bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1
2 **Fatherhood alters gene expression within the MPOA**
3
4 Adele M. H. Seelke[1], Jessica M. Bond[1], Trent C. Simmons[1], Nikhil Joshi[2], Matthew L.
5 Settles[2], Danielle Stolzenberg[1], Mijke Rhemtulla[1], and Karen L. Bales[1,3]
6
7
8 1. Department of Psychology, University of California, Davis
9 2. Bioinformatics Core Facility, University of California, Davis
10 3. California National Primate Research Center, University of California, Davis
11
12 **Running Title:** Fatherhood alters gene expression within the MPOA
13
14 **Key Words:** Prairie vole, monogamy, biparental care, paternal behavior, RNA
15 sequencing
16
17 **Corresponding Author:** Adele M.H. Seelke
18 Address: Department of Psychology, University of California, Davis, Davis, CA 95616
19 Telephone: 530-752-0668
20 Fax: 530-752-2087
21 Email: amseelke@ucdavis.edu
22
23
24 **Declarations of Interest:** none
25
26

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Abstract:**

2 Female parenting is obligate in mammals, but fathering behavior among mammals is rare.
3 Only 3-5% of mammalian species exhibit biparental care, including humans, and
4 mechanisms of fathering behavior remain sparsely studied. However, in species where it
5 does exist, paternal care is often crucial to the survivorship of offspring. The present
6 study is the first to identify new gene targets linked to the experience of fathering
7 behavior in a biparental species using RNA sequencing. In order to determine the pattern
8 of gene expression within the medial preoptic area that is specifically associated with
9 fathering behavior, we identified differentially expressed genes in male prairie voles
10 (*Microtus ochrogaster)* that experienced one of three social conditions: virgin males, pair
11 bonded males, and males with fathering experience. Differentially expressed genes from
12 each comparison (i.e., Virgin vs Paired, Virgin vs Fathers, and Paired vs Fathers) were
13 evaluated using the Gene Ontology enrichment analysis, and Kegg pathways analysis to
14 reveal metabolic pathways associated with specific differentially expressed genes. Using
15 these tools, we identified a group of genes that are differentially expressed in voles with
16 different amounts of social experience. These genes are involved in a variety of
17 processes, with particular enrichment in genes associated with immune function,
18 metabolism, synaptic plasticity, and the remodeling of dendritic spines. The identification
19 of these genes and processes will lead to novel insights into the biological basis of
20 fathering behavior.

21

22 **Keywords:** fathering, dendritic spines, plasticity, gene expression, RNA sequencing,
23 parenting

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **1. Introduction:**

2 Biparental care, where both mother and father contribute to the care of the
3 offspring, is displayed by a minority of mammalian species – usually cited as 3-5%
4 (Kleiman, 1977; Lukas and Clutton-Brock, 2013; Opie et al., 2013). Female parenting is
5 obligate because mammalian offspring need to nurse. Therefore, the presence of the
6 male, and particularly active participation of the male in fathering, is an unusual situation
7 seen only in our own and a limited number of other mammalian species (Gubernick and
8 Alberts, 1987; Mendoza and Mason, 1997; Runcie, 2000; Thomas and Birney, 1979;
9 Wynne-Edwards and Timonin, 2007). In species where it does exist, including humans,
10 paternal care is often crucial to the survivorship of offspring, or at the least has significant
11 and long-term impacts on growth as well as neural, reproductive and social development
12 (Bales and Saltzman, 2016; Cantoni and Brown, 1997); however little is known about the
13 specific neurobiological regulation of paternal care (Wynne-Edwards and Timonin,
14 2007). The vast majority of parenting research focuses on the mother, while the role of
15 the father is mostly considered in the context of paternal absence (Bales and Saltzman,
16 2016). Considering paternal care through the absence of the father in a biparental species
17 has drawbacks, however, since it is impossible to distinguish between the influence of the
18 quantitative absence of another caregiving individual and the qualitative absence of the
19 father in particular.

20 However, paternal absence is the most extreme situation. Although less studied,
21 individual variation in fathering can also have long-term effects on offspring (Bales and
22 Saltzman, 2016), and in the context of non-human mammals is always carried out in a
23 biparental care situation. In prairie voles, we have shown that natural variation in

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 biparental parenting behavior predicts pup development and juvenile social behavior
2 (Perkeybile et al., 2013), exploratory behavior and pair-bonding, and adult aggression
3 and stress responses (Arias Del Razo and Bales, 2016; Perkeybile and Bales, 2015a;
4 Perkeybile and Bales, 2015b). It is not always possible in a biparental care situation to
5 tell what outcomes in offspring are due to maternal care and what are due to paternal
6 care. However, some very interesting roles for the father have been observed. For
7 instance, in some species, males may compensate for poor maternal care (or allow
8 mothers to expend less energy on non-nutritive tasks like carrying)(Bales et al., 2002;
9 Perkeybile et al., 2013); or a paternal behavior such as retrievals (carrying pups back to
10 the nest or territory) may be directly linked to offspring display of retrievals and
11 aggression as an adult (Bester-Meredith and Marler, 2003; Frazier et al., 2007).

12 While little is known about the effects of paternal care on offspring, especially
13 when compared to maternal care, even less is known about the neural mechanisms
14 underlying fathering behavior. It has been hypothesized that similar neural circuits are
15 responsible for both maternal and paternal behaviors (Dulac et al., 2014). While
16 alterations in neural activity appear to be hormonally regulated in females, hormonal
17 manipulation in males does not have the same effect on paternal behavior (Saltzman and
18 Ziegler, 2014). This has led some to suggest that maternal and paternal behavior depends
19 upon non-homologous neuroendocrine circuits (Wynne-Edwards and Timonin, 2007),
20 and has raised the question of what factors are involved in the generation of these
21 behaviors.

22 Although the neuroendocrine contributions to parenting may vary by sex, it is
23 believed that the neural circuit governing parental behavior is conserved across sex (Kohl

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 et al., 2018). The MPOA is a central node in the neural circuit that regulates both
2 maternal and paternal care and has long been recognized as playing a critical role in the
3 generation and regulation of parental behavior (see (Bales and Saltzman, 2016; Kohl and
4 Dulac, 2018) for review). Paternal experience increases Fos immunoreactivity in the
5 MPOA of California mice (de Jong et al., 2009). Virgin male prairie voles that were
6 exposed to pups also showed an increase in Fos immunoreactivity within the MPOA
7 (Kirkpatrick et al., 1994). Lesions of the MPOA disrupt both maternal and paternal
8 behavior in biparental California mice (Lee and Brown, 2002), and maternal behavior in
9 rats (Numan et al., 1988) and mice (Tsuneoka et al., 2013).

10 The relationship between maternal behavior and gene expression has been
11 examined in a variety of species. The initiation of maternal behavior is reduced in
12 oxytocin knock-out mice (Rich et al., 2014). In humans, differences in maternal
13 responsiveness to babies and toddlers were associated with variants in serotonin
14 transporter and oxytocin receptor genes (Bakermans-Kranenburg and van Ijzendoorn,
15 2008; Feldman et al., 2012). Maternal experience in mice was linked to large scale
16 changes in gene expression in the lateral septum (Eisinger et al., 2013). And mice that
17 were bred for high or low levels of maternal aggression showed a wide range of
18 differentially expressed genes within the hypothalamus (Gammie et al., 2007). It should
19 be noted that these studies only examined mothers and the majority targeted a specific
20 gene or genes that were already implicated in parental behavior or attachment. As such,
21 they did not identify or examine novel gene targets that may also play a role in fathering
22 behavior.

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 The goal of this study was to identify novel gene targets and potential
2 mechanisms that may contribute to the production and regulation of paternal behavior.
3 We analyzed gene expression in three groups of adult male prairie voles: virgin males,
4 males who had formed a pair bond with a female, and males who had fathering
5 experience. Samples were taken from the medial preoptic area (MPOA), a region that is
6 central to the expression of both maternal and paternal behaviors (Dulac et al., 2014;
7 Kuroda and Numan, 2014; Rilling and Young, 2014; Stolzenberg and Champagne, 2016),
8 and RNA was extracted and sequenced.
9
10 **2. Materials and Methods:**
11 2.1 Subjects:
12 Subjects were 18 adult male prairie voles. Animals were born and housed in the
13 Psychology Department Vivarium at the University of California, Davis. These animals
14 were descendants of a wild stock originally caught near Champaign, Illinois. The animals
15 were weaned at 20 days of age and pair housed with an animal of the same sex (sibling if
16 available, similarly aged non-sibling if not) in small laboratory cages (27 x 16 x 13 cm)
17 in which food and water were available *ad libitum*. All animals were maintained at
18 approximately 70°F (21°C) on a 14:10 light/dark cycle with the lights on at 6 a.m. All
19 experiments were performed under National Institutes of Health guidelines for the care of
20 animals in research and were approved by the Institutional Animal Care and Use
21 Committee of the University of California, Davis.
22 At postnatal day (P) 42-45 subjects were placed in one of three groups of age-
23 matched males: 1) virgin males, 2) sexually experienced, “pair-bonded” males, or 3)

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 males with fathering experience (Figure 1). This was designed to dissociate alterations in
2 gene expression that were related to pair bonding from alterations related to paternal
3 behavior. Virgin males were housed with a male same-age conspecific, and they were
4 euthanized without engaging in sexual contact with females. Pair-bonded males were
5 housed with a same-age female conspecific for ~20 days, after which the males were
6 euthanized. Because mating and pregnancy strengthens pair bonds in prairie voles (Insel
7 et al., 1995; Williams et al., 1992; Winslow et al., 1993; Young and Wang, 2004), we
8 confirmed that females were pregnant. Pair-bonded males were euthanized before
9 females gave birth, ensuring they had no contact with pups. The third group consisted of
10 males which had ~3 days of paternal experience. These males were also housed with
11 female pair-mates with whom they presumably formed a pair-bond. The females gave
12 birth, and the males were permitted three days of contact with pups before they were
13 euthanized. Three days of parental experience was chosen to minimize age differences
14 between subjects. Furthermore, prairie vole fathers already exhibit large amount of
15 paternal care by postnatal day 3 (Oliveras and Novak, 1986; Perkeybile et al., 2013).
16 Subjects were anesthetized using isoflurane and euthanized via cervical
17 dislocation. Upon euthanasia, brains were removed and flash frozen. The brains were
18 sliced on a cryostat into 120 μm sections and mounted on slides. Punches were taken
19 from the MPOA using a 15.5-gauge blunt needle (Figure 2), and were stored in a -80
20 freezer until RNA extraction. The sequence of analyses for this study is outlined in
21 Figure 3.
22
23 2.2 RNA Extraction:

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Total RNA was isolated with Qiazol reagent (Qiagen) and purified with an RNeasy®
2 Plus Micro Kit (74004; Qiagen, Valencia, CA) as well as the optional DNase digestion
3 (Qiagen 129046). A Nanodrop™ Spectrophotometer was used to determine the quality
4 and quantity of the RNA. All samples had a 260/280 ratio > 1.8.
5
6 2.3 RNA Sequencing:
7 A total of 18 RNA-seq libraries were prepared from the RNA of the 18 male prairie voles
8 (Table 1). RNA sequencing and library preparation was performed by the DNA
9 Technologies and Expression Analysis Core in the Genome Center of the University of
10 California, Davis. Barcoded RNA-seq libraries were generated from 1 ug total RNA each
11 after poly-A enrichment using the Kapa Stranded RNA-seq kit (Kapa Biosystems, Cape
12 Town, South Africa) following the instructions of the manufacturer. The libraries were
13 generated on a Sciclone G3 liquid handler (Caliper Life Sciences, Alameda,
14 CA). Quality was verified with the Bioanalyzer 2100 instrument (Agilent, Santa Clara,
15 CA) and quantified by fluorometry on a Qubit instrument (LifeTechnologies, Carlsbad,
16 CA) and pooled in equimolar ratios. The pooled library was then quantified by qPCR
17 with a Kapa Library Quant kit (Kapa Biosystems) and sequenced on 1 lane of an Illumina
18 HiSeq 4000 (Illumina, San Diego, CA) with paired-end 150bp reads.
19 Raw sequencing data have been deposited at NCBI's Sequence Read Archive
20 (SRA) under study accession number SRP128134.
21
22 2.4 Bioinformatic analysis:

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Bioinformatic analysis was performed by the UC Davis Bioinformatics Core
2 Facility also in the Genome Center. Briefly, reads were trimmed for adapter
3 contamination and quality using scythe (version c128b19) and sickle (version
4 7667f147e6) respectively. The reads were then aligned to the prairie vole genome
5 (MicOch1.0) using bwa mem (version 0.7.13), after which featureCounts (version 1.5.0-
6 p1) was used to create the raw gene expressions counts. Finally, R (version 3.3.2) with
7 the edgeR and limma/voom packages were used to filter and transform (voom
8 transformation), and test for statistical significances between groups. Briefly, data were
9 prepared by first choosing to keep genes that achieved at least 0.5 count per million
10 (cpm) in at least five samples, normalization factors were calculated using trimmed mean
11 of M-value (TMM), and the voom transformation was applied. A completely randomized
12 design was implemented, comparisons of interest were extracted using contrasts, and
13 moderated statistics were computed using the empirical bayes procedure eBayes. Finally,
14 each gene was corrected for multiple testing using the Benjamini-Hochberg (BH) false
15 discovery rate correction.
16
17 2.5 Gene Ontology Analysis
18 Differential gene expression was directly compared between each pair of groups,
19 resulting in three comparisons: Virgin males vs Paired males (V vs P), Virgin males vs
20 Fathers (V vs F), and Paired males vs Fathers (P vs F). None of the differentially
21 expressed genes reached the level of statistical significance. In order to capture the genes
22 that were most likely to show functional differentiation between comparison groups we
23 performed gene ontology annotation enrichment analysis. The gene enrichment analysis

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

annotated the differentially expressed genes using one of three data sets: cellular component, molecular function, or biological process. Enrichment testing was conducted using Kolmogorov-Smirnov testing as implemented in the Bioconductor package topGO (Alexa and Rahnenfuhrer, 2016). We next examined the GO annotations that were significantly enriched (raw P value < 0.05) and selected the GO annotations in each comparison that were related to the brain or behavior, excluding unrelated annotations (i.e. GO:0003014, Renal system process or GO:0008354, Germ cell migration). We then categorized the remaining annotations based on gross function within each comparison group.

2.6 Kegg Pathways Analysis

We examined the functions with the highest fold enrichment (# observed genes/# expected genes, fold enrichment $>|1|$, $p < 0.05$), then identified individual genes associated with that function. We ran each individual gene through the Kegg Pathways database (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/) to identify molecular signaling pathways associated with that gene. A single gene may be involved in a number of different metabolic and biological pathways, so we then identified commonly recurring pathways associated with the individual differentially expressed genes. Pathways that were unrelated to brain function (for example, those that were involved in kidney, liver, or heart metabolism) were not included in the analysis. When a specific gene was associated with multiple pathways of interest it was identified as a candidate gene for further analysis. For example, *Grin2a* was associated with 6 pathways that are involved in neural plasticity.

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Using the qualitative procedures described previously, we identified 49 candidate
2 genes which display differential expression. We then averaged gene expression across
3 animals within each condition and transformed the data into ratios; the values we used for
4 all analyses were the ratios of gene expression in each condition relative to the virgin
5 condition. The expression ratio of genes in virgin animals was set at 1, a value >1
6 indicated that genes were more expressed relative to virgins, and a value <1 indicated that
7 genes were less expressed relative to virgins. Thus, we analyzed whether gene expression
8 for each gene varied between paired animals and fathers. Effect size was measured using
9 Cohen's *d*.
10
11 2.7 Assessment of gene interaction networks
12 After identifying each set of differentially expressed genes, we analyzed the connectivity
13 of the gene network using the STRING Database (string-db.org) (Szklarczyk et al.,
14 2017). The STRING database identifies protein-protein interactions between members of
15 a gene set, which allows the user to build a network of functional gene interactions.
16 STRING also measures the functional and interaction enrichments of the gene network,
17 calling upon GO Annotations, Kegg pathways, and connections between nodes.
18
19 2.8 NanoString Analysis
20 Following the identification of differentially expressed candidate genes, we performed a
21 quantitative analysis of the expression of 33 genes (30 target genes and 3 housekeeping
22 genes) using the nCounter SPRINT profiler (NanoString Technologies, Seattle, WA).
23 Genes were chosen to be included in the NanoString analysis based on their differential

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 expression values as determined by the raw expression data, as well as their functional
2 significance. One additional gene, *Bdnf*, was chosen due to previous studies indicating
3 that it plays a significant role in plasticity and parenting (i.e., *Bdnf*) (Pereira, 2016;
4 Tabbaa et al., 2017). The nCounter analysis assay was conducted using RNA that
5 remained after the completion of the sequencing experiment.

6 Briefly, NanoString is a medium-throughput method that can analyze many genes
7 within a single sample with comparable sensitivity and accuracy to quantitative real-time
8 RT-PCR (Geiss et al., 2008). NanoString designed and manufactured custom probes
9 corresponding to 33 genes we identified for quantitative analysis, consisting of 30 target
10 genes and 3 housekeeping genes (*Gusb*, *Pgk1*, and *Eif4a2*). A codeset specific to a 100-
11 base region of the target mRNA was designed using a 3' biotinylated capture probe and a
12 5' reporter probe tagged with a specific fluorescent barcode. Data were collected using
13 the nCounter Digital Analyzer by counting the number of individual barcodes.

14 Each transcript of interest was recognized by a capture probe and a reporter probe,
15 each containing 30–50 bases complementary to the target mRNA. To minimize assay
16 variability, the code sets also included negative and positive control reporter probes that
17 were developed by the External RNA Control Consortium (ERCC). Six positive control
18 reporter probes (ERCC-selected mRNA targets) were pre-mixed with (Spike-Ins) the
19 code set at a concentration range (0.125–128 fM), a range corresponding to the
20 expression levels of most mRNA of interest, to control for overall efficiency of probe
21 hybridization and determine the detection range for transcripts of interest in each assay. A
22 scaling factor was calculated for each sample, and a scaling factor outside the range of

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

0.3 to 3 indicated suboptimal hybridization. In our samples, the scaling factor always fell within the optimal range and was thus applied to all counts in the sample.

Quantitative expression data from the nCounter was downloaded and analyzed using the nSolver software package (NanoString Technologies, Seattle, WA). The raw counts for all transcripts were multiplied by the scaling factor to produce the adjusted counts. The relative expression was determined for each comparison group, and the effect size of the difference between expression values was determined using Cohen's *d*. Differential expression was also compared using t-tests, and p-values were adjusted for multiple comparisons in nSolver.

3. Results

3.1 Gene Ontology Analysis

Individual genes are associated with gene ontology annotations in order to describe the various functions of a particular gene product. The cellular component analysis describes the locations of gene expression, at the levels of subcellular structures. The molecular function analysis describes the function that each gene product performs within the cell. The Biological Process Analysis describes a recognized series of events or collection of molecular functions associated with a gene or gene product. Each analysis was completed for all differentially expressed genes in each of the three comparison groups, Virgin vs Paired (*V vs P*), Virgin vs Father (*V vs F*), and Paired vs Father (*P vs F*). Because each GO Annotation references many genes, in some instances the same GO Annotation was present in multiple comparison groups.

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 The initial GO enrichment analysis returned 209 GO Annotations in the *V vs P*
2 comparison, 222 annotations in the *V vs F* comparison, and 264 in the *P vs F* comparison
3 that were significantly enriched. Upon selecting the GO annotations in each comparison
4 that were related to the brain or behavior, we were left with 47 GO Annotations in the *V*
5 *vs P* comparison, 47 annotations in the *V vs F* comparison, and 61 annotations in the *P vs*
6 *F* comparison. (Tables 2-4). We then categorized these annotations based on gross
7 function (Figure 4). The functional categories of GO annotations were differentially
8 distributed across the three comparison groups. Annotations related to Neuropeptide
9 activity were only found in the *V vs P* comparison, whereas Immune Function
10 annotations were most predominant in the *V vs F* comparison. The P vs F comparison
11 contained the greatest number of annotations related to Plasticity,
12 DNA/RNA/Transcription, and Axon/Dendrite/Synapse.
13
14 3.2 Kegg Pathways Analysis:
15 In each comparison group we examined GO annotations with the highest fold enrichment
16 (# observed genes/# expected genes) as well as functions related to neuronal activity,
17 plasticity, or active biological processes, then identified individual genes associated with
18 that function. We ran each individual gene through the Kegg Pathways database (Kyoto
19 Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/) to identify molecular
20 signaling pathways associated with that gene. Not every gene was associated with a
21 molecular signaling pathway. We then identified commonly recurring pathways
22 associated with the individual differentially expressed genes.

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 In the *V vs P* comparison group, the commonly recurring pathways included:
2 Protein export, Protein processing in the endoplasmic reticulum, Thyroid hormone
3 synthesis, Antigen processing and presentation, Ras signaling, Rap1 signaling,
4 Neuroactive ligand-receptor pathway, Calcium signaling, and Regulation of the actin
5 cytoskeleton. In the *V vs F* comparison group, the commonly recurring pathways
6 included: Protein processing in the endoplasmic reticulum, Regulation of the actin
7 cytoskeleton, Ras signaling, Metabolic pathways, Axon guidance, Protein processing in
8 the endoplasmic reticulum, Thyroid hormone synthesis, and Antigen processing and
9 presentation. In the *P vs F* comparison group, the commonly recurring pathways
10 included: Ras signaling, Rap1 signaling, Neuroactive ligand-receptor pathway, Calcium
11 signaling, MAPK signaling, LTP, Glutamatergic pathways, Dopaminergic pathways, and
12 Regulation of the actin cytoskeleton.
13 We next identified genes that were associated with multiple Kegg pathways. By
14 excluding genes that were not associated with any Kegg pathways, or were associated
15 with pathways that were not related to brain function, we further narrowed the range of
16 genes of interest to 49 genes. Ultimately, in each comparison group we identified genes
17 with differential expression across social experience and that were linked to biological
18 pathways within the brain (Table 5). We standardized the expression of each gene
19 relative to its expression in virgin males then grouped genes that were associated with
20 nine commonly recurring Kegg pathways and compared the expression of those genes
21 across groups. Since this was an exploratory study, we did not perform statistical tests,
22 and instead used Cohen's *d* as a measure of effect size (Figure 5; Table 6). We found
23 large effects of differential expression in genes that were associated with long term

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 potentiation and long term depression (LTP and LTD) (d = 1.072), Neurotransmitters (d
2 = 0.911), and Ca^{2+} Signaling (d = 0.877). We found medium effects of differential
3 expression in genes that were associated with Oxytocin Signaling (d = 0.787), Protein
4 Processing in the Endoplasmic Reticulum (d = 0.599), and Ras/Rap1 Signaling (0.578).
5
6 3.3 STRING Database Analysis:
7 We used the STRING database to assess the network connectivity between the genes in
8 each comparison group that were identified as having a high degree of differential
9 expression and functional significance. For each analysis, the STRING database
10 constructed a network showing interactions between gene products, as well as the degree
11 of enrichment. The STRING database also performed an enrichment analysis using both
12 Gene Ontology Annotations and Kegg pathways, revealing statistically significant
13 interactions between these gene products.
14 The 11 differentially expressed genes from the *V vs P* comparison group produced
15 a network with 11 nodes and 11 edges, and a PPI enrichment p-value of 5.86×10^{-7}
16 (Figure 6A). Thus, the proteins expressed by these genes have significantly more
17 interactions than would be expected by chance, as defined as a random set of similarly
18 sized proteins selected from the genome. There was one cluster of 7 interacting proteins,
19 and the functions of these gene products were primarily related to functions of the
20 endoplasmic reticulum, as well as the cellular response to stimulation.
21 The 33 differentially expressed genes from the *V vs F* comparison group produced
22 a network with 32 nodes and 29 edges, and a PPI enrichment p-value of 6.99×10^{-15}
23 (Figure 6B), indicating that the proteins expressed by these genes have significantly more

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 interactions than would be expected by chance. These gene products produced one large
2 cluster of 9 interacting proteins, one medium cluster of 5 interacting proteins, and three
3 separate small clusters of 2 interacting proteins. The large cluster was predominantly
4 involved with the function of the endoplasmic reticulum. The medium cluster was
5 involved with process of neural plasticity, including signaling pathways and modification
6 of the actin cytoskeleton. The three small clusters were involved with the elongation of
7 fatty acid chains, the formation of cholinergic receptors, and GPI-anchor synthesis.
8 The 31 differentially expressed genes from the *P vs F* comparison group produced
9 a network with 31 nodes and 27 edges, and a PPI enrichment p-value of 1.36×10^{-12}
10 (Figure 6C), indicating that the proteins expressed by these genes have significantly more
11 interactions than would be expected by chance. These gene products produced one large
12 network consisting of 20 interacting proteins. The genes in this network were involved in
13 a variety of functions, including synaptic plasticity and neural transmission, ion
14 transmembrane transport, the cellular response to stimulus, and the structure of the
15 synapse and dendrite.
16
17 3.4 NanoString Analysis
18 A total of 33 genes (30 target genes and 3 housekeeping genes) were selected for
19 quantitative analysis using NanoString. The housekeeping genes (*Gusb*, *Pgk1*, and
20 *Eif4a2*) did not show differential levels of expression across conditions, confirming that
21 these genes can serve as a good baseline in prairie voles. A heat map analysis revealed
22 that 23 of our 30 target genes had lower expression levels in fathers than in either virgins
23 or paired males (Figure 7). Six genes had lower expression levels in virgins, and no gene

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

in any group appeared to show inordinately high levels of expression. A regression analysis revealed similar levels of gene expression across all experimental conditions (Figure 8A).

Expression data for each individual gene was compared across groups using t-tests, which were run and p-values were adjusted for multiple comparisons using nSolver software. Of the 30 target genes, 11 genes showed significant differential expression between comparison groups ($p < 0.05$; *Cckbr*, *Rgs14*, *Itpr1*, *Ddn*, *Baiap*, *Gabrd*, *Chrm1*, *Kcnj4*, *Ngef*, *Prkcg*, and *Cacna2d3*; Figure 8B-O; Table 7). We also calculated the effect sizes using Cohen's d, examining differential expression of each gene across groups (Figure 8B-O; Table 8). In the V vs P group, we saw a large effect (defined as $0.8 < d < 1.2$) in Tiam1. In the P vs F group, we saw large effects ($0.8 < d < 1.2$) in *Baiap2*, *Cacna2d3*, *Cckbr*, *Chrm1*, *Ddn*, *Dlg4*, *Gabrd*, *Itpr1*, *Kdr*, *P2rx3*, *Pde2a*, *Ptk2b*, and *Rasgrf2*. In the P vs F group we also saw very large effects (defined as $d > 1.2$) in *Grin2b*, *Ngef*, *Prkcg*, *Rgs14*, and Sipa1l1. In the V vs F group we saw large effects ($0.8 < d < 1.2$) in *Adora2a*, *Cacna2d3*, *Cckbr*, *Chrm1*, *Ddn*, *Gabrd*, *Grin2a*, *Grin2b*, *Itpr1*, *Kcnj4*, *Ngef*, *Prkcg*, *Ptk2b*, and Sipa1l1. In the V vs F group we also saw very large effects ($d > 1.2$) in *Baiap2*, *Rgs14*, and *Rin1*.

4. Discussion:

The transition to fatherhood is associated with a variety of environmental and behavioral changes. In this experiment we sought to identify alterations in central nervous system gene expression that are associated with fathering experience. To our knowledge, this is the first time that RNA sequencing has been performed on prairie vole

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 brain tissue. As such, we faced several technical challenges over the course of this study.
2 For instance, while the prairie vole genome has been sequenced (McGraw et al., 2010;
3 McGraw et al., 2011), its annotation is incomplete, leaving us to rely on the annotated
4 mouse genome (*Mus musculus*) for many of our analyses. In addition, it is important to
5 consider the consequences associated with working in an outbred rodent like prairie
6 voles. The individual differences associated with an outbred population may have masked
7 additional target genes associated with the onset of paternity. Regardless, we still
8 observed significantly altered expression on both the individual gene and system level.
9 These results suggest that paternity engages similar physiological mechanisms across
10 males despite genetic diversity.

11 Biparental care is rare in mammals, but prairie voles are not the only rodents who
12 exhibit this behavior. The males of several species of *Peromyscus*, including *Peromyscus*
13 *californicus* and *Peromyscus polionotus*, exhibit paternal care, while other species,
14 including *Peromyscus maniculatus*, do not. This behavioral distinction allowed Bendesky
15 and colleagues to investigate genetic differences between *P. polionotus* and *P.*
16 *maniculatus* that are linked to parenting behavior (Bendesky et al., 2017). In a series of
17 experiments, they identified several quantitative trait loci that were linked to specific
18 behaviors of interest, including nest building. Further analysis revealed that the gene for
19 arginine vasopression (AVP) was directly related to nest building, and when AVP was
20 administered intracerebroventricularly there was a significant decrease in the quality of
21 nest building (Bendesky et al., 2017). Unlike the study by Bendesky and colleagues, we
22 did not find changes implicating AVP. However, there are several differences between
23 the two experiments. In this study, we specifically examined gene expression within one

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 hypothalamic nucleus, the MPOA. Our study was in a different species and used males
2 that had very specific social experiences: virgin males, pair bonded males, and males
3 with fathering experience.

4 We targeted the MPOA specifically because it has long been understood to play a
5 role in maternal behavior, and is also believed to be involved in paternal behavior.
6 Lesions to the MPOA disrupt parental behavior in both male and female California mice
7 (Lee and Brown, 2002). In California mouse males, testosterone levels within the MPOA
8 vary in response to parental status (Trainor et al., 2003). Likewise, male California mice
9 with fathering experience show increased Fos-like immunoreactivity in the MPOA
10 following pup exposure (de Jong et al., 2009). To our knowledge, this is the first time that
11 gene expression in the MPOA has been analyzed in the context of fathering behavior.

12 RNA sequencing is a powerful technique that allows us to identify alterations in
13 gene expression that are associated with behavioral and other phenotypic changes (Wang
14 et al., 2009). The greatest challenge with this technique, however, is the large amount of
15 data it produces. There is no one agreed upon analysis that most effectively identifies
16 specific genes of interest (Conesa et al., 2016; Zhang et al., 2014). Thus, in this study we
17 used several techniques to reveal novel gene targets to further our understanding of
18 paternal behavior. We believe that this is a strength rather than a weakness. All of the
19 target genes identified in this experiment are associated with the experimental differences
20 in social experience. The ultimate goal of this experiment was to increase our
21 understanding of the alterations that occur within the MPOA following exposure to
22 different social contexts in male prairie voles. As such, we have identified a set of genes
23 and their associated pathways that we can use to further explore male parenting behavior.

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Our quantitative assessment of gene expression revealed an overall decrease in
2 the expression of many genes in fathers relative to both virgins and pair-bonded males.
3 The specific genes of interest that we identified were involved in a range of physiological
4 processes, including metabolism, stress responsiveness, and plasticity. However, most of
5 the genes that showed significant differential expression, and specifically decreased
6 expression, were associated with synaptic transmission and dendritic spine motility
7 (Table 9). For example, several genes involved in the production and maintenance of
8 receptors (including *Cckbr*, *Chrm1*, *Gabrd*, *Grin2b*, and *Itpr1*) and ion channels
9 (including *Cacna2d3*, *Kcnj4*, and *P2rx3*) were significantly downregulated. These results
10 suggest that GABA, glutamate, and cholinergic systems are all affected by fathering
11 experience, as are calcium and potassium channels. Other genes that exhibited significant
12 downregulation in fathers were involved with the actin cytoskeleton, dendritic spine
13 motility, and other components of the physical plasticity of dendrites. We emphasize that
14 this is not an exhaustive list of differentially expressed genes, however, these results
15 suggest that synaptic plasticity may be diminished in the MPOA of male prairie voles
16 with fathering experience.
17 We were surprised by the lack of differential expression of oxytocin and
18 vasopressin related genes, however this finding is not unique within the literature. In a
19 series of experiments, Kenkel and colleagues examined the neuroendocrine correlates of
20 pup exposure in male prairie voles that were virgins or had fathering experience (Kenkel
21 et al., 2012; Kenkel et al., 2014). They saw changes in OT immunoreactivity in
22 PVN/BNST, but there were no changes to OT/AVP in the MPOA. Another study
23 examined OT immunoreactive cells in male prairie voles that were virgins, had

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 established pair bonds, or had fathering experience (Wang et al., 2015). They saw an
2 increase in the number of OT immunoreactive cells in the MPOA of paired males and
3 fathers compared to virgin males, but there was a greater increase of OT-ir cells in the
4 PVN of fathers compared to paired and virgin males. It is likely that examination of gene
5 expression in the PVN would show alterations in OT gene expression. In future studies
6 we hope to examine these and other brain regions.

7 Fatherhood also seems to be associated with structural alterations in neural
8 plasticity, as measured by changes in the number and density of dendritic spines. Mice
9 with fathering experience show increased survival of newborn neurons and increased
10 dendritic spine density within the hippocampus (Glasper et al., 2016; Hyer et al., 2016).
11 Male marmosets show an increase in dendritic spine density in the prefrontal cortex after
12 fathering experience (Kozorovitskiy et al., 2006). However, other studies have shown
13 reductions in the survival of adult-generated neurons in the amygdala and hippocampus
14 (Glasper et al., 2011; Lieberwirth et al., 2013). It seems clear that the effects of
15 fatherhood vary across brain regions, but we do not yet know what is causing these
16 changes in neural plasticity.

17 The lower gene expression related to dendritic spines, shown to be associated
18 with fatherhood in the present study, is evocative of similar changes seen in a recent
19 study of the MPOA of mother rats (Parent et al., 2017). *Rem2*, a gene associated with
20 reduction of dendritic branching but increases in spine density (Ghiretti et al., 2014;
21 Ghiretti and Paradis, 2011) was increased in the MPOA of high licking/grooming rats,
22 but only in lactating mothers (not in virgins). This increase was accompanied by
23 decreased dendritic complexity. *Rem2* is involved with GTPase activity and GTP

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 binding. While we did not see alterations in *Rem2* expression in this study, we found
2 differential expression of several genes that are involved in Ras and Rap1 signaling. Both
3 Ras and Rap1 are GTPases that play an important role in the structural and functional
4 plasticity of cells (Cahill et al., 2016; Zhu et al., 2002).

5 The down-regulation of genes associated with dendritic complexity in the present
6 study, as well as the study by Parent and colleagues, is similar to what one would expect
7 in an animal that had experienced high amounts of stress. It is well established that stress,
8 mediated by corticotropin-releasing hormone, results in a loss of dendritic spines (Chen
9 et al., 2008; Chen et al., 2013; Leuner and Shors, 2013; Liao et al., 2014; Radley et al.,
10 2006). Interestingly, rat mothers show a decrease in the number and density of dendritic
11 spines in the amygdala and stria terminalis four days after birth (Matsuo et al., 2017), and
12 an increase in dendritic spine density in the hippocampus during the postpartum period
13 (Kinsley et al., 2006). This suggests that alterations in dendritic spine density in mothers
14 are both transient and region specific. More studies must be done to determine if the same
15 holds true for vole fathers.

16 In many species, the transition to fatherhood is associated with a suite of behavioral
17 and hormonal changes, including those indicative of stress. In California mice
18 (*Peromyscus californicus*), fathers exhibit attenuated anxiety-like behavior approximately
19 two weeks after pups are born (Glasper et al., 2016; Hyer et al., 2016). Human males
20 show a peak in cortisol levels during the transition to fatherhood (Storey et al., 2000), but
21 this is highly variable across studies (Gordon et al., 2010). Prairie vole fathers show
22 increased anxiety-like behavior, and chronic pup exposure results in an increase in basal
23 CORT levels (Lieberwirth et al., 2013). In an open field test, fathers spent more time in

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 corner squares, and in an elevated plus maze, fathers spent less time in open arms. In
2 forced swim tests, fatherhood decreased the latency to immobility, and increased the
3 number and duration of immobility bouts (Lieberwirth et al., 2013). Additionally, males
4 with fathering experience showed fewer BrdU-labeled cells in the amygdala,
5 hippocampus, and ventromedial hypothalamus than virgin males (Lieberwirth et al.,
6 2013). In the long term, fatherhood may be beneficial for male health, but the transition
7 to fatherhood is a tremendously stressful period (Bartlett, 2004).

8 In male voles with fathering experience we also see the upregulation of genes
9 related to protein processing in the endoplasmic reticulum. The endoplasmic reticulum is
10 instrumental in managing the protein folding process, including disposing of misfolded
11 proteins (Zhang and Kaufman, 2008). Homeostatic imbalances, including stress, can alter
12 the functioning of the endoplasmic reticulum, leading to the initiation of the unfolded-
13 protein response, which can in turn lead to apoptosis (Banhegyi et al., 2007; Mandl et al.,
14 2009). This may be one mechanism by which physiological stress can result in
15 homeostatic perturbations (Walter and Ron, 2011; Zhang and Kaufman, 2006), including
16 some of the changes that are evident in vole fathers, such as weight loss (Campbell et al.,
17 2009; Kenkel et al., 2014).

18 While many of the changes we saw in gene expression may be partially
19 attributable to stress, there are likely many other additional factors at play. Fathers in
20 many species show systematic endocrine changes (Saltzman and Ziegler, 2014).
21 Environmental factors, including changes in the types and amount of sensory stimulation,
22 or the amount of parental care they received, may play a role as well (Braun and

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Champagne, 2014; Champagne, 2016). Much more work must be done to tease apart
2 these many factors.
3 In this study we saw the most varied and interesting differences between the
4 paired males and males with fathering experience. This was surprising, as we expected
5 that the greatest differences would be between the virgin males and fathers. However,
6 examination of the quantitative results begins to clarify these findings (Figs 5 and 8). The
7 expression of genes of interest is slightly elevated in paired animals relative to virgins,
8 but the expression in fathers is decreased relative to virgins. Thus, while the expression
9 levels of some genes do not significantly differ between virgins and paired males, and
10 virgins and fathers, we found significant differences between paired males and fathers.
11 This may suggest that the experience of fathering is functionally distinct from any other
12 type of social interactions that these animals have encountered.
13
14 *4.1 Conclusions*
15 The purpose of this study was to explore how gene expression changed across the
16 transition to fatherhood, and to identify novel targets to allow for deeper investigation of
17 male parenting behavior. The use of RNA sequencing confirmed that there are
18 differences in gene expression between voles that had different social experiences,
19 including virgin males, males that had formed a pair bond with a female, and males with
20 parenting experience. The genes identified in this study suggest novel processes that are
21 related to paternal behavior and offer new targets for the further exploration of fathering
22 behavior.
23

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Acknowledgments:

2 The sequencing was carried out at the DNA Technologies and Expression Analysis Cores
3 at the UC Davis Genome Center, supported by NIH Shared Instrumentation Grant
4 1S10OD010786-01, and was made possible by a pilot grant from the UC Davis Genome
5 Center. Thanks to Chris Harshaw for helpful analytical suggestions. Thanks to Cindy
6 Clayton and Rhonda Oates-O'Brien for husbandry and veterinary care of the prairie vole
7 colony. And special thanks to the anonymous reviewers for their helpful and constructive
8 comments on this manuscript.

9

10

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Table 1: RNA data
2

ID	Raw Reads	Trimmed Reads	% Reads Kept	% Aligned	% Aligned to rRNA
V1	25764898	25657648	99.58373598	95.1	5.67
V2	20747099	20665472	99.60656186	95.99	2.63
V3	23633483	23500072	99.43550005	96.13	2.07
V4	17827384	17709907	99.34103063	96.06	2.38
V5	22459377	22360940	99.56171091	95.59	4.09
V6	22690769	22591612	99.56300732	96.09	2.78
P1	22451193	22317681	99.40532336	92.7	8.72
P2	23931482	23837973	99.60926365	95.84	2.26
P3	21258757	21155922	99.51626993	94.97	5.00
P4	20114070	20011415	99.48963586	95.86	3.16
P5	21770575	21677246	99.57130668	96.11	2.06
P6	19282593	19185302	99.49544649	95.73	3.28
F1	20833672	20702987	99.3727222	95.14	4.82
F2	21680207	21568888	99.48654088	95.99	2.53
F3	20565896	20479195	99.57842342	96.06	1.65
F4	19759347	19680046	99.59866589	96.17	2.27
F5	21472445	21367346	99.51054014	95.95	3.06
F6	22055401	21961628	99.57482977	96.31	2.32

3
4

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Table 2: Virgin vs Paired Gene Ontology Annotations
2

GO ID	GO Annotation	# genes	Raw P Value
GO:0032286	central nervous system myelin maintenance	4	0.003
GO:0044224	juxtaparanode region of axon	6	0.0034
GO:0019933	cAMP-mediated signaling	61	0.0056
GO:0045597	positive regulation of cell differentiation	475	0.006
GO:0050790	regulation of catalytic activity	800	0.0063
GO:0048406	nerve growth factor binding	4	0.0076
GO:0035749	myelin sheath adaxonal region	4	0.0078
GO:0042043	neurexin family protein binding	5	0.008
GO:0008277	regulation of G-protein coupled receptor protein signaling pathway	53	0.0081
GO:0061002	negative regulation of dendritic spine morphogenesis	4	0.0083
GO:0007218	neuropeptide signaling pathway	16	0.0092
GO:0071277	cellular response to calcium ion	28	0.0106
GO:0042102	positive regulation of T cell proliferation	38	0.0117
GO:0098656	anion transmembrane transport	58	0.012
GO:0042048	olfactory behavior	6	0.0129
GO:0044548	S100 protein binding	9	0.0132
GO:0043679	axon terminus	29	0.0138
GO:0048485	sympathetic nervous system development	13	0.014
GO:0061014	positive regulation of mRNA catabolic process	24	0.0145
GO:0006401	RNA catabolic process	107	0.0147
GO:0005184	neuropeptide hormone activity	5	0.0152
GO:0043220	Schmidt-Lanterman incisure	8	0.0158
GO:0002052	positive regulation of neuroblast proliferation	12	0.0158
GO:1902711	GABA-A receptor complex	4	0.016
GO:1900271	regulation of long-term synaptic potentiation	11	0.0168
GO:0051965	positive regulation of synapse assembly	49	0.0176
GO:2000144	positive regulation of DNA-templated transcription, initiation	10	0.018
GO:0022851	GABA-gated chloride ion channel activity	3	0.018
GO:0000123	histone acetyltransferase complex	56	0.0186
GO:0035976	transcription factor AP-1 complex	5	0.019
GO:0008626	granzyme-mediated apoptotic signaling pathway	3	0.0194
GO:0021879	forebrain neuron differentiation	38	0.0198
GO:0048011	neurotrophin TRK receptor signaling pathway	15	0.0202
GO:2000147	positive regulation of cell motility	235	0.0212
GO:0071933	Arp2/3 complex binding	4	0.0216
GO:0005125	cytokine activity	15	0.0217

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

GO:0007271	synaptic transmission, cholinergic	7	0.0228
GO:0035176	social behavior	31	0.0249
GO:0035198	miRNA binding	11	0.0255
GO:0070723	response to cholesterol	10	0.0256
GO:0005856	cytoskeleton	919	0.0257
GO:0005272	sodium channel activity	23	0.0263
GO:0042391	regulation of membrane potential	194	0.0276
GO:0050775	positive regulation of dendrite morphogenesis	13	0.0283
GO:0008188	neuropeptide receptor activity	15	0.0299
GO:0006814	sodium ion transport	69	0.0316
GO:0008021	synaptic vesicle	52	0.0355

1

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Table 3: Virgin vs Father Gene Ontology Annotations
2

GO ID	GO Annotation	# genes	Raw P Value
GO:0001975	response to amphetamine	11	0.00033
GO:0016310	phosphorylation	981	0.00097
GO:0007191	adenylate cyclase-activating dopamine receptor signaling pathway	6	0.00172
GO:1903861	positive regulation of dendrite extension	16	0.00206
GO:0043278	response to morphine	7	0.00239
GO:0060391	positive regulation of SMAD protein signal transduction	8	0.00258
GO:0005254	chloride channel activity	29	0.0027
GO:0000082	G1/S transition of mitotic cell cycle	95	0.00378
GO:0005516	calmodulin binding	36	0.0038
GO:0042110	T cell activation	210	0.00406
GO:0008091	spectrin	3	0.006
GO:0043406	positive regulation of MAP kinase activity	98	0.00781
GO:0071277	cellular response to calcium ion	28	0.0082
GO:0019228	neuronal action potential	12	0.0093
GO:0097440	apical dendrite	4	0.0098
GO:0030857	negative regulation of epithelial cell differentiation	23	0.01001
GO:0051098	regulation of binding	214	0.01046
GO:0007626	locomotory behavior	143	0.01116
GO:0014002	astrocyte development	18	0.01447
GO:0010862	positive regulation of pathway-restricted SMAD protein phosphorylation	20	0.0151
GO:0007249	I-kappaB kinase/NF-kappaB signaling	104	0.01513
GO:0048681	negative regulation of axon regeneration	9	0.01835
GO:0005815	microtubule organizing center	413	0.0185
GO:0048715	negative regulation of oligodendrocyte differentiation	10	0.0188
GO:0001963	synaptic transmission, dopaminergic	19	0.01883
GO:0001726	ruffle	80	0.0191
GO:0008023	transcription elongation factor complex	29	0.0194
GO:0019233	sensory perception of pain	38	0.0198
GO:0060158	phospholipase C-activating dopamine receptor signaling pathway	4	0.02016
GO:0043235	receptor complex	178	0.0212
GO:0002407	dendritic cell chemotaxis	6	0.02176
GO:0005912	adherens junction	151	0.0224
GO:0019538	protein metabolic process	2271	0.02608
GO:0017146	NMDA selective glutamate receptor complex	8	0.0274

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

GO:0042098	T cell proliferation	88	0.03093
GO:0031340	positive regulation of vesicle fusion	4	0.03095
GO:0003909	DNA ligase activity	3	0.0332
GO:0002682	regulation of immune system process	531	0.03451
GO:0005921	gap junction	9	0.0375
GO:0000778	condensed nuclear chromosome kinetochore	4	0.0399
GO:0071144	heteromeric SMAD protein complex	2	0.0451
GO:0035240	dopamine binding	4	0.0455
GO:0099604	ligand-gated calcium channel activity	19	0.0469
GO:0032444	activin responsive factor complex	2	0.0471
GO:0005247	voltage-gated chloride channel activity	2	0.0475
GO:0001591	dopamine neurotransmitter receptor activity, coupled via Gi/Go	2	0.0479
GO:0001042	RNA polymerase I core binding	2	0.0493

1

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Table 4: Paired vs Father Gene Ontology Annotations
2

GO ID	GO Annotation	# genes	Raw P Value
GO:0005955	calcineurin complex	4	0.00104
GO:0050840	extracellular matrix binding	33	0.00172
GO:0046959	habituation	4	0.0031
GO:0007626	locomotory behavior	144	0.0032
GO:2001223	negative regulation of neuron migration	7	0.004
GO:0046330	positive regulation of JNK cascade	64	0.0043
GO:0060079	excitatory postsynaptic potential	36	0.0043
GO:0060391	positive regulation of SMAD protein signal transduction	8	0.0047
GO:0015116	sulfate transmembrane transporter activity	3	0.00473
GO:0070723	response to cholesterol	10	0.0048
GO:0001696	gastric acid secretion	6	0.005
GO:0051281	positive regulation of release of sequestered calcium ion into cytosol	20	0.005
GO:0060395	SMAD protein signal transduction	38	0.0051
GO:0042755	eating behavior	10	0.0057
GO:0033192	calmodulin-dependent protein phosphatase activity	4	0.00583
GO:0000403	Y-form DNA binding	4	0.00644
GO:0010001	glial cell differentiation	118	0.0065
GO:0048407	platelet-derived growth factor binding	11	0.0071
GO:0017134	fibroblast growth factor binding	11	0.00772
GO:0016575	histone deacetylation	32	0.008
GO:0045893	positive regulation of transcription, DNA-templated	783	0.009
GO:0005516	calmodulin binding	36	0.01032
GO:0007616	long-term memory	18	0.0104
GO:0005882	intermediate filament	30	0.01192
GO:0061014	positive regulation of mRNA catabolic process	24	0.0122
GO:0060080	inhibitory postsynaptic potential	9	0.0124
GO:0035418	protein localization to synapse	22	0.0141
GO:0008009	chemokine activity	4	0.01592
GO:0007015	actin filament organization	169	0.0166
GO:0070410	co-SMAD binding	8	0.01861
GO:0007212	dopamine receptor signaling pathway	20	0.0198
GO:0001973	adenosine receptor signaling pathway	5	0.0199
GO:0005102	signaling receptor binding	554	0.02035
GO:0000978	RNA polymerase II proximal promoter sequence-specific DNA binding	232	0.02098

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

GO:0005736	DNA-directed RNA polymerase I complex	7	0.02166
GO:0004930	G-protein coupled receptor activity	110	0.02186
GO:0050882	voluntary musculoskeletal movement	6	0.0224
GO:0000307	cyclin-dependent protein kinase holoenzyme complex	27	0.02343
GO:0030374	ligand-dependent nuclear receptor transcription coactivator activity	24	0.02383
GO:0097110	scaffold protein binding	35	0.02393
GO:0008622	epsilon DNA polymerase complex	3	0.02522
GO:0005881	cytoplasmic microtubule	29	0.02579
GO:0000118	histone deacetylase complex	28	0.02583
GO:0099061	integral component of postsynaptic density membrane	2	0.02667
GO:0044309	neuron spine	40	0.02738
GO:0044295	axonal growth cone	7	0.02809
GO:0071144	heteromeric SMAD protein complex	2	0.02824
GO:0043197	dendritic spine	37	0.02853
GO:0043235	receptor complex	179	0.0291
GO:0015271	outward rectifier potassium channel activity	5	0.0317
GO:0042805	actinin binding	16	0.03185
GO:0003700	DNA binding transcription factor activity	385	0.03189
GO:0019905	syntaxin binding	25	0.03526
GO:0098831	presynaptic active zone cytoplasmic component	2	0.03658
GO:0005794	Golgi apparatus	562	0.04007
GO:0000976	transcription regulatory region sequence-specific DNA binding	368	0.04263
GO:0017016	Ras GTPase binding	161	0.04338
GO:0030864	cortical actin cytoskeleton	34	0.04449
GO:0014069	postsynaptic density	77	0.04529
GO:0060053	neurofilament cytoskeleton	2	0.04637
GO:0008076	voltage-gated potassium channel complex	41	0.04825

1

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Table 5: Differentially expressed genes
2

Comparison	Genes
V vs P	Cckar, Dnajc3, Enah, Hspa5, Hyou1, Pak3, Pdia3, Pdia4, Rala, Sorbs1, Th
V vs F	Arpc5, Baiap2, Cbl, Chrm1, Chrna1, Cyp2s1, Derl1, Dnajc3, Elovl1, Elovl6, Enah, Epha2, Erp29, Faah, Glra3, Hspa5, Itgb4, Kcnj4, Ksr1, Lamtor3, Nf2, Pdia3, Pdia4, Pdia6, Pigh, Pigo, Pla2g16, Pomgnt2, Prkgc, Pvrl3, Rdx, Tram1, Txndc5
P vs F	Adcy4, Adora2a, Atp2b1, Baiap2, Cacna2d3, Cacnb3, Cckbr, Chrm1, Ddn, Dlg4, Gabrd, Gpr156, Grin2a, Grin2b, Ifngr1, Itpr1, Kcnj2, Kcnj4, Kcnn3, Kdr, Lama2, Ngef, P2rx3, Park2, Prkcg, Ptk2b, Rasgrf2, Rgs14, Rin1, Sipa1l1, Tiam1

3
4

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Table 6: Pathways associated with differentially expressed genes
2

Neurotransmitters	Adcy4, Adora2a, Cacna2d3, Cacnb3, Cckar, Cckbr, Chrm1, Dlg4, Gabrd, Gpr156, Grin2a, Grin2b, Itpr1, Kcnj2, Kcnj4, P2rx3, Prkcg, Rin1
Ca signaling	Adcy4, Adora2a, Cckar, Cckbr, Chrm1, Grin2a, Grin2b, Itpr1, P2rx3, Ptk2b, Rin1
OT signaling	Adcy4, Cacna2d3, Cacnb3, Kcnj2, Kcnj4, Itpr1, Prkcg
Regulation of actin cytoskeleton	Arpc5, Baiap2, Chrm1, Enah, Itgb4, Rdx, Tiam1, Enah, Pak3
Ras/Rap1 signaling	Adcy4, Adora2a, Epha2, Grin2a, Grin2b, Kdr, Ksr1, Pak3, Pla2g16, Prkgc, Rala, Rasgrf2, Rgs14, Rin1, Sipa1l1, Tiam1
Protein processing in ER	Derl1, Dnajc3, Erp29, Hspa5, Hyou1, Pdia3, Pdia4, Pdia6, Tram1, Txndc5
Thyroid hormone synthesis	Adcy4, Itpr1, Prkcg, Pdia4, Hspa5
LTP/LTD	Grin2a, Grin2b, Itpr1, Rin1

3
4

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Table 7: P-values
2

Gene Name	F vs P	F vs V	P vs V
Adora2a	0.1014	0.2444	0.9891
Baiap2	**0.0014**	0.0776	0.7946
Bdnf	0.6273	0.2702	0.4012
Cacna2d3	**0.0460**	**0.0326**	0.9266
Cacnb3	0.4862	0.5735	0.2516
Cckbr	**0.0120**	0.0767	0.7745
Chrm1	**0.0050**	0.0876	0.7353
Ddn	**0.0021**	0.0829	0.6383
Dlg4	0.2146	0.6877	0.2711
Gabrd	**0.0018**	0.0803	0.5384
Gal	0.6783	0.2519	0.1944
Gpr156	0.8590	0.7849	0.8507
Grin2a	0.2294	0.1292	0.7381
Grin2b	0.0778	0.0877	0.7291
Itpr1	**0.0500**	0.0906	0.8406
Kcnj2	0.3466	0.1956	0.7144
Kcnj4	**0.0474**	0.2362	0.8533
Kdr	0.2402	0.3649	0.8613
Negr1	0.5673	0.8722	0.4501
Ngef	**0.0061**	0.1282	0.5444
P2rx3	0.0605	0.3671	0.2208
Pde2a	0.0677	0.2581	0.6827
Prkcg	**0.0427**	**0.0390**	0.8554
Ptk2b	0.0729	0.0996	0.9024
Rasgrf2	0.1027	0.1289	0.8217
Rgs14	**0.0002**	**0.0237**	0.4867
Rin1	0.1646	0.0696	0.2535
Sipa1l1	0.0612	0.1155	0.5573
Th	0.4437	0.4343	0.1845
Tiam1	0.7299	0.2976	0.2510

3
4 **Bold** indicates significant values
5

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Table 8: Cohen's d values
2

Gene Name	F vs P	F vs V	P vs V
Adora2a	0.5380	**0.9243**	0.3863
Baiap2	**0.9039**	***1.2258***	0.3220
Bdnf	0.2066	0.6260	0.4194
Cacna2d3	**1.0766**	**1.1004**	0.0239
Cacnb3	0.4467	0.3052	0.7520
Cckbr	**0.9881**	**0.9884**	0.0003
Chrm1	**0.9371**	**1.0050**	0.0679
Ddn	**1.0688**	**1.0685**	0.0003
Dlg4	**0.8759**	0.1721	0.7037
Gabrd	**0.9480**	**0.9667**	0.0187
Gal	0.2902	0.4687	0.7589
Gpr156	0.0103	0.2844	0.2947
Grin2a	0.6531	**0.8749**	0.2218
Grin2b	***1.2351***	**1.0075**	0.2276
Itpr1	**0.8004**	**1.0156**	0.2152
Kcnj2	0.5576	0.7754	0.2178
Kcnj4	0.6183	**0.8591**	0.2407
Kdr	**0.8634**	0.6950	0.1683
Negr1	0.3141	**0.1085**	0.4227
Ngef	**1.1987**	**0.9933**	0.2054
P2rx3	**1.1447**	0.4699	0.6748
Pde2a	**0.9381**	0.7403	0.1978
Prkcg	***1.2933***	**1.1543**	0.1389
Ptk2b	**0.8524**	**0.9836**	0.1313
Rasgrf2	**0.9509**	0.7855	0.1654
Rgs14	***1.5018***	***1.2370***	0.2648
Rin1	0.7439	***1.4011***	0.6572
Sipa1l1	***1.2207***	**0.9080**	0.3127
Th	0.3234	0.4063	0.7297
Tiam1	0.3216	0.5269	**0.8484**

3 **Bold** indicates large effect (0.8 < d < 1.2)
4 ***Bold italics*** indicates very large effect (d > 1.2)
5

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Table 9:** Genes of interest

Gene ID	Gene Name	Function	GO Annotations
Baiap2	Brain-specific angiogenesis inhibitor 1-Associated protein 2	Insulin receptor tyrisone kinase substrate	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite, Dendritic Spine, Synapse
Cacna2d3	Calcum voltage gated channel, auxiliary subunit alpha 2 delta 3	Voltage gated calcium channel	Ion Channel Activity
Cckbr	Cholecystokinin B receptor	Multipass transmembrane receptor protein	Signaling, Regulation of Biological Quality
Chrm1	Cholinergic receptor, muscarinic 1	Muscarinic receptor	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite, Synapse
Ddn	Dendrin	plasma membrane surrounding dendritic spine	Membrane Part
Gabrd	GABA A receptor, subunit delta	GABA receptor	Signaling, Dendrite, Synapse, Ion Channel Activity
Grin2b	Glutamate receptor, ionotropic, NMDA 2b	NMDA receptor	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite, Dendritic Spine, Synapse, Ion Channel Activity
Itpr1	inositol 1,4,5-triphosphate receptor 1	Calcium channel	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite, Synapse, Ion Channel Activity
Kcnj4	potassium voltage gated channel subfamily J member 4	potassium channels - ion homeostasis	Membrane Part, Dendrite, Synapse, Ion Channel Activity
Ngef	Neuronal guanine nucleotide exchange factor	dendritic spine morphogenesis	Signaling, Membrane Part
P2rx3	purinergic receptor p2x, ligand gated ion channel	ATP receptor	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite, Dendritic Spine, Synapse, Ion Channel Activity
Pde2a	Phosphodiesterase 2a	2nd messenger signaling/dendritic spines	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite
Prkcg	protein kinase c gamma	signaling protein	Signaling, Membrane Part, Dendrite, Synapse
Ptk2b	protein tyrosine kinase 2 beta	ion channel regulation; MapK signaling	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite, Dendritic Spine, Synapse, Ion Channel Activity
Rgs14	regulator of g-protein signaling 14	scaffold protein	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite, Dendritic Spine, Synapse
Rin1	Ras and Rab interactor 1	Ras effector	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Sipa1l1	signal induced proliferation associate 1 like 1	Ras effector	Signaling, Regulation of Biological Quality, Membrane Part, Dendrite, Dendritic Spine, Synapse

1
2

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Figure Legends:**

2 **Figure 1:** Timeline of social interactions across the lifespan. All subjects were separated
3 from their parents and removed from their home cage at postnatal day (P) 20 and housed
4 with a same-sex conspecific. Around P42, males assigned to the “paired” and “father”
5 groups were rehoused with an opposite-sex conspecific, while males in the “virgin”
6 group remained with their same-sex conspecific. 20 days later (~P62), the subjects in the
7 “virgin” and “paired” groups were euthanized and their brains were removed. One day
8 later (~P63), pups were born to the males in the “father” group, and these males were
9 euthanized 2-3 days later (~P65).

10

11 **Figure 2:** A schematic representing the area from which tissue samples were taken. The
12 circumference of the tissue punch is delineated by a circle, and the MPOA is outlined in
13 black. The tissue punches removed the entirety of the MPOA, as well as small portions of
14 adjacent hypothalamic tissue.

15

16 **Figure 3:** Analysis plan for RNA sequencing experiment. Following the collection of
17 brain tissue, RNA was extracted from the MPOA and sequenced at the UC Davis
18 Expression Analysis Core facility. The resulting sequences were linked to specific genes
19 and analyzed for relative frequency at the UC Davis Bioinformatics Core facility. The top
20 500 differentially expressed genes in each comparison group were analyzed using the
21 Gene Ontology database (geneontology.org) and Kegg Pathways analysis (www.kegg.jp).
22 These analyses allowed us to identify a subset of genes of interest and their associated
23 biological processes that were differentially expressed in each comparison group. We

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 then analyzed the interrelatedness of these groups of genes using STRING Database
2 analysis (string-db.org). Finally, the expression of 30 genes of interest was quantitatively
3 expressed using the NanoString Analysis.
4
5 **Figure 4:** Enrichment of Gene Ontology Annotations across comparison groups. The
6 gene enrichment analysis grouped the differentially expressed genes using gene ontology
7 annotations data. We selected significantly enriched GO annotations and identified the
8 annotations that were involved in brain or behavioral processes. Those annotations were
9 then categorized by function within each comparison group. We identified 9 functional
10 groups: plasticity (red), DNA/RNA/Transcription (blue), Axon/Dendrite/Synapse
11 (yellow), Ion channel/Receptor (purple), Neuropeptides (green), Immune function
12 (orange), Metabolism (brown), Glia (white), and Other (gray). We saw differences in the
13 relative distribution of GO annotation functional groups across the comparison groups.
14 Neuropeptides were only seen in the *V vs P* group, whereas the *V vs F* group showed a
15 high number of annotations related to Immune Function. The *P vs F* group contained the
16 largest number of annotations related to Plasticity, DNA/RNA/Transcription, and
17 Axon/Dendrite/Synapse.
18
19 **Figure 5:** Gene expression in paired males and fathers relative to virgin males. Using the
20 Kegg Pathways analysis, we identified nine pathways of biological or behavioral
21 significance and their associated differentially expressed genes. The mean expression of
22 genes associated with each pathway in fathers was averaged and compared against
23 expression in virgin males. On the whole, gene expression was decreased in fathers

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 relative to both virgins and paired males. Of the nine pathways, only one showed an
2 increase in gene expression in fathers (protein processing in the endoplasmic reticulum),
3 while five showed decreases in gene expression in fathers (Ras/Rap1 signaling, Oxytocin
4 signaling, Neurotransmitters, Calcium signaling, and LTP/LTD). The overall average
5 gene expression is indicated by black stars. Values that exhibited medium effect sizes
6 (0.5 < Cohen's d < 0.8) are indicated by blue squares and values that exhibited large
7 effect sizes (Cohen's d > 0.8) are indicated by red triangles).
8
9 **Figure 6:** STRING Database analysis of gene product interaction networks. Selected
10 differentially expressed genes were run through the STRING database of gene product
11 interactions, and networks were generated for each comparison. A) Paired vs Virgin
12 network. B) Virgin vs Father. C) Paired vs Father.
13
14 **Figure 7:** Heat map representing the relative expression of individual genes in virgin
15 males, paired males, and males with fathering experience. Gene enrichment is encoded in
16 the heat map ranging from low (green) to high (red). Genes that show similar expression
17 patterns are clustered together, as indicated by the dendrogram to the left of the heat map.
18
19 **Figure 8**: Quantitative analysis of differential gene expression. A) A scatterplot showing
20 B-O) Box and whisker plots showing the expression of genes in virgins, paired males,
21 and fathers. The whiskers represent 1.5x the interquartile range. While we quantified 30
22 genes, here we show the 14 genes that exhibited significantly different levels of
23 expression across groups or exhibited large effect size. In each gene of these genes,

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 expression in fathers was lower than expression in paired males, and in three cases
2 (Rgs14, Prkcg, and Cacna2d3) expression in fathers was also significantly lower than in
3 virgin males. * - Significantly differs from fathers ($p < 0.05$). † - Large effect size
4 compared to fathers (Cohen's $d > 0.8$).
5
6

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **References:**
2

3 Alexa A, Rahnenfuhrer J. 2016. topGo: Enrichment analysis for gene ontology. Version
4 R package version 2.32.0.
5 Arias Del Razo R, Bales KL. 2016. Exploration in a dispersal task: Effects of early
6 experience and correlation with other behaviors in prairie voles (Microtus
7 ochrogaster). Behavioural processes 132:66-75.
8 Bakermans-Kranenburg MJ, van Ijzendoorn MH. 2008. Oxytocin receptor (OXTR) and
9 serotonin transporter (5-HTT) genes associated with observed parenting. Soc
10 Cogn Affect Neurosci 3(2):128-134.
11 Bales K, French JA, Dietz JM. 2002. Explaining variation in maternal care in a
12 cooperatively breeding mammal. Animal Behaviour 63:453-461.
13 Bales KL, Saltzman W. 2016. Fathering in rodents: Neurobiological substrates and
14 consequences for offspring. Horm Behav 77:249-259.
15 Banhegyi G, Baumeister P, Benedetti A, Dong D, Fu Y, Lee AS, Li J, Mao C, Margittai
16 E, Ni M, Paschen W, Piccirella S, Senesi S, Sitia R, Wang M, Yang W. 2007.
17 Endoplasmic reticulum stress. Annals of the New York Academy of Sciences
18 1113:58-71.
19 Bartlett E. 2004. The effects of fatherhood on the health of men: A review of the
20 literature. The Journal of Men's Health and Gender 1(2-3):159-169.
21 Bendesky A, Kwon YM, Lassance JM, Lewarch CL, Yao S, Peterson BK, He MX, Dulac
22 C, Hoekstra HE. 2017. The genetic basis of parental care evolution in
23 monogamous mice. Nature.
24 Bester-Meredith JK, Marler CA. 2003. Vasopressin and the transmission of paternal
25 behavior across generations in mated, cross-fostered Peromyscus mice.
26 Behavioral Neuroscience 117(3):455-463.
27 Braun K, Champagne FA. 2014. Paternal influences on offspring development:
28 behavioural and epigenetic pathways. Journal of neuroendocrinology 26(10):697-
29 706.
30 Cahill ME, Bagot RC, Gancarz AM, Walker DM, Sun H, Wang ZJ, Heller EA, Feng J,
31 Kennedy PJ, Koo JW, Cates HM, Neve RL, Shen L, Dietz DM, Nestler EJ. 2016.
32 Bidirectional Synaptic Structural Plasticity after Chronic Cocaine Administration
33 Occurs through Rap1 Small GTPase Signaling. Neuron 89(3):566-582.
34 Campbell JC, Laugero KD, Van Westerhuyzen JA, Hostetler CM, Cohen JD, Bales KL.
35 2009. Costs of pair-bonding and paternal care in male prairie voles (Microtus
36 ochrogaster). Physiology & behavior 98(3):367-373.
37 Cantoni D, Brown RE. 1997. Paternal investment and reproductive success in the
38 California mouse, Peromyscus californicus. Animal behaviour 54(2):377-386.
39 Champagne FA. 2016. Epigenetic legacy of parental experiences: Dynamic and
40 interactive pathways to inheritance. Dev Psychopathol 28(4pt2):1219-1228.
41 Chen Y, Dube CM, Rice CJ, Baram TZ. 2008. Rapid loss of dendritic spines after stress
42 involves derangement of spine dynamics by corticotropin-releasing hormone. J
43 Neurosci 28(11):2903-2911.
44 Chen Y, Kramar EA, Chen LY, Babayan AH, Andres AL, Gall CM, Lynch G, Baram
45 TZ. 2013. Impairment of synaptic plasticity by the stress mediator CRH involves

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

selective destruction of thin dendritic spines via RhoA signaling. Mol Psychiatry 18(4):485-496.

Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, Szczesniak MW, Gaffney DJ, Elo LL, Zhang X, Mortazavi A. 2016. A survey of best practices for RNA-seq data analysis. Genome Biol 17:13.

de Jong TR, Chauke M, Harris BN, Saltzman W. 2009. From here to paternity: neural correlates of the onset of paternal behavior in California mice (Peromyscus californicus). Horm Behav 56(2):220-231.

Dulac C, O'Connell LA, Wu Z. 2014. Neural control of maternal and paternal behaviors. Science 345(6198):765-770.

Eisinger BE, Zhao C, Driessen TM, Saul MC, Gammie SC. 2013. Large scale expression changes of genes related to neuronal signaling and developmental processes found in lateral septum of postpartum outbred mice. PloS one 8(5):e63824.

Feldman R, Zagoory-Sharon O, Weisman O, Schneiderman I, Gordon I, Maoz R, Shalev I, Ebstein RP. 2012. Sensitive parenting is associated with plasma oxytocin and polymorphisms in the OXTR and CD38 genes. Biological psychiatry 72(3):175-181.

Frazier CR, Trainor BC, Cravens CJ, Whitney TK, Marler CA. 2007. Paternal behavior influences development of aggression and vasopressin expression in male California mouse offspring. Hormones and Behavior 50:699-707.

Gammie SC, Auger AP, Jessen HM, Vanzo RJ, Awad TA, Stevenson SA. 2007. Altered gene expression in mice selected for high maternal aggression. Genes Brain Behav 6(5):432-443.

Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, James JJ, Maysuria M, Mitton JD, Oliveri P, Osborn JL, Peng T, Ratcliffe AL, Webster PJ, Davidson EH, Hood L, Dimitrov K. 2008. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol 26(3):317-325.

Ghiretti AE, Moore AR, Brenner RG, Chen LF, West AE, Lau NC, Van Hooser SD, Paradis S. 2014. Rem2 is an activity-dependent negative regulator of dendritic complexity in vivo. J Neurosci 34(2):392-407.

Ghiretti AE, Paradis S. 2011. The GTPase Rem2 regulates synapse development and dendritic morphology. Developmental neurobiology 71(5):374-389.

Glasper ER, Hyer MM, Katakam J, Harper R, Ameri C, Wolz T. 2016. Fatherhood contributes to increased hippocampal spine density and anxiety regulation in California mice. Brain Behav 6(1):e00416.

Glasper ER, Kozorovitskiy Y, Pavlic A, Gould E. 2011. Paternal experience suppresses adult neurogenesis without altering hippocampal function in Peromyscus californicus. J Comp Neurol 519(11):2271-2281.

Gordon I, Zagoory-Sharon O, Leckman JF, Feldman R. 2010. Oxytocin, cortisol, and triadic family interactions. Physiology & behavior 101(5):679-684.

Gubernick DJ, Alberts JR. 1987. The biparental care system of the California mouse, Peromyscus californicus. J Comp Psychol 101(2):169-177.

Hyer MM, Hunter TJ, Katakam J, Wolz T, Glasper ER. 2016. Neurogenesis and anxiety-like behavior in male California mice during the mate's postpartum period. The European journal of neuroscience 43(5):703-709.

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Insel TR, Preston S, Winslow JT. 1995. Mating in the monogamous male: behavioral
2 consequences. Physiology & behavior 57(4):615-627.
3 Kenkel WM, Paredes J, Yee JR, Pournajafi-Nazarloo H, Bales KL, Carter CS. 2012.
4 Neuroendocrine and behavioural responses to exposure to an infant in male
5 prairie voles. Journal of neuroendocrinology 24(6):874-886.
6 Kenkel WM, Suboc G, Carter CS. 2014. Autonomic, behavioral and neuroendocrine
7 correlates of paternal behavior in male prairie voles. Physiology & behavior
8 128:252-259.
9 Kinsley CH, Trainer R, Stafisso-Sandoz G, Quadros P, Marcus LK, Hearon C, Meyer
10 EA, Hester N, Morgan M, Kozub FJ, Lambert KG. 2006. Motherhood and the
11 hormones of pregnancy modify concentrations of hippocampal neuronal dendritic
12 spines. Horm Behav 49(2):131-142.
13 Kirkpatrick B, Kim JW, Insel TR. 1994. Limbic system fos expression associated with
14 paternal behavior. Brain Res 658(1-2):112-118.
15 Kleiman DG. 1977. Monogamy in mammals. Quarterly Review of Biology 52:39-69.
16 Kohl J, Babayan BM, Rubinstein ND, Autry AE, Marin-Rodriguez B, Kapoor V,
17 Miyamishi K, Zweifel LS, Luo L, Uchida N, Dulac C. 2018. Functional circuit
18 architecture underlying parental behaviour. Nature 556(7701):326-331.
19 Kohl J, Dulac C. 2018. Neural control of parental behaviors. Curr Opin Neurobiol
20 49:116-122.
21 Kozorovitskiy Y, Hughes M, Lee K, Gould E. 2006. Fatherhood affects dendritic spines
22 and vasopressin V1a receptors in the primate prefrontal cortex. Nat Neurosci
23 9(9):1094-1095.
24 Kuroda KO, Numan M. 2014. The medial preoptic area and the regulation of parental
25 behavior. Neurosci Bull 30(5):863-865.
26 Lee AW, Brown RE. 2002. Medial preoptic lesions disrupt parental behavior in both
27 male and female California mice (Peromyscus californicus). Behavioral
28 neuroscience 116(6):968-975.
29 Leuner B, Shors TJ. 2013. Stress, anxiety, and dendritic spines: what are the connections?
30 Neuroscience 251:108-119.
31 Liao XM, Yang XD, Jia J, Li JT, Xie XM, Su YA, Schmidt MV, Si TM, Wang XD.
32 2014. Blockade of corticotropin-releasing hormone receptor 1 attenuates early-life
33 stress-induced synaptic abnormalities in the neonatal hippocampus. Hippocampus
34 24(5):528-540.
35 Lieberwirth C, Wang Y, Jia X, Liu Y, Wang Z. 2013. Fatherhood reduces the survival of
36 adult-generated cells and affects various types of behavior in the prairie vole
37 (Microtus ochrogaster). The European journal of neuroscience 38(9):3345-3355.
38 Lukas D, Clutton-Brock TH. 2013. The evolution of social monogamy in mammals.
39 Science 341(6145):526-530.
40 Mandl J, Meszaros T, Banhegyi G, Hunyady L, Csala M. 2009. Endoplasmic reticulum:
41 nutrient sensor in physiology and pathology. Trends Endocrinol Metab 20(4):194-
42 201.
43 Matsuo S, Matsuda KI, Takanami K, Mori T, Tanaka M, Kawata M, Kitawaki J. 2017.
44 Decrease in neuronal spine density in the postpartum period in the amygdala and
45 bed nucleus of the stria terminalis in rat. Neurosci Lett 641:21-25.

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 McGraw LA, Davis JK, Lowman JJ, ten Hallers BF, Koriabine M, Young LJ, de Jong PJ,
2 Rudd MK, Thomas JW. 2010. Development of genomic resources for the prairie
3 vole (Microtus ochrogaster): construction of a BAC library and vole-mouse
4 comparative cytogenetic map. BMC genomics 11:70.
5 McGraw LA, Davis JK, Young LJ, Thomas JW. 2011. A genetic linkage map and
6 comparative mapping of the prairie vole (Microtus ochrogaster) genome. BMC
7 Genet 12:60.
8 Mendoza SP, Mason WA. 1997. Attachment relationships in New World primates.
9 Annals of the New York Academy of Sciences 807:203-209.
10 Numan M, Corodimas KP, Numan MJ, Factor EM, Piers WD. 1988. Axon-sparing
11 lesions of the preoptic region and substantia innominata disrupt maternal behavior
12 in rats. Behavioral neuroscience 102(3):381-396.
13 Oliveras D, Novak M. 1986. A comparison of paternal behaviour in the meadow vole
14 Microtus pennsylvanicus, the pine voles, M. pinetorum and the prairie vole M.
15 ochrogaster. Animal behaviour(34):519-526.
16 Opie C, Atkinson QD, Dunbar RI, Shultz S. 2013. Male infanticide leads to social
17 monogamy in primates. Proc Natl Acad Sci U S A 110(33):13328-13332.
18 Parent C, Wen X, Dhir SK, Ryan R, Diorio J, Zhang TY. 2017. Maternal care associates
19 with differences in morphological complexity in the medial preoptic area.
20 Behavioural brain research 326:22-32.
21 Pereira M. 2016. Structural and functional plasticity in the maternal brain circuitry. In:
22 Rutherford HJV, Mayes LC, editors. Maternal brain plasticity: Preclinical and
23 human research and implications for intervention New Directions for child and
24 adolescent development. p 23-46.
25 Perkeybile AM, Bales KL. 2015a. Early rearing experience is associated with vasopressin
26 immunoreactivity but not reactivity to an acute non-social stressor in the prairie
27 vole. Physiology & Behavior 147:149-156.
28 Perkeybile AM, Bales KL. 2015b. Early rearing experience is related to altered
29 aggression and vasopressin production following chronic social isolation in the
30 prairie vole. Behavioural Brain Research 283:37-46.
31 Perkeybile AM, Griffin LL, Bales KL. 2013. Natural variation in early parental care
32 correlates with social behaviors in adolescent prairie voles (*Microtus*
33 *ochrogaster*). Frontiers in Behavioral Neuroscience 7:21.
34 Radley JJ, Rocher AB, Miller M, Janssen WG, Liston C, Hof PR, McEwen BS, Morrison
35 JH. 2006. Repeated stress induces dendritic spine loss in the rat medial prefrontal
36 cortex. Cereb Cortex 16(3):313-320.
37 Rich ME, deCardenas EJ, Lee HJ, Caldwell HK. 2014. Impairments in the initiation of
38 maternal behavior in oxytocin receptor knockout mice. PloS one 9(6):e98839.
39 Rilling JK, Young LJ. 2014. The biology of mammalian parenting and its effect on
40 offspring social development. Science 345(6198):771-776.
41 Runcie MJ. 2000. Biparental care and obligate monogamy in the rock-haunting possum,
42 Petropseudes dahli, from tropical Australia. Animal behaviour 59(5):1001-1008.
43 Saltzman W, Ziegler TE. 2014. Functional significance of hormonal changes in
44 mammalian fathers. Journal of neuroendocrinology 26(10):685-696.

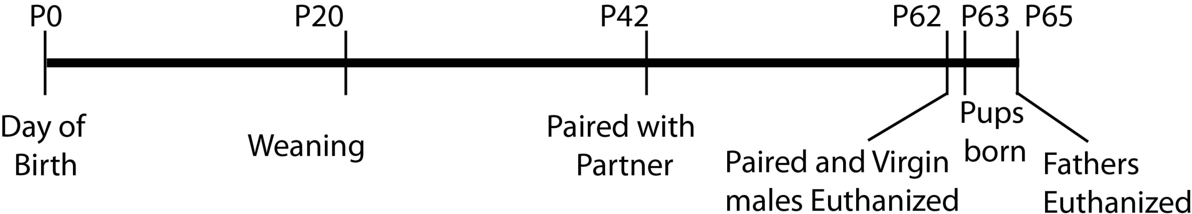
bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

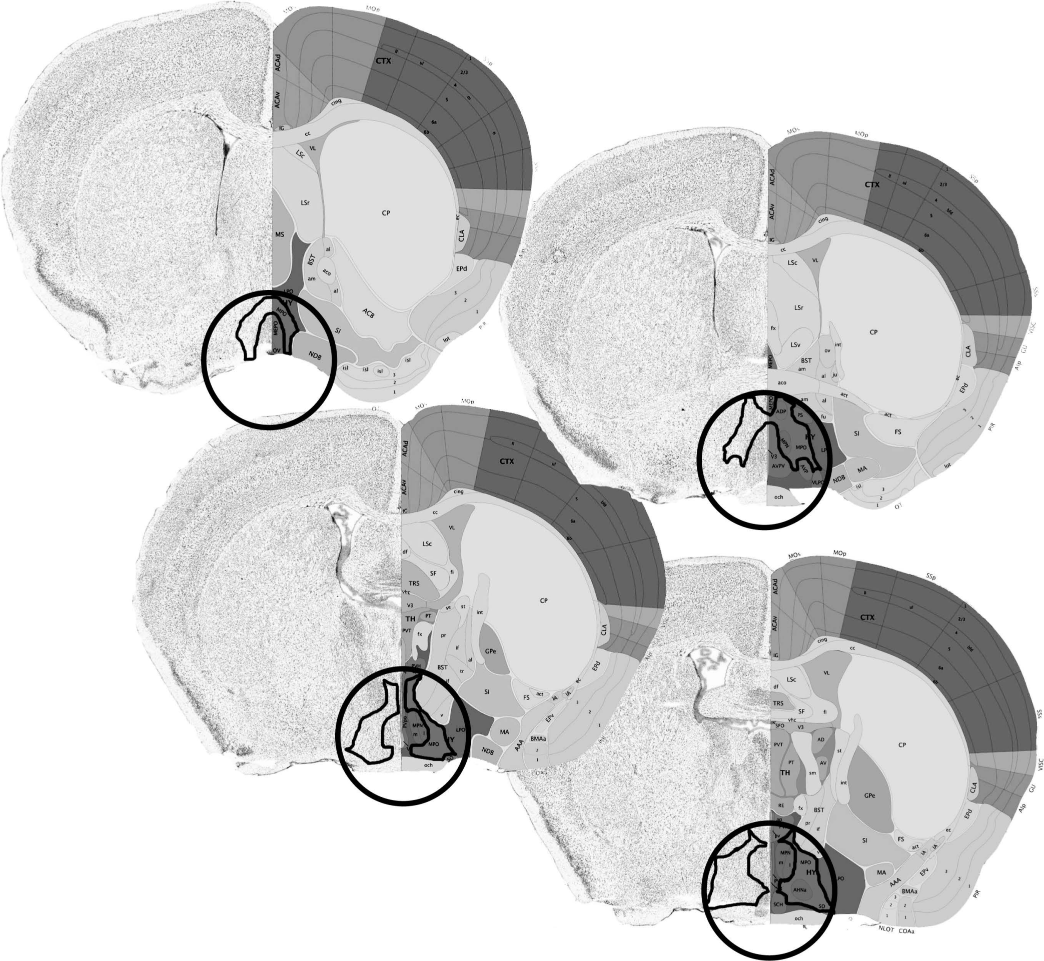
1 Stolzenberg DS, Champagne FA. 2016. Hormonal and non-hormonal bases of maternal
2 behavior: The role of experience and epigenetic mechanisms. Horm Behav
3 77:204-210.
4 Storey AE, Walsh CJ, Quinton RL, Wynne-Edwards KE. 2000. Hormonal correlates of
5 paternal responsiveness in new and expectant fathers. Evol Hum Behav 21(2):79-
6 95.
7 Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, Santos A,
8 Doncheva NT, Roth A, Bork P, Jensen LJ, von Mering C. 2017. The STRING
9 database in 2017: quality-controlled protein-protein association networks, made
10 broadly accessible. Nucleic acids research 45(D1):D362-D368.
11 Tabbaa M, Lei K, Liu Y, Wang Z. 2017. Paternal deprivation affects social behaviors and
12 neurochemical systems in the offspring of socially monogamous prairie voles.
13 Neuroscience 343:284-297.
14 Thomas JA, Birney EC. 1979. Parental Care and Mating System of the Prairie Vole,
15 Microtus-Ochrogaster. Behav Ecol Sociobiol 5(2):171-186.
16 Trainor BC, Bird IM, Alday NA, Schlinger BA, Marler CA. 2003. Variation in aromatase
17 activity in the medial preoptic area and plasma progesterone is associated with the
18 onset of paternal behavior. Neuroendocrinology 78(1):36-44.
19 Tsuneoka Y, Maruyama T, Yoshida S, Nishimori K, Kato T, Numan M, Kuroda KO.
20 2013. Functional, anatomical, and neurochemical differentiation of medial
21 preoptic area subregions in relation to maternal behavior in the mouse. J Comp
22 Neurol 521(7):1633-1663.
23 Walter P, Ron D. 2011. The unfolded protein response: from stress pathway to
24 homeostatic regulation. Science 334(6059):1081-1086.
25 Wang B, Li Y, Wu R, Zhang S, Tai F. 2015. Behavioral responses to pups in males with
26 different reproductive experiences are associated with changes in central OT, TH
27 and OTR, D1R, D2R mRNA expression in mandarin voles. Horm Behav 67:73-
28 82.
29 Wang Z, Gerstein M, Snyder M. 2009. RNA-Seq: a revolutionary tool for
30 transcriptomics. Nat Rev Genet 10(1):57-63.
31 Williams JR, Catania KC, Carter CS. 1992. Development of Partner Preferences in
32 Female Prairie Voles (Microtus-Ochrogaster) - the Role of Social and Sexual
33 Experience. Horm Behav 26(3):339-349.
34 Winslow JT, Hastings N, Carter CS, Harbaugh CR, Insel TR. 1993. A role for central
35 vasopressin in pair bonding in monogamous prairie voles. Nature 365(6446):545-
36 548.
37 Wynne-Edwards KE, Timonin ME. 2007. Paternal care in rodents: weakening support for
38 hormonal regulation of the transition to behavioral fatherhood in rodent animal
39 models of biparental care. Horm Behav 52(1):114-121.
40 Young LJ, Wang Z. 2004. The neurobiology of pair bonding. Nat Neurosci 7(10):1048-
41 1054.
42 Zhang K, Kaufman RJ. 2006. The unfolded protein response: a stress signaling pathway
43 critical for health and disease. Neurology 66(2 Suppl 1):S102-109.
44 Zhang K, Kaufman RJ. 2008. From endoplasmic-reticulum stress to the inflammatory
45 response. Nature 454(7203):455-462.

bioRxiv preprint doi: https://doi.org/10.1101/258111; this version posted May 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

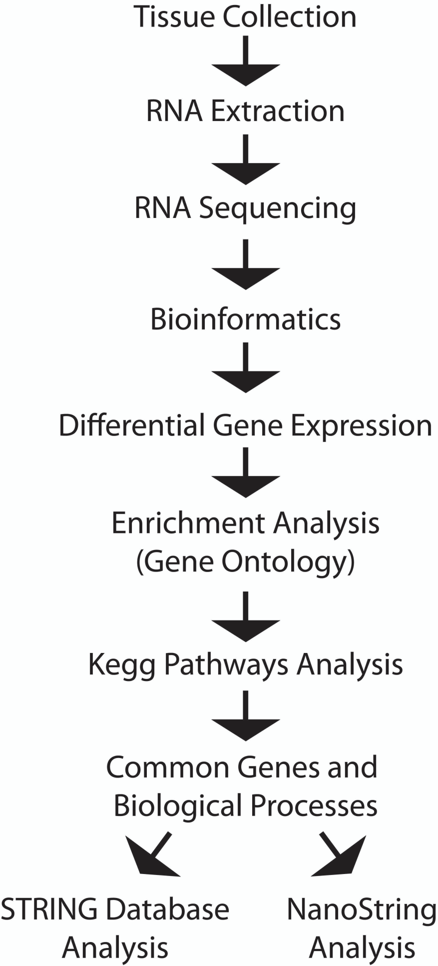
1 Zhang ZH, Jhaveri DJ, Marshall VM, Bauer DC, Edson J, Narayanan RK, Robinson GJ,
2 Lundberg AE, Bartlett PF, Wray NR, Zhao QY. 2014. A comparative study of
3 techniques for differential expression analysis on RNA-Seq data. PloS one
4 9(8):e103207.
5 Zhu JJ, Qin Y, Zhao M, Van Aelst L, Malinow R. 2002. Ras and Rap control AMPA
6 receptor trafficking during synaptic plasticity. Cell 110(4):443-455.
7

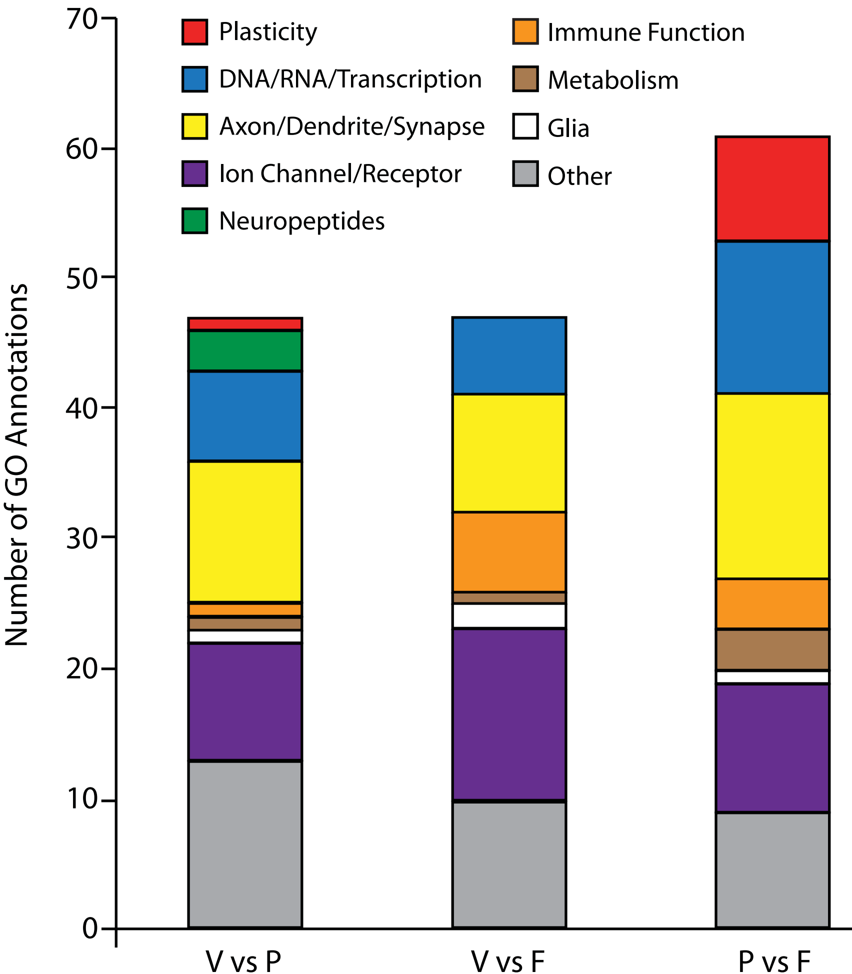
Experiment Timeline

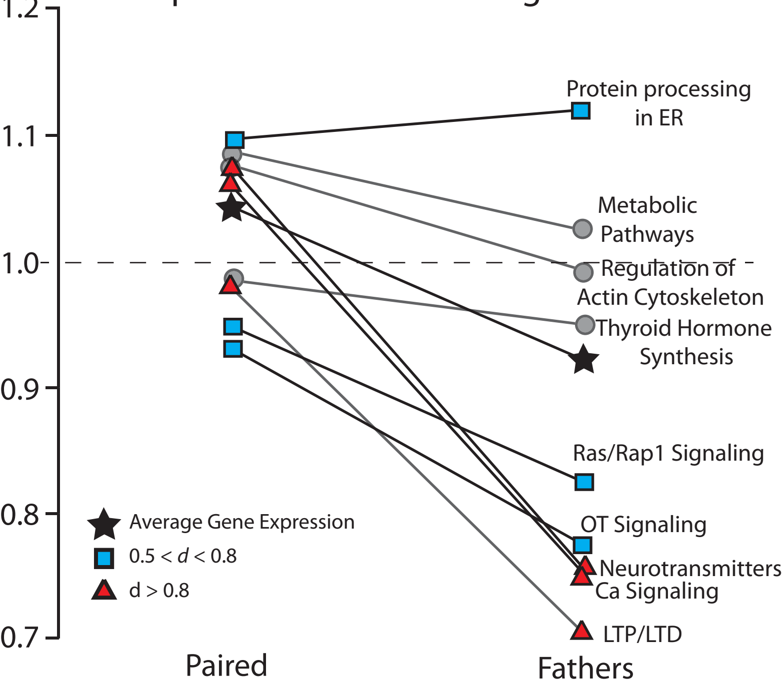




MOs
MOp
CTX
SSp
ACAd
ACAv
cing
cc
IG
LSc
VL
LSr
CP
CLA
MS
BST
aco
al
am
EPd
LPO
HY
MPO
MEPO
ACB
SI
NDB
ov
lot
isl
PIR
fx
LSv
ov
int
ju
act
ADP
PS
MPN
AVPV
AVP
V3
VLPO
FS
MA
och
OT
df
SF
TRS
vhc
TH
PT
PVT
st
pr
GPe
PVH
tr
if
IA
ec
EPv
AAA
BMAa
SO
PVpo
SFO
AD
AV
sm
RE
SCH
AHNa
NLOT
COAa
VISC
GU
AIp
SSs

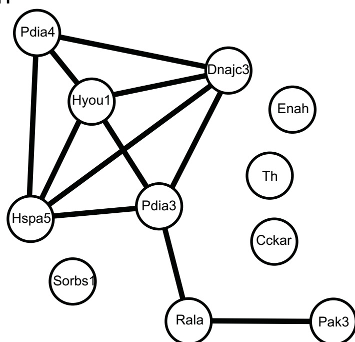

Tissue Collection
RNA Extraction
RNA Sequencing
Bioinformatics
Differential Gene Expression
Enrichment Analysis
(Gene Ontology)
Kegg Pathways Analysis
Common Genes and
Biological Processes
STRING Database
Analysis
NanoString
Analysis


Gene Ontology Annotations
Number of GO Annotations
70
60
50
40
30
20
10
0
Plasticity
DNA/RNA/Transcription
Axon/Dendrite/Synapse
Ion Channel/Receptor
Neuropeptides
Immune Function
Metabolism
Glia
Other
V vs P
V vs F
P vs F

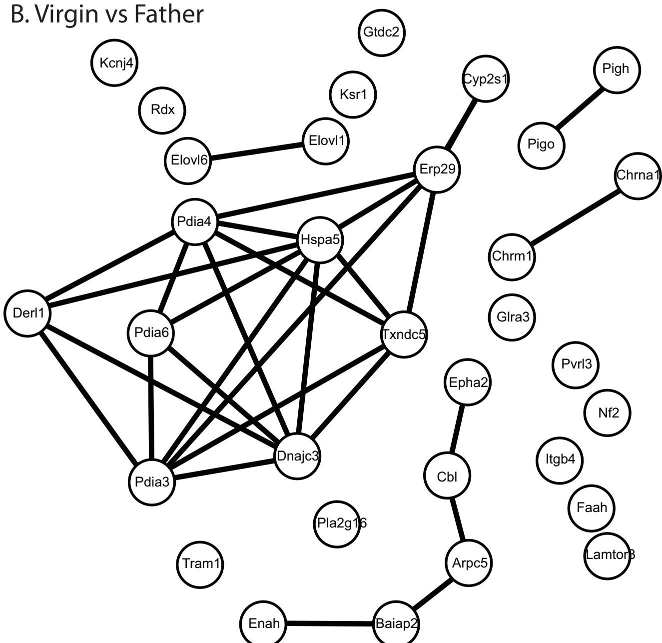


Expression Relative to Virgin Males
1.2
1.1
1.0
0.9
0.8
0.7
Protein processing in ER
Metabolic Pathways
Regulation of Actin Cytoskeleton
Thyroid Hormone Synthesis
Ras/Rap1 Signaling
OT Signaling
Neurotransmitters
Ca Signaling
LTP/LTD
Average Gene Expression
0.5 < d < 0.8
d > 0.8
Paired
Fathers

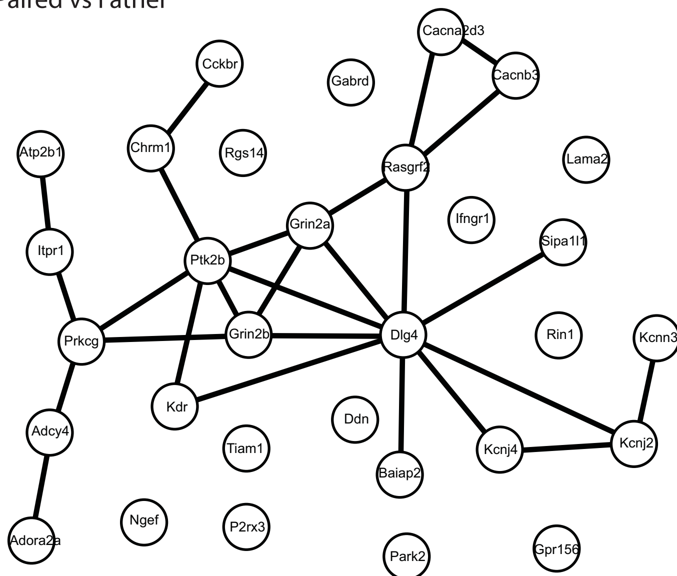
A. Paired vs Virgin

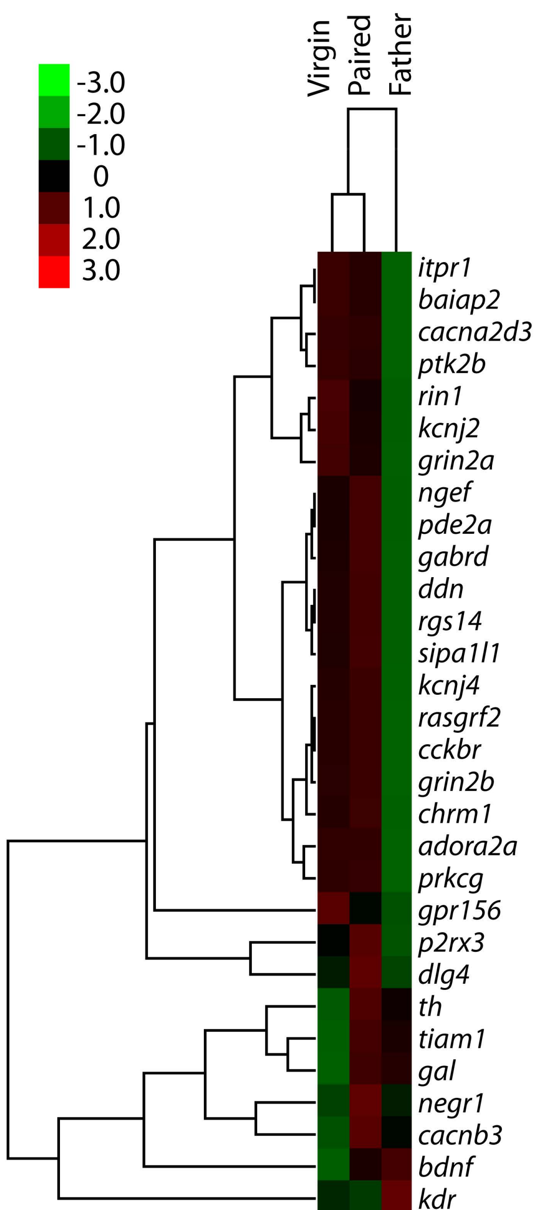


B. Virgin vs Father

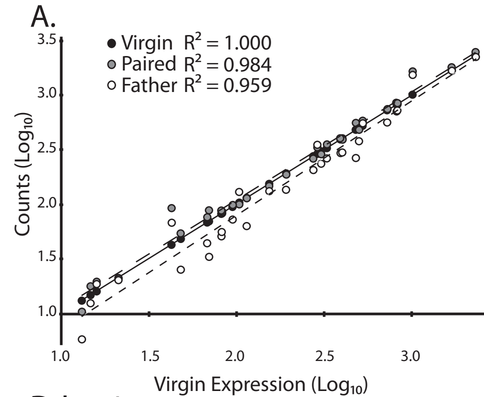


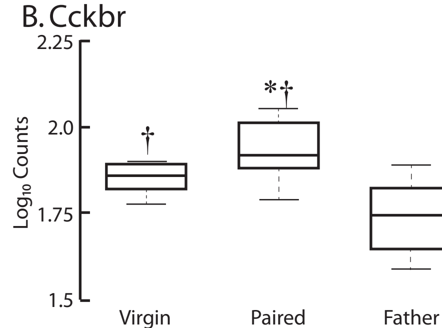
C. Paired vs Father

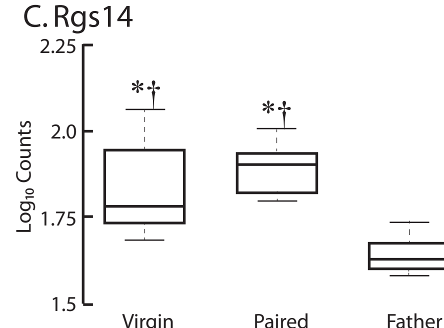


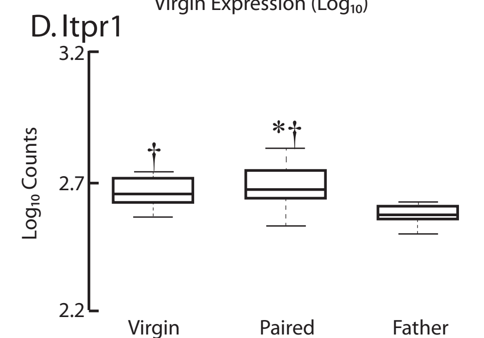


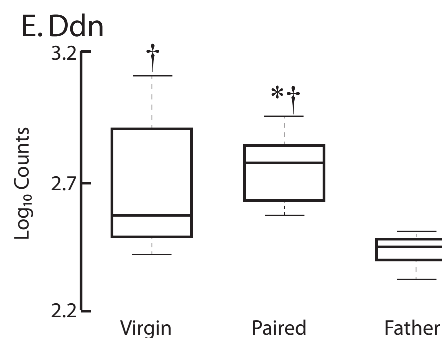
-3.0
-2.0
-1.0
0
1.0
2.0
3.0
Virgin
Paired
Father
itpr1
baiap2
cacna2d3
ptk2b
rin1
kcnj2
grin2a
ngef
pde2a
gabrd
ddn
rgs14
sipa1l1
kcnj4
rasgrf2
cckbr
grin2b
chrm1
adora2a
prkcg
gpr156
p2rx3
dlg4
th
tiam1
gal
negr1
cacnb3
bdnf
kdr

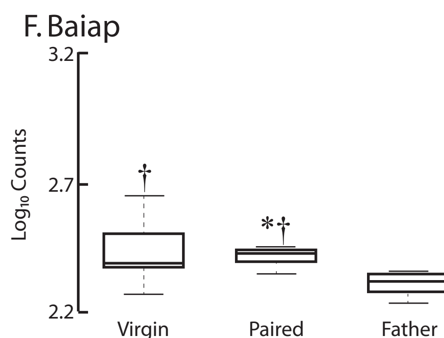

A.
Virgin R^2 = 1.000
Paired R^2 = 0.984
Father R^2 = 0.959
Counts (Log10)
Virgin Expression (Log10)

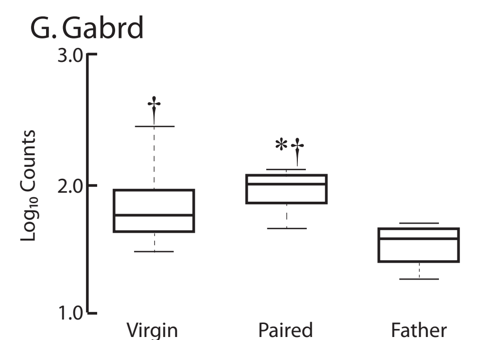

B. Cckbr
Log10 Counts
Virgin
Paired
Father

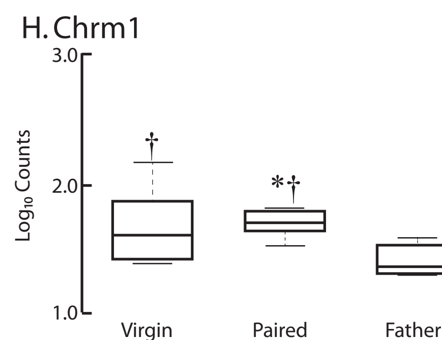

C. Rgs14
Log10 Counts
Virgin
Paired
Father

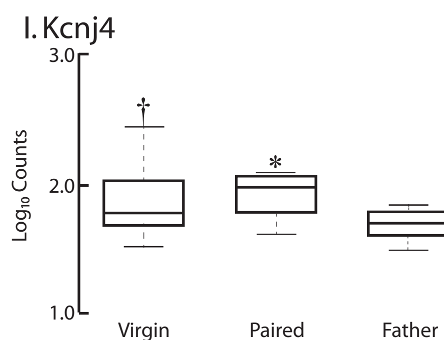

D. Itpr1
Log10 Counts
Virgin
Paired
Father

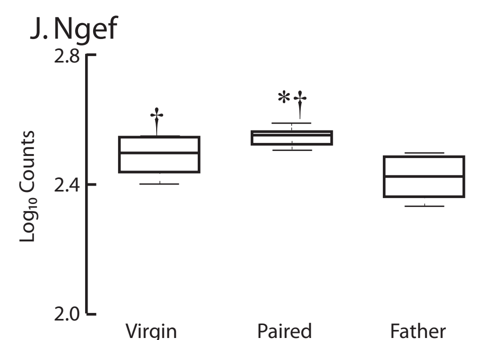

E. Ddn
Log10 Counts
Virgin
Paired
Father

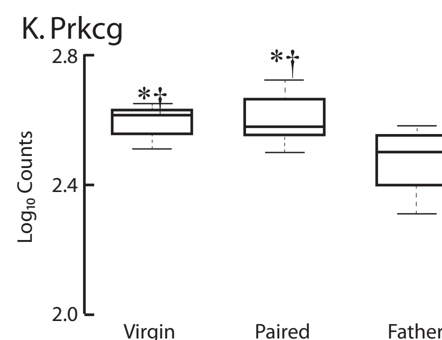

F. Baiap
Log10 Counts
Virgin
Paired
Father

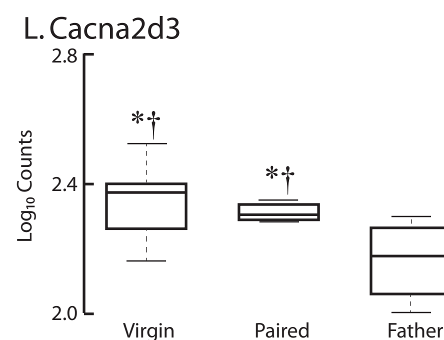

G. Gabrd
Log10 Counts
Virgin
Paired
Father

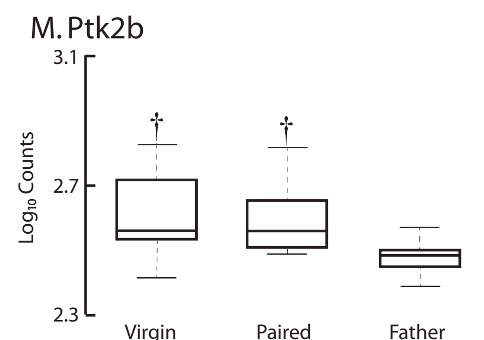

H. Chrm1
Log10 Counts
Virgin
Paired
Father

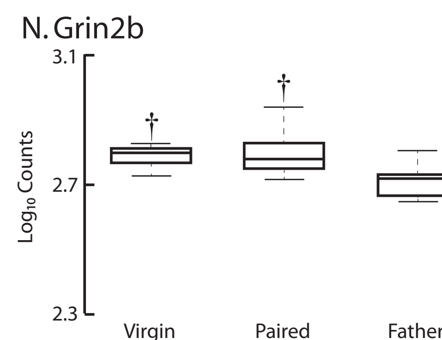

I. Kcnj4
Log10 Counts
Virgin
Paired
Father

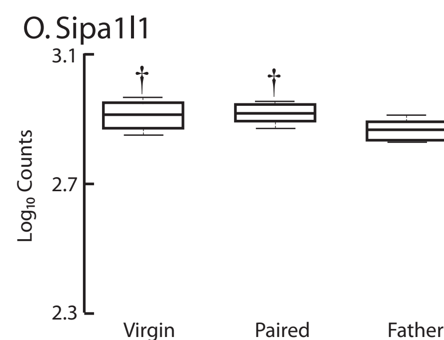

J. Ngef
Log10 Counts
Virgin
Paired
Father


K. Prkcg
Log10 Counts
Virgin
Paired
Father


L. Cacna2d3
Log10 Counts
Virgin
Paired
Father


M. Ptk2b
Log10 Counts
Virgin
Paired
Father


N. Grin2b
Log10 Counts
Virgin
Paired
Father


O. Sipa1l1
Log10 Counts
Virgin
Paired
Father