), and PND 16 fathers ($p = 0.001$). *OTr* mRNA receptor expression did not differ among any groups ($p = 0.335$; Figure 1D).

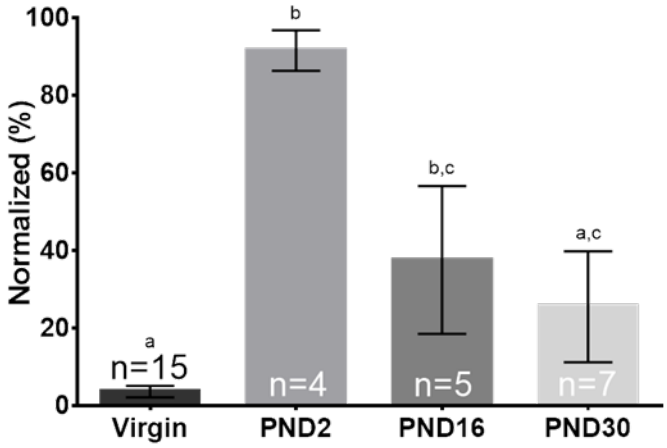

Fig. 2. Circulating estradiol was altered across the postpartum period in male California mice. At PND 2, fathers had increased circulating estradiol levels compared to virgins and PND 16 fathers. At PND 16, circulating estradiol was elevated in fathers compared to virgins. There was no difference between virgins and PND 30 fathers in circulating estradiol levels. Bars reflect mean ± SEM. b, significantly different from virgins. c, significantly different from PND 2 ($p < 0.05$).

Estradiol. One-way ANOVA revealed a significant effect of postnatal day on circulating estradiol concentrations ($F_{3,24} = 9.755$; $p < 0.001$; Figure 2). Post-hoc analysis indicated that compared to virgins, estradiol concentrations were significantly increased at PND 2 ($p < 0.001$) and PND 16 ($p = 0.02$). Lower estradiol concentrations were observed at PND 16 and PND 30, compared to PND 2 ($p = 0.009$ and $p = 0.001$, respectively). No differences were observed in circulating estradiol levels between PND 30 and PND 16 fathers ($p = 0.437$) nor between PND 30 fathers and virgins ($p =0.087$).

Experiment 2

Paternal Care. Daily administration of TMX did not alter paternal care behaviors, as a one-way ANOVA revealed no significant differences among handled, SAL, and TMX treated fathers ($p > 0.05$; Table 2). Latency to first, frequency of, and duration of huddling, retrieving, grooming, and nest building were not different among groups.

Table 2. Paternal care behaviors were not altered by treatment.

Behavior	Measure	Treatment		
		Handled	**SAL**	**TMX**
Huddling	Bouts	5.33 ± 4.37	7.00 ± 1.92	10.22 ± 3.88
	Latency (s)	582.70 ± 308.90	368.50 ± 157.50	571.10 ± 151.30
	Duration (s)	20.00 ± 17.53	91.03 ± 35.29	330.10 ± 137.80
Grooming	Bouts	10.00 ± 5.03	26.89 ± 4.83	26.22 ± 4.50
	Latency (s)	61.12 ± 14.78	34.70 ± 13.60	60.10 ± 26.47
	Duration (s)	28.03 ± 25.47	54.60 ± 14.07	114.00 ± 60.13
Nest Building	Bouts	3.67 ± 1.33	6.89 ± 2.80	9.11 ± 3.10
	Latency (s)	162.20 ± 64.53	371.10 ± 162.60	344.00 ± 162.50
	Duration (s)	4.98 ± 1.18	21.38 ± 9.76	39.81 ± 22.28
Pup Retrieval	Bouts	0.33 ± 0.33	0.00 ± 0.00	0.11 ± 0.11
	Latency (s)	1134.00 ± 65.79	1200.00 ± 0.00	1141.00 ± 59.10
	Duration (s)	0.30 ± 0.30	0.00 ± 0.00	0.19 ± 0.19

EPM. Two-way ANOVA did not reveal a significant main effect of paternal experience or an interaction between paternal experience and treatment on EPM behavior ($p > 0.05$; Figure 3). There was a non-significant main effect of treatment on time spent on the open arms of the EPM ($p = 0.079$, Figure 3A). Total number of entries into the open and closed arms did not differ among groups ($p > 0.05$, for both open and closed arms; Figure 3B). No differences were observed in total distance moved ($p > 0.05$; Figure 3C).

BrdU. Two-way ANOVA showed a significant interaction between paternal experience and treatment on the number of BrdU-labeled cells in the DG ($F_{2,37} = 4.727$; $p = 0.015$; Figure 4). Post-hoc comparison revealed that TMX treated fathers had fewer BrdU-labeled cells in the DG compared to handled fathers ($p = 0.035$), SAL treated

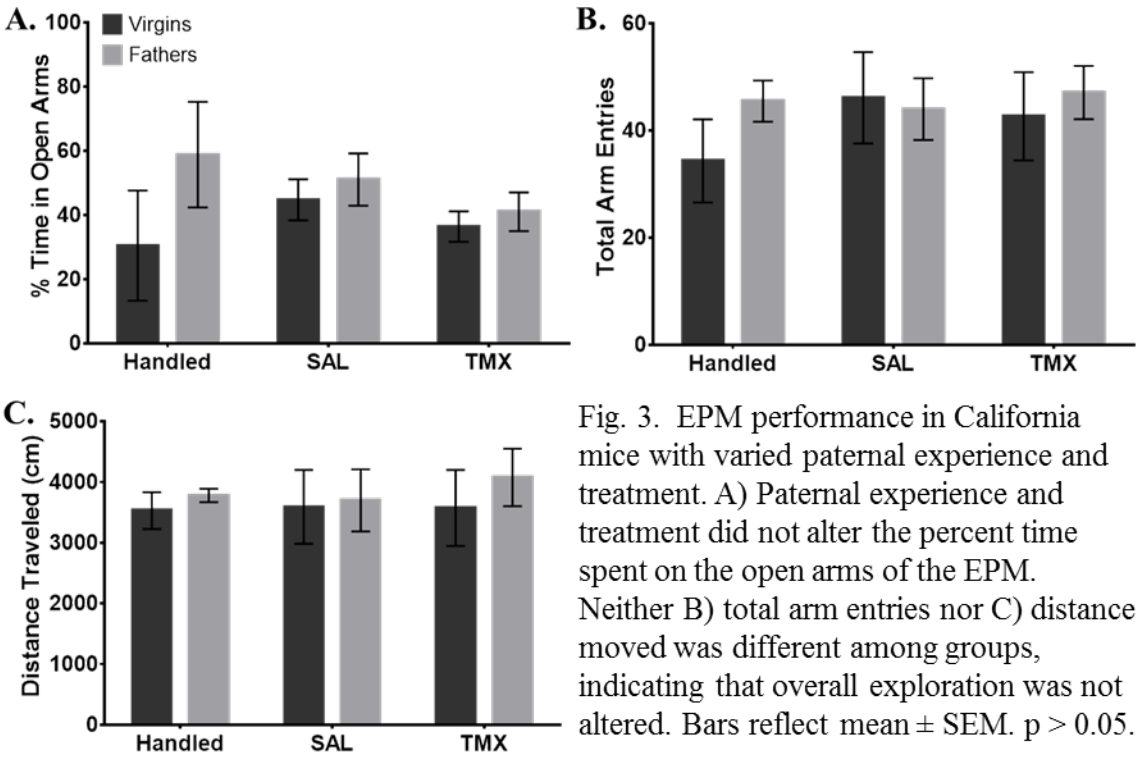

Fig. 3. EPM performance in California mice with varied paternal experience and treatment. A) Paternal experience and treatment did not alter the percent time spent on the open arms of the EPM. Neither B) total arm entries nor C) distance moved was different among groups, indicating that overall exploration was not altered. Bars reflect mean ± SEM. $p > 0.05$.

fathers ($p = 0.019$), and TMX treated virgins ($p = 0.006$). There was no main effect of paternal experience or treatment on BrdU-labeling in the DG ($p > 0.05$; Figure 4).

Tuj-1. Two-way ANOVA revealed a significant interaction between paternal experience and treatment on the number of BrdU+ cells that co-expressed the immature neuronal marker Tuj-1 ($F_{2,37} = 3.570$; $p = 0.038$; Figure 5). Post-hoc analysis revealed that SAL fathers had more BrdU+ cells that co-labeled with Tuj-1 compared to handled virgins ($p = 0.045$), SAL virgins ($p = 0.004$), TMX virgins ($p = 0.006$), and TMX treated fathers ($p = 0.001$). No significant main effect of paternal experience ($p = 0.077$) or treatment ($p = 0.065$) on BrdU+ cells that were Tuj-1+ was observed (Figure 5).

Weight. Two-way ANOVA revealed a main effect of time-point ($F_{3, 70} = 3.290$, $p = 0.026$; Figure 6B) and treatment ($F_{2, 70} = 4.738$, $p = 0.012$; Figure 6B) on weight gain in fathers but not virgins ($p > 0.05$; Figure 6A). Post-hoc analyses indicated that SAL

treated fathers weighed more on the day of EPM testing and perfusion (PND 16) compared to handled fathers ($p = 0.034$).

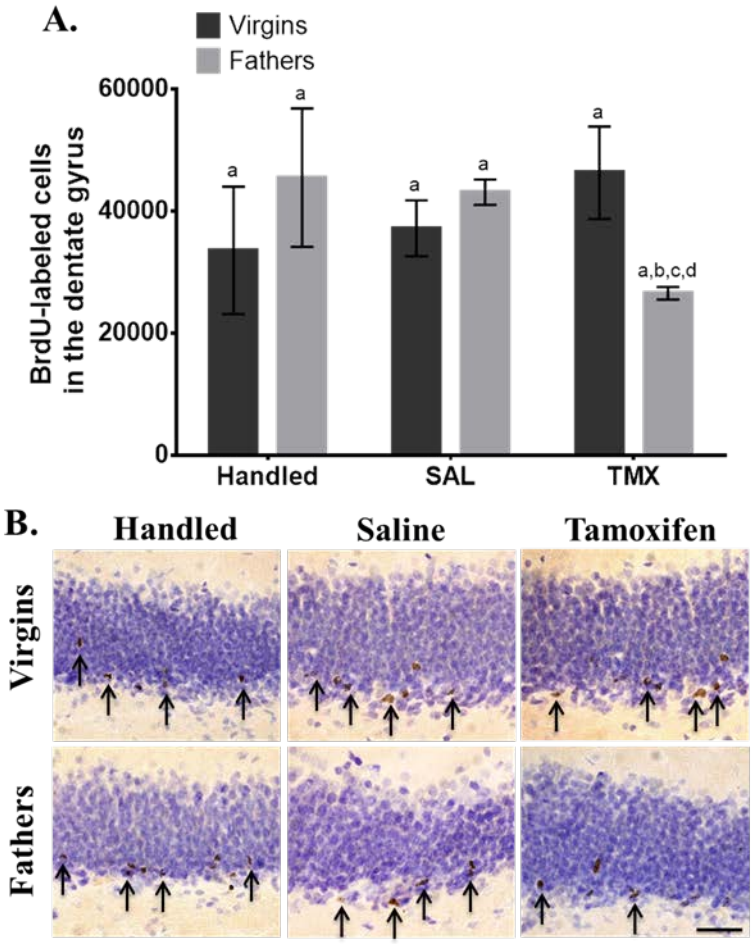

Fig. 4. Paternal experience and treatment altered BrdU-labeling in the DG of male California mice. A) TMX treated fathers had fewer BrdU-labeled cells compared to handled fathers, SAL treated fathers, and TMX treated virgins. Bars reflect mean ± SEM. b, significantly different from handled fathers. c, significantly different from SAL fathers. d, significantly different from TMX virgins ($p < 0.05$). B) Photomicrographs (40x oil) of representative BrdU-labeled cells in the DG of each group. Scale bar = 40µm. Arrows point to BrdU-labeled cell clusters.

Discussion

The findings from this study characterized the role of estradiol and Erβ in paternal experience-induced alterations of anxiety-like behavior and adult neurogenesis in male California mice. We observed that *Erβ* mRNA expression is temporally elevated in the hippocampus of California mouse fathers at PND 16. Circulating estradiol is increased at PND 2 and remains elevated at PND 16 before returning to baseline by PND 30 in fathers. Previous work has indicated that fathers engage in more

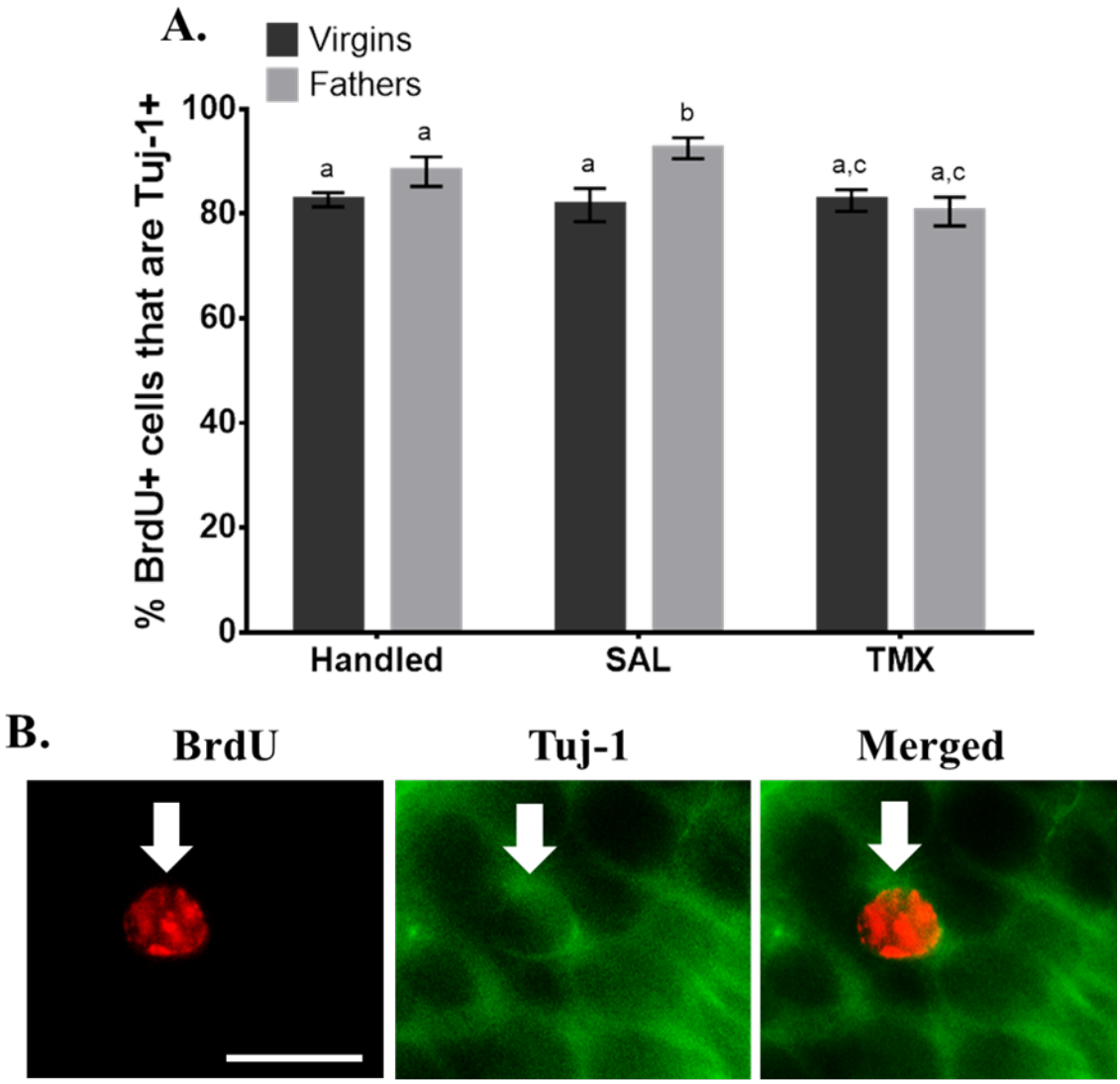

Fig. 5. The percentage of BrdU+ cells that were Tuj-1+ in male California mice. A) SAL treated fathers had more BrdU+, Tuj-1+ cells compared to handled virgins and SAL treated virgins. TMX treated fathers had fewer BrdU+ cells that were Tuj-1+ compared to SAL fathers. Bars reflect mean ± SEM. b, significantly different from handled virgins and SAL virgins. c, different from SAL fathers ($p < 0.05$). B) Photomicrographs (40x oil) of a BrdU-labeled cell in the DG that co-labeled with the immature neuronal marker Tuj-1. Scale bar = 20µm. Arrows point to BrdU-labeled cell location.

active paternal care (increased pup retrievals) around PND 16 (Bester-Meredith et al., 1999), as offspring become ambulatory and begin to venture outside the nest (Vieira and Brown, 2002). Concomitant with this shift in paternal care strategy, fathers show reduced anxiety-like behavior (Glasper et al., 2016; Hyer et al., 2016) and maintained survival of

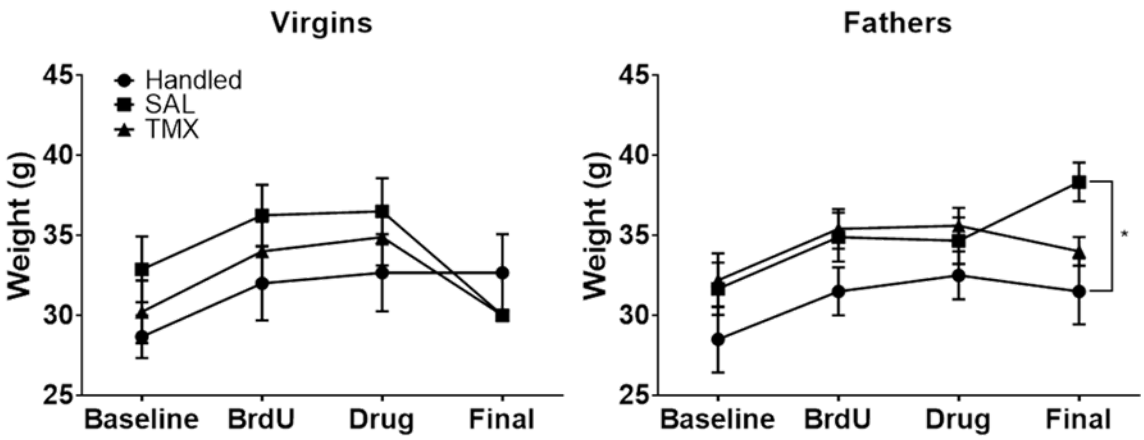

Fig. 6. Weights of male California mice at significant points across the experimental procedure. A) Weight was unchanged in virgin males across time. $p > 0.05$. B) SAL treated fathers had a significant increase in weight on the final day of testing compared to handled fathers. *$p < 0.05$. Lines reflect mean ± SEM.

adult born neurons (Hyer et al., 2016). As estradiol and Erβ were elevated at PND 16, it was possible that this neuroendocrine mechanism was influencing hippocampal plasticity at this time. When treated with TMX, a SERM with selective affinity for inhibiting Erβ (Watanabe et al., 1997), the number of BrdU-labeled cells is suppressed in fathers but not virgins. Additionally, inhibition of Erβ, via daily TMX administration, reduces the number of BrdU+ cells that co-label with the immature neuronal marker Tuj-1, indicating that short-term survival of new neurons is selectively impaired in fathers following inhibition of Erβ. This, however, does not alter anxiety-like behavior in fathers or virgins. Given these data, it appears that changes to Erβ expression in fathers occur specifically when the paternal care strategy becomes more active. The increase in Erβ may alter the available binding sites for estradiol, thus altering the impact that this steroid hormone can have on new hippocampal structural plasticity.

Paternal care in California mouse fathers is driven by increased aromatase activity, which converts testosterone to estradiol, in the MPOA (Trainor and Marler, 2002; Trainor et al., 2003). Even brief offspring exposure is enough to elevate circulating

estradiol (Trainor and Marler, 2002). We observed that circulating estradiol was elevated by PND 2 and remained higher than virgin levels at PND 16 before returning to baseline at PND 30. These findings parallel previous work (Trainor and Marler, 2002; Trainor et al., 2003) indicating that estradiol mediates paternal care in the California mouse. The current data are the first to characterize the trajectory of estradiol across the postpartum period in fathers. As estradiol levels decreased in a step-wise pattern from PND 2 to PND 16 and finally returned to baseline at PND 30, these data suggest that estradiol may promote paternal care behaviors earlier in the postpartum period and are less necessary near weaning. Elevated estradiol can attenuate anxiety-like behavior (for reviews see Walf & Frye 2006; Galea et al. 2006) and promote survival of adult born neurons (Blurton-Jones et al., 2004; Ormerod et al., 2004) through activation of Erβ. Given this, we investigated Erβ mRNA expression in the hippocampus and observed a significant increase in fathers at PND 16, concomitant with increased estradiol, and previously observed reductions in anxiety-like behavior and maintained adult neurogenesis (Hyer et al., 2016). Taken together, these data suggest that elevated circulating estradiol and enhanced expression of Erβ in the hippocampus provide a mechanism by which estradiol can alter hippocampal plasticity.

We observed an effect of TMX treatment on adult neurogenesis in fathers at PND 16. Following 8 days of subcutaneous TMX injections from PND 9-16, the number of BrdU-labeled cells in the DG is suppressed compared to SAL treated fathers. In addition, TMX treatment suppresses the number of BrdU+ cells that co-expressed the immature neuron marker Tuj-1 indicating, that TMX impaired survival of adult born neurons specifically. No effect of TMX treatment on BrdU-labelling or Tuj-1 expression in the

DG was observed in virgin males. Previous work has shown that estradiol increases cell survival in the male meadow vole. Estradiol treatment promotes cell survival if given 6-10 days following BrdU injection, concomitant with axon extension (Ormerod et al., 2004). Estrogen's effects on adult neurogenesis appear to be specific to survival as proliferation is not altered with estradiol treatment (Ormerod et al. 2004). Hyer et al. (2016) observed that adult neurogenesis was maintained in fathers from PND 9 to PND 16 while non-fathers experienced a significant decline in surviving cells. Proliferation was not altered by paternal experience. The present finding are the first to elucidate a possible role for estradiol in promoting neuron survival in fathers that may be dependent on activation of Erβ.

Immunoreactivity for Erβ and Erα is evident in proliferating cells while Erβ has also been observed in the granule cell layer (Isgor & Watson 2005; reviewed in Galea et al. 2006). This suggests that Erβ activation may promote cell survival while Erα promotes cell proliferation. In the current study, estradiol was elevated from PND 2 until PND 16. Treatment with TMX began on PND 9, one week after BrdU administration, at the time when these labeled cells were extending their axons and estradiol was increased paralleling the methods used by Ormerod and colleagues (2004). Thus, any affect that estradiol could have on adult neurogenesis by activating Erβ was inhibited. As Hyer et al. (2016) observed no changes in cell proliferation with fatherhood at any time-point, it is likely that any effect of estradiol on adult neurogenesis in California mouse fathers is specific to survival of adult born cells and not alterations in the proliferative population. The cellular mechanism by which this occurs is possibly through disinhibition of inhibitory interneurons. Estradiol binding to Erβ activates the metabotropic glutamate

receptor 2/3 (mGLur2/3) on inhibitory interneurons (Boulware et al., 2005). Erβ is co-localized with parvalbumin inhibitory interneurons within the hilus region of the DG (Blurton-Jones et al., 2004). It is possible that by activating Erβ, estradiol disinhibits these interneurons allowing growth factors, such as brain derived neurotrophic factor (BDNF), to enhance the survival of adult born neurons (Blurton-Jones et al., 2004). Future studies are required to confirm the specificity of this pathway.

Earlier work has indicated that hippocampal functional plasticity, specifically anxiety-like behavior, is altered at varying points across the postpartum period in California mouse fathers (Franssen et al., 2011; Glasper et al., 2011, 2016; Chauke et al., 2012; Hyer et al., 2016). In the early postpartum period (PND 3), no changes in anxiety-like behavior, as measured by EPM performance, is observed in California mouse fathers (Chauke et al., 2012) - an effect that is also absent by PND 9 (Hyer et al., 2016). However, by PND 16 (Hyer et al., 2016) and PND 19 (Glasper et al., 2016), anxiety-like behavior is attenuated in *P. californicus* fathers. As these changes occur when paternal care becomes more active (Bester-Meredith et al., 1999) due to more mobile offspring (Vieira and Brown, 2002), it is possible that the type of father-offspring interaction may contribute to altered anxiety-like behavior in this species. In further support of this hypothesis, at the end of the postpartum period, when offspring require little parental care (Bester-Meredith et al., 1999; Vieira and Brown, 2002), anxiety-like behavior, as measured by the open field task, has returned to baseline levels (Glasper et al., 2011). Previous work in both fathers and mothers has indicated that offspring contact can reduce negative affect in the parents. California mouse fathers exposed to a pup show reduced stress responsivity in a novel environment (Bardi et al., 2011). Even virgin males of

biparental species, including the California mouse (Bardi et al., 2011) and the prairie vole (Kenkel et al., 2012), show reduced negative affect after pup contact. Taken together, these findings indicate that offspring contact can reduce negative affect in males of biparental species.

In the current study, we observed no changes in time spent on the open arms of the EPM with fatherhood or with TMX treatment. Glasper et al. (2015) found that fathers exhibited more time spent on the open arms of the EPM at PND 19 compared to non-fathers. Hyer et al. (2016) replicated this effect in fathers at PND 16. In both studies, at these respective time-points, fathers spent about 30% more time on the open arms than non-fathers – resulting in a significant reduction of anxiety-like behavior expressed by California mouse fathers. In the current study, regardless of paternal experience or drug treatment, mice spent only 30-40% of their time on the open arms of the EPM mirroring the performance of non-fathers in Glasper et al. (2015) and Hyer et al. (2016).

A likely possibility that could have contributed to this effect is an activation of the stress response following eight days of subcutaneous injections prior to EPM testing. As anxiety-like behavior is responsive to these types of stressors, injections may have activated the HPA axis and increased anxiety-like behavior (reviewed in McEwen et al. 2015) in fathers specifically. Furthermore, the handled control groups showed a trend towards fathers spending more time on the open arms of the EPM compared to virgins. However, this finding was not significant and handled fathers still only spent around 60% of their time on the open arms (compared to 80% previously observed in fathers (Glasper et al., 2016; Hyer et al., 2016). This finding suggests that while eliminating injections,

eight days of handling alone may slightly increase anxiety-like behavior in California mouse fathers.

In mothers, stress-induced responsivity of the HPA axis is suppressed. This is evident through decreases in corticotropin-releasing hormone, adrenocorticotropic releasing hormone, and suppressed glucocorticoid release. These hormonal changes are accompanied by lowered behavioral expression of anxiety and fear to promote pup contact (for reviews see Kinsley & Lambert 2008; Slattery & Neumann 2008; Macbeth & Luine 2010). HPA axis function has been investigated in California mouse fathers and suggests that HPA responsivity is generally not impacted by fatherhood. New fathers compared to virgins and mated males show no changes in diurnal corticosterone rhythm in response to predator odor or pharmacological manipulation of corticosterone (Harris and Saltzman, 2013). While five minutes of restraint stress early in the active cycle significantly increases corticosterone in virgin males and females (Harris et al., 2012), a single injection of corticosterone administered on PND 3 does not appear to impair paternal care (Harris et al., 2011). However, exposure to chronic variable stress for 7 days increases basal corticosterone in California mouse males regardless of mating and/or paternal experience (De Jong et al., 2013; Harris et al., 2013). In the current study, mice were injected for eight days, similar to the chronic variable stress time-frame used previously (De Jong et al., 2013; Harris et al., 2013). It is also possible that injection treatment altered food intake which could have altered EPM performance. Saline treated fathers showed a significant gain in weight after eight days of injections treatment. TMX treated fathers did not show this increase mirroring previous work which has found that chronic TMX treatment results in weight loss and low caloric efficiency (Lampert et al.,

2013). Taken together, these findings suggest that while fatherhood may reduce HPA reactivity in California mice to mild stressors (Harris et al., 2012; De Jong et al., 2013), chronic stress exposure can circumvent this buffer.

In addition to the changes observed in *Erβ* mRNA expression, we observed that *PRLr* mRNA expression is reduced in fathers regardless of postpartum time-point. Prolactin, an anterior pituitary hormone, appears to have similar effects on anxiety and adult neurogenesis as estradiol. Like female rats (Torner et al., 2001), intracerebral infusions of prolactin in males reduces anxiety-like behavior on the EPM in a dose-dependent manner. The prolactin-induced reduction of anxiety-like behavior on the EPM in rats is attenuated with down-regulation of PRLr expression (Torner et al., 2001). Prolactin has been shown to mediate adult neurogenesis in C57BL6 mouse fathers. Delivery of prolactin to these fathers enhances adult neurogenesis in the DG. These newborn cells co-labeled with PRLr expression while PRLr knockout fathers had reduced adult neurogenesis (Mak and Weiss, 2010). Given these findings, it was somewhat surprising to observe a fatherhood-induced reduction of PRLr in California mice at PND 16. Circulating prolactin is elevated in California mouse males two days postpartum (Gubernick and Nelson, 1989), however, the persistence of this increase is unknown. More recent work has implicated prolactin as an initial promoter of paternal care as opposed to being heavily involved in maintaining paternal behavior throughout the postpartum period (for reviews see Wynne-Edwards & Timonin 2007; Saltzman & Ziegler 2014). Thus, it is possible that prolactin and its receptor may be more involved in paternal care in the early postpartum period as opposed to the time-point of interest in the current study.

Neither *OTr* or *V1ar* mRNA in the hippocampus of California mouse fathers was altered at PND 16. The lack of an effect on *OTr* or *V1ar* expression at this time is somewhat unsurprising. While circulating oxytocin is increased following copulation, it returns to baseline in the postpartum period in California mouse males (Gubernick et al., 1995). OTr is decreased in the BNST in fathers and no different from virgins in the amygdala and MPOA suggesting it may not play a role in fatherhood-induced plasticity (Perea-Rodriguez et al., 2015). While circulating vasopressin may correlate with paternal care in fathers (Bester-Meredith and Marler, 2003), the expression of *V1ar* mirrors the pattern of *OTr* in the paternal brain (Perea-Rodriguez et al., 2015). While oxytocin and vasopressin can influence anxiety (for review see Neumann & Landgraf 2012) and adult neurogenesis (Leuner et al., 2012), it is unlikely that they are playing a role in California mouse fathers at this time-point as the expression of both receptors was unaltered. V1ar expression in the hippocampus was down-regulated at the end of the postpartum period. As vasopressin is associated with aggression in the California mouse (Frazier et al., 2006), it is possible that the reduction of this vasopressin receptor is important for suppressing aggressive behavior prior to the birth of a new litter (Gubernick, 1988).

The data from the current study shed light on a possible neuroendocrine mechanism underlying paternal experience-induced hippocampal plasticity in the California mouse. These findings suggest that estradiol and Erβ are elevated concomitantly with previously observed alterations to anxiety-like behavior and adult neurogenesis in the DG (Glasper et al., 2016; Hyer et al., 2016) at a time when offspring need requires a higher degree of active father engagement (Bester-Meredith et al., 1999). Furthermore, inhibition of this receptor using the SERM TMX impairs short term

survival of adult born neurons in the DG in fathers, while adult neurogenesis remains unaltered in non-paternal males. Together, these findings suggest that activation of Erβ may underlie at least structural hippocampal plasticity in California mouse fathers. The need to elucidate mechanisms driving paternal experience-induced plasticity is important for promoting a healthy father-offspring relationship that will facilitate strong offspring development and the well-being of the father. Overall, this study has narrowed the gap in the literature on characterizing neuroendocrine function and neuroplasticity in males of a biparental species.

Chapter 6: General Discussion and Future Directions

The hippocampus is a brain region that mediates behaviors that facilitate reproductive fitness. These functions include aspects of emotional responsivity, learning, and memory. It is evident in both mothers and fathers that the hippocampus undergoes functional and structural plasticity in the postpartum period (Pawluski and Galea, 2006; Leuner et al., 2007; Glasper et al., 2011). As the hippocampus is a brain region rich in hormone receptors (McEwen, 1999), hormones associated with reproduction can drive hippocampal plasticity. In mothers, the hormones associated with pregnancy, parturition, and lactation can influence the hippocampus (Gould et al., 1990; Galea et al., 2006; Leuner et al., 2007). As fathers do not experience the same physiological changes associated with reproduction as mothers, the neuroendocrine mechanisms underlying plasticity of the hippocampus are more elusive. By investigating paternal experience-induced hippocampal plasticity and the hormones associated with paternal care, it is possible to determine how the hippocampus is altered postpartum and what neuroendocrine mechanisms may drive these changes in fathers.

Human fathers experience a number of changes in hormone levels as parturition approaches as well as into the early postpartum period, including decreased testosterone (Storey et al., 2000), increased estradiol (Berg and Wynne-Edwards, 2001), and increased prolactin (Storey et al., 2000). Additionally, human fathers experience increased activation (Swain et al., 2007) and increased gray matter (Kim et al., 2014) in brain regions associated with parental care, such as the hypothalamus, striatum, and amygdala. Often, these hormonal and neural changes in human fathers are associated with infant contact (Kim et al., 2014). In mothers, similar changes in neural activation (Kim et al.,

2010) and hormone levels (Stolzenberg and Champagne, 2016) occur. These changes, as well as increased infant contact, are associated with reduced negative affect in mothers (Horowitz and Goodman, 2005). Data shows that 6-12% of new fathers will develop postpartum depression (Ramchandani et al., 1992; Paulson et al., 2016) and up to 16% will experience postpartum anxiety disorders (Bögels et al., 2008), however, the mechanism(s) underlying altered emotional responsivity in fathers is unknown. This indicates that, while the relationship between maternal experience and emotional dysregulation has been studied (Glasheen et al., 2010), paternal experience-induced emotional changes also deserve attention (Cummings et al., 2005; Paulson et al., 2006). As fathers can be emotionally vulnerable during the postpartum period, the function and structure of brain regions related to parenting and emotionality, such as the hippocampus, and the neuroendocrine factors that can influence this region, may be underlying altered affective state in fathers.

Emotional responsivity during the paternal postpartum period

The hippocampus can act as a modulator of emotional responsivity. Studies investigating hippocampal activation have found that when the ventral portion of the rodent dentate gyrus (DG) is excited, anxiety-like behavior is reduced (Kheirbek et al., 2013). With lesions of this region anxiety-like behavior increases (Kjelstrup et al., 2002). Research investigating anxiety-like behavior in rodent fathers has shown a varying pattern across the postpartum period. In California mouse fathers (*Peromyscus californicus*), a biparental species (Dudley, 1974), anxiety-like behavior, measured by the elevated plus maze (EPM), is not altered on postnatal day (PND) 2-3 compared to virgins (Chauke et al., 2012). These findings were replicated in the current work as California

mouse fathers mirrored performance of non-father controls on the EPM at PND 2. This same effect was observed at PND 9, indicating that alterations in anxiety-like behavior, at least on the EPM, do not occur in the early postpartum period in this species (Chapter 3, Figure 1; Hyer et al., 2016). Fathers of other biparental species show altered anxiety-like behavior shortly following parturition. Prairie vole fathers (*Michrotus ochrogaster*) exhibit increased anxiety-like behavior in the open field task and on the EPM on PND 6 and 7, respectively (Lieberwirth et al., 2013). Both California mice (Gubernick and Alberts, 1987) and prairie voles (Ahern et al., 2011) exhibit paternal care of altricial offspring. Prairie vole litters can include up to seven offspring (Ahern et al., 2011) while California mice only have up to four pups per litter (Gubernick, 1988). Thus, the increase in anxiety-like behavior in prairie vole fathers may be due to higher litter size. Regardless of this difference, a lack of attenuated anxiety-like behavior early postpartum may facilitate the father's presence in the nest in both species, whereas reduced anxiety-like behavior may encourage extra-next exploration. By remaining in the nest, the father provides more offspring care through huddling and grooming behavior (Bester-Meredith and Marler, 2003) of young, altricial offspring (Gubernick and Alberts, 1987; Rosenfeld et al., 2013).

The mid-postpartum period in California mice is characterized by a change in the paternal care strategy to accommodate more developed offspring. Paternal behavior shifts from passive (i.e. huddling and grooming) to active care (i.e. pup retrievals; Bester-Meredith et al., 1999; Frazier et al., 2006) when offspring become ambulatory (Vieira and Brown, 2002) and are able to thermoregulate (Rosenfeld et al., 2013) around PND 15. As this time-point represents a change in the type of father-offspring interaction, it

was hypothesized that this time-point may be significant for potential offspring contact-induced alterations in hippocampal plasticity. Supporting this hypothesis, California mouse fathers exhibited reduced anxiety-like behavior on the EPM at PND 16 (Chapter 3, Figure 1; Hyer et al., 2016) and PND 19, compared to non-fathers (Chapter 1, Figure 1; Glasper et al., 2016). These data, along with the lack of alterations in anxiety-like behavior prior to this time-point, suggest that the degree of active father-offspring interaction may be an important mediator of anxiety-like behavior. As PNDs 15-21 are characterized by more active paternal care (Bester-Meredith et al., 1999; Frazier et al., 2006) to accommodate ambulatory offspring that are wandering from the nest (Vieira and Brown, 2002; Rosenfeld et al., 2013), this time-point may benefit from reduced anxiety-like behavior in the father. A less anxious phenotype in the father may promote willingness to leave the safety of the nest to retrieve offspring – an important factor in promoting offspring survival and thus, reproductive success.

Paternal anxiety-like behavior at the end of the postpartum period, near weaning, has been observed in the California mouse. At weaning (PND 35), there is no apparent change in anxiety-like behavior in the open field task (OFT) in California mouse fathers compared to virgins (Glasper et al., 2011). As offspring have reached stable body temperatures (Rosenfeld et al., 2013), require limited nursing (Gubernick and Alberts, 1987), and have full range of mobility (Vieira and Brown, 2002), offspring care is relatively undemanding for both the father and mother at this time-point (Bester-Meredith et al., 1999). However, the California mouse is one of few monogamous species that undergoes postpartum estrus (Gubernick, 1988). Almost immediately following parturition, the male California mouse will attempt to mate with the female (Gubernick,

1988; Rosenfeld et al., 2013). If successful, this will result in a second litter gestating throughout the postpartum period of the preceding litter. Thus, at the weaning period (PND 35) of the primary litter, a second litter will very shortly be born. As anxiety-like behavior has returned to baseline levels at this time (Glasper et al., 2011), it is more likely that the father will remain in the nest, avoiding exploratory opportunities. With the imminent arrival of a new, altricial litter, the presence of the father in the nest will facilitate the birth and survival of these new offspring (Gubernick et al., 1993; Rosenfeld et al., 2013). Taken together, these novel data on anxiety-like behavior across the postpartum period in fathers reflects a pattern of behavior that facilitates paternal offspring care as it relates to the development of, and contact with, the pups.

While evidence for direct offspring contact-induced changes in fathers is somewhat limited, virgin males of biparental species show reduced negative affect following exposure to pups. Specifically, California mouse virgins that have been exposed to pups for only three days show reduced stress reactivity when exposed to a novel environment (Bardi et al., 2011). Similarly, prairie vole virgins exposed to pups show a buffered stress response to handling (Kenkel et al., 2012) and the forced swim task (FST) – a measure of passive stress coping behavior (Bales et al., 2006). Given these data and the findings that California mouse fathers have reduced anxiety-like behavior (Chapter 1, Figure 1; Glasper et al., 2016; Chapter 3, Figure 1; Hyer et al., 2016) concomitant with the shift to an active paternal care strategy (Bester-Meredith et al., 1999) for more developed offspring (Vieira and Brown, 2002), it was likely that offspring contact played a role in the affective state of fathers. When California mouse fathers were separated from their offspring for 21 days, they showed increased passive coping in the

FST (Chapter 2, Figure 1; Hyer and Glasper, revise and resubmit). While mate separation alone increased passive-stress coping in this task, separation from both offspring and mate exacerbated this effect. Taken together, these findings, combined with the reduction in anxiety-like behavior mid-postpartum, indicate that offspring contact may provide a buffer against negative affect in California mouse fathers. Importantly, fatherhood alone, without persistent offspring exposure, does not appear to be enough to induce this effect. These data parallel findings in maternal rats (Lonstein, 2005) and human mothers (Horowitz and Goodman, 2005; Kim et al., 2010) showing that offspring contact can improve emotional responsivity in parents.

The temporal nature of these changes along with the fluctuations that occur in the paternal postpartum hormonal milieu, suggest that a neuroendocrine mechanism may be driving these changes. In the California mouse, estradiol plays an integral role in paternal care. The conversion of testosterone to estradiol via aromatization in the medial preoptic area (MPOA), a brain region associated with parental care, is necessary for California mouse fathers to exhibit paternal care behaviors (Trainor and Marler, 2001, 2002; Trainor et al., 2003). Along with its role in paternal care, estradiol can alter anxiety-like behavior (Walf and Frye, 2006). Gonadectomized male rats treated with estradiol show a rapid reduction in anxiety-like behavior in the open field task (Filova et al., 2015). Similarly, in female ovariectomized rats estradiol administered directly to the hippocampus is anxiolytic (Walf and Frye, 2005). Estradiol's effect on anxiety-like behavior appears to be through activation of the beta estrogen receptor (Erβ). When treated with testosterone metabolites 3α-diol or 3β-diol, which can be converted to estradiol, wild type male mice show reduced anxiety-like behavior on the OFT and EPM. However, Erβ knockout mice

do not show any treatment-induced attenuations in anxiety-like behavior (Frye et al., 2008). Female rats treated with the selective estrogen receptor modulator (SERM) tamoxifen (TMX), which acts as an Erβ antagonist and slight Erα agonist (Watanabe et al., 1997), do not exhibit an anxiolytic response to estradiol treatment (Walf and Frye, 2005). Given these data linking estradiol, paternal care, and anxiety, it is possible that estradiol activating Erβ plays a role in altering negative affect in California mouse fathers.

To determine estradiol's role in functional hippocampal plasticity in fathers, circulating estradiol and Erβ mRNA expression in the hippocampus were characterized at three time-points across the postpartum period - PND 2, 16, and 30. Circulating estradiol was elevated in California mouse fathers compared to virgin males beginning at PND 2 (Chapter 5, Figure 2). Previous work investigating estradiol in California mouse fathers showed that elevated estradiol correlated with increase paternal care after a single exposure to a newborn pup (Trainor and Marler, 2002). These data suggest that initial increases in estradiol likely promote offspring care. By PND 16, estradiol was reduced compared to PND 2 but remained elevated above virgin levels (Chapter 5, Figure 2). In males, testosterone is converted to estradiol via aromatization. California mouse fathers show increased aromatase activity in the MPOA 2-3 weeks postpartum, suggesting that increased estradiol production in this brain region facilitates paternal care at this time-point (Trainor et al., 2003). By PND 30, estradiol had returned to baseline levels (Chapter 5, Figure 2). Overall, these findings are consistent with the pattern of estradiol fluctuations previously observed in this species and show for the first time that estradiol levels decrease at the end of the postpartum period in fathers. To assess the extent to

which Erβ is altered in the hippocampus of California mouse fathers across the postpartum period, mRNA expression was determined in whole hippocampi using quantitative polymerase chain reaction (qPCR). At PND 16, fathers exhibited increased Erβ expression compared to PND 2 and PND 9 fathers (Chapter 5, Figure 1). These data are the first to show that a hormone receptor is temporally altered across the postpartum period in fathers. Taken together, these data indicate that estradiol and Erβ are elevated concomitant with changes in anxiety-like behavior in California mouse fathers, suggesting a possible neuroendocrine mechanism driving these changes.

California mouse fathers were treated daily from PND 9 to PND 16 with the Erβ antagonist TMX to determine if Erβ activation was underlying altered hippocampal function. On PND 16, when fathers have shown reduced anxiety-like behavior (Chapter 3, Figure 1; Hyer et al., 2016), performance on the EPM was determined. Following eight days of treatment, handled fathers showed slightly reduced anxiety-like behavior compared to handled virgin males (Chapter 5; Figure 1), similar to previous observations (Glasper et al., 2016; Hyer et al., 2016). This fatherhood-induced attenuation of anxiety-like behavior was eliminated following eight days of injections as saline treated fathers mirrored saline treated males in EPM performance. TMX treatment did not further alter anxiety-like behavior in fathers. As saline treatment alone prevented the reduction in anxiety-like behavior observed in fathers, the effect of Erβ activation on this form of hippocampal plasticity in fathers is difficult to determine. Previous work investigating the effects of chronic variable stress on California mouse fathers found that seven days of mild stressors increased corticosterone levels (Harris and Saltzman, 2013). It is possible that eight days of injections heightened stress responsivity in California mouse fathers

preventing the attenuation of anxiety-like behavior previously seen during this time-point (Chapter 1, Figure 1; Glasper et al., 2016; Chapter 3, Figure 1; Hyer et al., 2016). Future studies using alternate methods of manipulating Erβ activation are necessary to determine this receptor's role in anxiety-like behavior in fathers.

Structural plasticity in the postpartum period of California mouse fathers

The hippocampus is unique in that it is one of only two brain regions that undergoes extensive adult neurogenesis, the birth of new neurons in adulthood, in addition to remodeling of existing cells (reviewed in Gould, 2007). Parental care in both fathers and mothers is accompanied by altered structural plasticity in the hippocampus. In the early postpartum period, rat dams have reduced proliferation of new cells in the DG on PND 2 and PND 8 (Leuner et al., 2007). Using bromodeoxyuridine (BrdU), a synthetic thymidine analogue that labels adult born neurons, proliferation of new cells was determined in California mouse fathers. Contrary to maternal rats, fathers showed no deficits in proliferation at this early time-point (Chapter 3, Figure 2; Hyer et al., 2016). A similar pattern continues into the mid-postpartum period where rat dams have reduced cell proliferation at PND 8 (Leuner et al., 2007) but California mouse fathers remain at baseline on PND 9 (Chapter 3, Figure 2; Hyer et al., 2016). These findings are consistent with evidence from prairie vole fathers indicating that cell proliferation within the DG is not altered in multiparous fathers on PND 1 of their second litter (Lieberwirth et al., 2013). The discrepancies between fathers and mothers at this early postpartum time-point are likely due to hormonal mechanisms. In rat dams, the suppression of adult neurogenesis in the early postpartum period is directly linked to lactation-induced

increases in glucocorticoids (Leuner et al., 2007). As fathers do not lactate, these differences are not unexpected.

Changes in adult neurogenesis in maternal rats are related mother-offspring interaction (lacatation; Leuner et al., 2007), thus it is possible that altered father-offspring interaction may also influence paternal adult neurogenesis. As father-offspring interaction shifts from passive to active paternal care by PND 16 (Bester-Meredith et al., 1999) to accommodate more developed, ambulatory offspring exploring outside the nest (Vieira and Brown, 2002), fathers may experience an alteration in adult neurogenesis at this time. At PND 16, despite more active paternal care, California mouse fathers still show baseline levels of proliferation (Chapter 3, Figure 2; Hyer et al., 2016). The effect of offspring exposure on cell proliferation has also been investigated in prairie voles with similar results. Mated males exposed to a pup for 20 minutes (acute) or for 10 days (chronic) show no change in proliferation of new cells in the DG (Lieberwirth et al., 2013). Overall, these data suggest that cell proliferation in the DG is not altered in the postpartum period of fathers. As adult born neurons take at least two weeks to show any functional relevance (Kempermann et al., 2008), it may not be energetically advantageous to invest in cell proliferation while caring for offspring.

While cell proliferation in fathers appears not to be modified in the postpartum period, the survival of new cells may be differentially affected by fatherhood. In mothers, survival of adult born neurons is depressed throughout the postpartum period (Leuner et al., 2007; Pawluski and Galea, 2007; Glasper et al., 2011). Like proliferation, this suppression is a result of lactation-induced glucocorticoids (Leuner et al., 2007). To determine the extent to which adult neurogenesis was modified across the postpartum

period as a result of altered father-offspring interaction, California mouse males were injected with BrdU on PND 2 (time-matched for non-father controls) to label the proliferating population. In fathers, one-week survival of BrdU-labeled cells was impaired at PND 9 compared to non-fathers (Chapter 3, Figure 2; Hyer et al., 2016). While the suppression of adult neurogenesis in maternal rats is a result of lactation-dependent increases in glucocorticoids (Leuner et al., 2007), fathers do not lactate and, when undisturbed, they show blunted corticosteroid responsivity (Harris et al., 2012). Given this, glucocorticoids may not underlie this effect in males. Like cell proliferation, it is possible that fathers are shunting metabolic energy away from survival in favor of other energetic demands for offspring care at this time. Future studies are required to investigate a possible mechanism underlying this reduction.

While non-fathers have increased BrdU-labeling at PND 9, they experienced a significant decline in the number of surviving cells from one to two weeks (PND 9-16). Fathers, on the other hand, maintained the number of BrdU-labeled cells from PND 9 to PND 16 (Chapter 3, Figure 2; Hyer et al., 2016) – at a time when paternal care becomes more active (Bester-Meredith et al., 1999). Importantly, BrdU+ cells in fathers were more likely to be co-labeled with Tuj-1, a neuron-specific marker, at PND 9 and 16 compared to non-fathers (Chapter 3, Figure 3; Hyer et al., 2016). These findings indicate that during the time when the paternal care strategy becomes more active (Bester-Meredith et al., 1999) to accommodate ambulatory offspring (Vieira and Brown, 2002), adult neurogenesis is maintained. This maintenance is significant as these cells are two weeks old and are thus showing initial functional activity (Kempermann et al., 2008), therefore they may be contributing to hippocampus function. This change in structural plasticity

occurs concomitant to attenuated anxiety-like behavior (Chapter 1, Figure 1; Glasper et al., 2016; Chapter 3, Figure 1; Hyer et al., 2016). It is possible that the surviving new neurons contribute to hippocampal functional plasticity to alter emotional responsivity and improve paternal care in fathers.

This enhancement in adult neurogenesis disappears by the end of the postpartum period as California mouse fathers show a reduction in the number of four-week old cells at PND 35 compared to virgin controls (Glasper et al., 2011). This findings is similar to evidence from prairie vole fathers as they show impaired cell survival after six weeks (Lieberwirth et al., 2013). These data suggest that adult neurogenesis is differentially altered across the postpartum period in California mouse fathers. Proliferation of adult born cells within the DG does not appear altered in fathers (Lieberwirth et al., 2013; Chapter 3, Figure 2; Hyer et al., 2016) yet survival of adult born cells for one week just after parturition is suppressed (Chapter 3, Figure 2; Hyer et al., 2016). However, survival of adult born neurons is maintained (Chapter 3, Figure 2; Hyer et al., 2016) at a time when paternal care is more active (Bester-Meredith et al., 1999) and anxiety-like behavior is attenuated (Chapter 1, Figure 1; Glasper et al., 2016; Chapter 3, Figure 1; Hyer et al., 2016). Finally, long-term survival of adult born neurons at PND 35 is suppressed in fathers of this species concomitant with a return to baseline anxiety-like behavior (Glasper et al., 2011). Like the change in anxiety-like behavior observed across the postpartum period, it is possible that this reduction is significant for promoting paternal care of subsequent offspring.

In addition to alterations in adult neurogenesis, the postpartum period is similarly accompanied by changes in existing cell structure in the hippocampus. To determine the

trajectory of dendritic spine alterations across the postpartum period, Golgi-cox analysis was used to determine spine density in the DG of California mouse fathers. At PND 2, spine density was not altered along DG granule cells in fathers (Figure 1). This is contrary to findings in rat dams that show increased spine density in area CA1 of the hippocampus in the early postpartum period (PND 5-6) (Kinsley et al., 2006). However, in fathers, DG dendritic spine density is enhanced by PND 16 compared to virgin males (Figure 1). These findings indicate that acute offspring exposure may not be enough to alter spine density within this region in fathers and that a longer duration of offspring contact (> two weeks) may be necessary. Importantly, after two weeks, offspring become more ambulatory (Vieira and Brown, 2002) requiring fathers to become more active in their paternal care strategy (Bester-Meredith et al., 1999). Additionally, anxiety-like behavior is decreased (Chapter 1, Figure 1; Glasper et al., 2016; Chapter 3, Figure 1; Hyer et al., 2016) and adult neurogenesis is maintained at this time-point (Chapter 3, Figure 2; Hyer et al., 2016). It is possible that the increase in spine density may contribute to these temporal-specific changes in behavior.

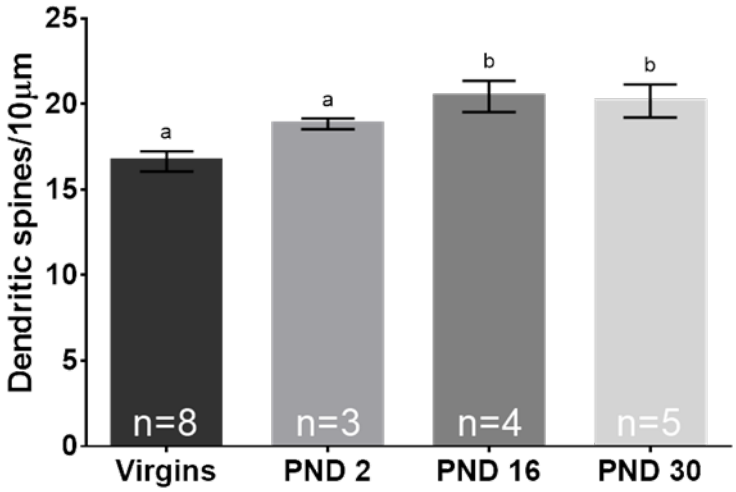

Fig. 1. Spine density in the dentate gyrus (DG) was increased across the postpartum period in fathers. At postnatal day (PND) 16 and PND 30 fathers had a higher density of dendritic spines along DG granule cells compared to virgin males. No differences were evident between virgins and fathers at PND 2. Bars represent mean ± SEM. Differing letters represent significant differences ($p < 0.05$).

Spine density in the DG remains elevated until the end of the postpartum period in California mouse fathers (Chapter 1, Figure 2; Glasper et al., 2016; Figure 1). Similarly, spine density is increased within multiple regions of the maternal hippocampus, including the DG, on PND 20 (Leuner and Gould, 2010a). Multiparous mothers show an even further increase in spine density in area CA1 along basal dendrites (Pawluski and Galea, 2006). Interestingly, when fathers were permanently separated from their offspring on PND 1, these changes in DG spine density were no longer evident – DG spine density in separated fathers was reduced compared to control fathers (Chapter 2, Figure 2; Hyer and Glasper, revise and resubmit). These findings indicate that at the end of the postpartum period, spine density is enhanced in the hippocampus – specifically within the DG. As separation from offspring eliminates this increase, it is apparent that fatherhood alone is not enough to maintain this region-specific type of structural plasticity (Chapter 2, Figure 2; Hyer and Glasper, revise and resubmit). Taken together, these data indicate that plasticity of existing cells within the hippocampus is altered in California mouse fathers postpartum. As these changes are not evident shortly following parturition (Figure 1), are present during the point when paternal care becomes more active (Figure 1; Bester-Meredith et al., 1999), and are eliminated after separation from offspring (Chapter 2, Figure 2; Hyer and Glasper; under review), it is likely that these changes are dependent on regular interaction with offspring. These data are the first to show the significant impact that offspring interaction can have on plasticity of existing hippocampal cells in fathers and that maintaining this contact is essential for DG dendritic plasticity.

The evidence above indicates that, in California mouse fathers, structural plasticity is differentially altered across the postpartum period. These changes appear

specific to the pattern of paternal care. As postpartum alterations in the hormonal milieu drive paternal care, neuroendocrine mechanisms are likely underlying structural hippocampal plasticity in fathers. In the California mouse, estradiol promotes paternal care (Trainor and Marler, 2002; Trainor et al., 2003). As reported above, estradiol is increased at PND 2 and 16 in fathers. Estradiol can alter adult neurogenesis in the hippocampus. Peripheral estradiol treatment given one week post-BrdU injection, at the time of axon extension, enhances survival of adult born neurons without altering cell proliferation in male meadow voles (Ormerod et al., 2004). Along with the observed increase in circulating estradiol, Erβ is elevated in California mouse fathers at PND 16. Characterization of Erα and Erβ expression in the hippocampus has shown distinct receptor localization, with some conflict, indicating that Erβ activation may be important for estradiol's effects on adult neurogenesis specifically. In ovariectomized females, Erα does not appear to be expressed in the granule cell layer of the dentate gyrus, but is present in the subgranular zone (SGZ) and hilus. Erα fails to co-label with H-thymidine positive cells in female rats (Tanapat et al., 2005) or with BrdU-labeled cells in prairie and meadow voles (Fowler et al., 2005) suggesting it is not involved in adult neurogenesis. In intact female rats, Erβ positive cells are evident in the ventral hippocampus within the granule cell layer suggesting they could contribute to adult neurogenesis (Blurton-Jones et al., 2004). Despite these anatomical findings, agonists for both receptors increase cell proliferation in female rats when given four hours before BrdU injection (Mazzucco et al., 2006).

As the above work suggests that estradiol (Ormerod et al., 2004) and Erβ are associated with adult neurogenesis (reviewed in Walf & Frye 2006; Duarte-Guterman et

al. 2015; Galea et al. 2013), activation of Erβ is a likely target mechanism underlying the observed changes in fatherhood-induced alterations to adult neurogenesis in California mice. California mouse fathers and virgins were treated with daily, subcutaneous injections of TMX beginning on PND 9 until PND 16 to determine if Erβ activation was underlying the maintenance of adult neurogenesis in fathers. This time-course was significant as previous work has shown that when estradiol is given at the time of axon extension (one week old cells) survival is enhanced a week later (Ormerod et al., 2004). This corresponded with the present experimental design as BrdU was given on PND 2 and then, one week later, TMX treatment began. TMX treatment impaired two-week cell survival in fathers alone. Virgins did not show any effect of TMX treatment on BrdU-labeling. Additionally, while saline treated fathers showed more neuron specific labeling of BrdU cells compared to virgins, TMX treatment significantly inhibited this effect in fathers alone. Taken together, these finding suggest that inhibition of Erβ impairs short-term survival of adult neurogenesis in California mouse fathers. These findings are consistent with previous work (Ormerod et al., 2004) indicating that, by inhibiting estradiol specifically at the time when adult born neurons are extending their axons, cell survival is impaired in males. Based on the current work, estradiol's influence on adult neurogenesis occurs through an Erβ-dependent mechanism.

A potential model describing the mechanisms underlying fatherhood-induced hippocampal plasticity in California mice.

The above data describe functional and structural plasticity across the postpartum period in California mouse fathers. The postpartum changes in hippocampal plasticity reflect a pattern of alterations that likely facilitates paternal care and, thus, improves

reproductive fitness. Generally, father-offspring interaction appears to alter anxiety-like behavior, adult neurogenesis, and spine density through an estradiol-dependent mechanism acting through Erβ. This proposed model shows the significance of hippocampal plasticity across the postpartum period for fathers and highlights the important role that father-offspring interaction plays in these alterations.

After only a brief time spent with offspring (PND 2 Chapter 5, Figure 2; 20 minute exposure Trainor and Marler, 2002), estradiol is increased in California mouse fathers. This increase promotes the initiation of paternal behaviors (Trainor et al., 2003). At this same time, no change is evident in adult neurogenesis (Hyer et al., 2016) or DG spine density (Figure 1) indicating that acute offspring exposure does not alter these aspects of hippocampal functional plasticity. Finally, anxiety-like behavior is not altered by PND 2. While estradiol is elevated to promote paternal care, the lack of alterations in the hippocampus shortly after parturition likely serves an important function. By remaining at a baseline anxious phenotype, fathers may be more likely to remain in the nest rather than engaging in exploration outside of the nest. The presence of the father at this early time point is significant. The altricial offspring do not yet have the ability to thermoregulate (Rosenfeld et al., 2013) and the father spends a significant amount of time huddling over the nest providing warmth (Bester-Meredith et al., 1999). Indeed the presence of the father significantly increases chances of offspring survival (Gubernick et al., 1993; Rosenfeld et al., 2013).

As the offspring develop into the mid-postpartum period, hippocampal plasticity in the father begins to alter. Adult neurons born in the DG at parturition begin to extend axons and dendritic projections by PND 9 (Kempermann et al., 2008). Elevated estradiol,

evident from PND 2-16 in fathers, can act to facilitate the survival of these cells (Ormerod et al., 2004) through Erβ activation (Blurton-Jones et al., 2004; Mazzucco et al., 2006). Erβ is co-localized with parvalbumin inhibitory interneurons (Blurton-Jones et al., 2004). The hilus region within the DG is composed of parvalbumin inhibitory interneurons (Bergami et al., 2015) and Erβ has been extensively localized within this region (Blurton-Jones et al., 2004). Erβ activation initiates the mGluR2/3 signaling cascade resulting in a downregulation of calcium mediated CREB phosphorylation (Boulware et al., 2005). This likely results in disinhibition of parvalbumin inhibitory interneurons. Disinhibition of these inhibitory interneurons likely facilitates survival of immature adult born neurons by allowing activation of these new cells. It is also possible that disinhibition of parvalbumin interneurons enhances BDNF release which can increase adult neurogenesis (Blurton-Jones et al., 2004). The estradiol-driven survival of these neurons in fathers is evident at PND 16 as the number of cells at PND 9 and 16 are consistent compared to a significant decline observed in non-fathers (Hyer et al., 2016).

At PND 15, fathers alter their paternal care strategy. Offspring have developed the ability to thermoregulate (Rosenfeld et al., 2013) thus requiring less parental huddling for warmth. Additionally, pups become ambulatory near this time. These changes in pup development facilitate their exploration around and outside of the nest. Fathers respond by shifting their paternal behavior from passive care (i.e. huddling and grooming) to active care (i.e. pup retrievals). Estradiol signaling in the MPOA at this time-point is essential for exhibition of paternal care behaviors (Trainor et al., 2003) and circulating estradiol remains elevated from their early postpartum period (Chapter 5, Figure 2). Alterations in functional and structural hippocampal plasticity accompany these changes

in paternal care. Elevated estradiol levels have contributed to increased survival of adult born neurons at PND 16 (Chapter 5, Figure 4). Similarly, spine density is elevated at PND 16 (Figure 1) – likely also a result of elevated estradiol (Gould et al., 1990; Woolley et al., 1990b). These enhancements in structural plasticity are accompanied by attenuated anxiety-like behavior in fathers (Glasper et al., 2016; Hyer et al., 2016). Both adult neurogenesis (Santarelli et al., 2003; Snyder et al., 2011) and spine density (Adamec et al., 2012; Wang et al., 2013) contribute to reduced negative affect. Given this, the estradiol mediated enhancements in structural plasticity may contribute to the reduced anxiety-like behavior evident at this time-point. While non-attenuated anxiety-like behavior in the early postpartum period was important for facilitating the father's presence in the nest, the opposite pattern is observed mid-postpartum. Mobile offspring exploring outside the nest require a less anxious father to emerge from the nest and retrieve the wandering pups.

By the end of the postpartum period, offspring require very little parental attention as they near weaning around PND 35. As the California mouse is a monogamous species that undergoes postpartum estrus, (Gubernick, 1988) the birth of a second litter is imminent by PND 35. Circulating estradiol has decreased to baseline levels by PND 30. Concomitantly, adult neurogenesis has decreased below levels observed in virgin controls and anxiety-like behavior has returned to baseline levels by this time-point in California mouse fathers (Glasper et al., 2011). The regression of adult neurogenesis and anxiety-like behavior prior to the birth of a new litter enhances the likelihood that the father will remain in the nest, avoiding exploratory opportunities. His presence in the nest will promote the survival of these new offspring (Gubernick et al.,

1993; Rosenfeld et al., 2013). Interestingly, spine density in the DG remains elevated at this time-point (Glasper et al., 2016). As estradiol is down-regulated, this neuroendocrine mechanism is not likely contributing to this increase at this time. These findings are consistent with data from multiparous maternal rats who show enhancements in spine density along CA1 basal dendrites beyond the increases observed in primiparous mothers (Pawluski and Galea, 2006). Thus, it is possible that multiparity may have an effect on hippocampal plasticity in fathers beyond what is observed in primiparous fathers.

Conclusions

The model outlined here reflects the pattern of paternal care, estradiol signaling, and hippocampal plasticity across the postpartum period in fathers. The interaction between estradiol and hippocampal plasticity facilitate aspects of paternal care which promote offspring survival. The development of the pups and the interaction between father and offspring play a critical role in shaping this pattern of care. In fact, offspring contact is essential for enhancements in spine density and reducing negative affect in fathers (Hyer and Glasper, under review). Taken together, this model highlights the importance of investigating neural, behavioral, and hormonal changes in the postpartum father as, like mothers, they experience many important changes.

The current findings narrow the gap in the literature on fatherhood-induced alterations to hippocampal function and structure. Furthermore, this work contributes to the literature on postpartum hormonal alterations in fathers, which is still quite controversial (Saltzman and Ziegler, 2014; Bales and Saltzman, 2016). The hippocampus is a brain region known for its involvement in aspects of emotional regulation, learning and memory, and stress responsivity. These behaviors, while not direct parental care, play

an important role in successfully rearing offspring. In biparental species, paternal care can significantly impact the well-being of the offspring. Therefore it is important to characterize the changes that the father undergoes that will impact his behavior and thus his paternal care and offspring investment. By helping to elucidate these changes and a possible mechanism driving them, this work has shed light on paternal experience-induced alterations and some of the factors that influence these changes. The findings from these studies may help to elucidate postpartum function in fathers, with implications for postpartum mental health. In fact, human fathers exhibit a similar enhancement of estradiol immediately postpartum (Berg and Wynne-Edwards, 2001) like California mouse fathers. Thus, it is possible that estradiol in human fathers may initiate similar changes in the hippocampus to facilitate offspring care, as it does in the California mouse. As human paternal care is more complex than in non-human mammals, the role of the hippocampus in fatherhood may be even more significant in humans. Finally, reduced negative affect in humans is associated with decreased hippocampal function and structure. Since fathers have a 16% chance of exhibiting postpartum anxiety (Bögels et al., 2008), and this behavior can negatively impact the child, investigating how estradiol may interact with the human male hippocampus in the postpartum period is significant.

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