Fatherhood alters gene expression within the MPOA

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Abstract:

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

Introduction:

Biparental care is displayed by a minority of mammalian species – usually cited as 3-5% (1-3). Female parenting is obligate because mammalian offspring need to nurse. Therefore, the presence of the male, and particularly active participation of the male in fathering, is an unusual situation seen only in our own and a limited number of other mammalian species (4-8). In species where it does exist, including humans, paternal care is often crucial to the survivorship of offspring, or at the least has significant and long-term impacts on growth as well as neural, reproductive and social development (9, 10). In biparental species, both mothers and fathers contribute to the care of offspring, however little is known about the specific neurobiological regulation of paternal care (6). The vast majority of parenting research focuses on the mother, while the role of the father is mostly considered in the context of paternal absence (9).

Father absence has been extensively studied in both human and animal models, and paternal deprivation has been a commonly used manipulation to study the effects of male parenting in animal models (11-13). Female prairie voles that were raised without a father displayed less alloparenting (care of offspring not their own, usually siblings) (14); took longer to form a pair-bond (14, 15); and exhibited less parenting towards their own offspring (15). Father absence in mandarin voles has been shown to affect a host of behavioral and neuroendocrine variables in ways that build on the prairie vole findings, including negative effects on pair-bonding (16), social recognition (17), and play behavior (18). Long-term neuroendocrine effects on offspring include changes in oxytocin and vasopressin systems (14, 17, 18), estrogen receptors (17), stress systems (19-21), and dopaminergic metabolism (16, 22).

However, paternal absence is the most extreme situation. Although less studied, individual variation in fathering can also have long-term effects on offspring (23), and in the context of non-human mammals is always carried out in a biparental care situation. In prairie voles, we have shown that natural variation in biparental parenting behavior predicts pup development and juvenile social behavior (24), exploratory behavior and pair-bonding, and adult aggression and stress responses (25-27). It is not always possible in a biparental care situation to tell what outcomes in offspring are due to maternal care and what are due to paternal care. However, some very interesting roles for the father have been observed. For instance, in some species, males may compensate for poor maternal care (or allow mothers to expend less energy on non-nutritive tasks like carrying)(24, 28); or a paternal behavior such as retrievals (carrying pups back to the nest or territory) may be directly linked to offspring display of retrievals and aggression as an adult (29, 30).

While little is known about the effects of paternal care on offspring, even less is known about the neural mechanisms underlying fathering behavior. It has been hypothesized that similar neural circuits are responsible for both maternal and paternal behaviors (31). While alterations in neural activity appear to be hormonally regulated in females, hormonal manipulation in males does not have the same effect on paternal behavior (32). This has led some to suggest that male parenting is not hormonally regulated (6), and has raised the question of what factors are involved in the generation of these behaviors.

The goal of this study was to identify novel gene targets and potential mechanisms that contribute to the production and regulation of paternal behavior. We

analyzed gene expression in three groups of male prairie voles: virgin males, males who have formed a pair bond with a female, and males who had fathering experience. Samples were taken from the medial preoptic area (MPOA), a region that is central to the expression of both maternal and paternal behaviors (31, 33-35), and RNA was extracted and sequenced. There are many methods by which one can extract meaning from RNA sequencing data; here we use several analytical techniques to identify genes that are differentially expressed in voles with and without fathering experience.

Results

Gene Ontology Analysis

Differential gene expression was directly compared across groups, resulting in three comparisons: Virgin males vs Paired males (*V vs P*), Virgin males vs Fathers (*V vs F*), and Paired males vs Fathers (*P vs F*). Each comparison exhibited over 16,000 differentially expressed genes. We performed an enrichment analysis on the top 500 differentially expressed genes (thus, approximately the top 3%) from each comparison using the Gene Ontology (GO) database (http://www.geneontology.org/). The gene enrichment analysis annotated the differentially expressed genes using one of three functional analyses: cellular component, molecular function, or biological process.

Cellular Component Analysis: The cellular component analysis describes the locations of gene expression, at the levels of subcellular structures. This analysis was completed for the top 500 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*). Table S1 lists

the fold enrichment and p-values of GO Annotations identified in each of the three comparison groups.

Because each GO Annotation references many genes, in some instances the same GO Annotation was present in multiple comparison groups. The *P vs F* group contained 28 GO Annotations, and the *V vs F* group contained 10 GO Annotations. Four GO Annotations were found in both *V vs F* and *V vs P* groups, and 9 GO Annotations were seen in all three groups.

The GO Annotations identified in the Cellular Component Analysis were sorted into categories based on function. We examined which functions were associated with each comparison group. The *P vs F* group contained GO Annotations that fit into three categories: neuron (3 GO Annotations), dendrite/synapse (13 GO Annotations), and cellular anatomy (12 GO Annotations). All three groups contained GO Annotations that related to cellular anatomy (9 GO Annotations), as did the *V vs F and V vs P* groups (4 GO Annotations). The *V vs F* group contained GO Annotations that fit into two categories: vesicle (3 GO Annotations) and cellular anatomy (7 GO Annotations).

Molecular Function Analysis: The molecular function analysis describes the function that each gene product performs within the cell. As with the Cellular Component Analysis, the Molecular Function Analysis was completed for the top 500 differentially expressed genes in each of the three comparison groups. Table S2 lists the fold enrichment and p-values of GO Annotations identified in each of the three comparison groups. The *P vs F* comparison group contained 23 GO Annotations. Four GO Annotations were found in both *P vs F and V vs F* groups, and all three groups had 1 GO annotation in common.

As described above, the GO Annotations identified in the Molecular Function Analysis were sorted into categories based on function and then associated with each comparison group. The *P vs F* group contained GO Annotations that fit into seven categories: binding (which involves the interactions of a molecule with one or more sites on another molecule; 4 GO Annotations), ion transport (7 GO Annotations), membrane transport (2 GO Annotations), receptors (5 GO Annotations), catalysis (2 GO Annotations), and transduction (1 GO Annotation). GO Annotations related to binding were found in the *V vs F and P vs F* group (4 GO Annotations), as well as the *V vs F, V vs P, and F vs P* group (1 GO Annotation).

Biological Process Analysis: The Biological Process Analysis describes a recognized series of events or collection of molecular functions associated with a gene or gene product. The Biological Process Analysis was completed for the top 500 differentially expressed genes in each of the three comparison groups. Table S3 lists the fold enrichment and p-values of GO Annotations identified in each of the three comparison groups. The *V vs F* comparison group contained 2 GO Annotations, the *P vs F* comparison contained 75 GO Annotations, and the *V vs P* comparison contained 1 GO Annotation. The *V vs F and P vs F* groups had 6 GO Annotations in common, while the *P vs F and V vs P* groups had 2 GO Annotations in common. All three comparison groups had 1 GO Annotation in common.

The GO Annotations identified in the Biological Processes Analysis were sorted into categories based on function and then associated with each comparison group. The *V vs F* contained 2 GO Annotations related to binding. The *P vs F* group contained GO

Annotations that fit into six categories: neuronal plasticity (9 GO Annotations), ion transport/homeostasis (17 GO Annotations), development (22 GO Annotations), regulation (24 GO Annotations), metabolic processes (2 GO Annotations), and localization (1 GO Annotation). The *V vs P* group contained 1 GO Annotation related to localization. GO Annotations related to cellular processes were found in the *V vs F and P vs F* group (6 GO Annotations), and GO Annotations related to actin were found in the *P vs F and V vs P* group (2 GO Annotations). All three groups shared one GO Annotation related to cellular processes.

Kegg Pathways Analysis:

In each comparison group we examined functions with the highest fold enrichment (# observed genes/# expected genes) as well as functions related to neuronal activity, plasticity, or active biological processes, 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. Not every gene was associated with a molecular signaling pathway. We then identified commonly recurring pathways associated with the individual differentially expressed genes.

In the *V vs P* comparison group, the commonly recurring pathways included: Protein export, Protein processing in the endoplasmic reticulum, Thyroid hormone synthesis, Antigen processing and presentation, Ras signaling, Rap1 signaling, Neuroactive ligand-receptor pathway, Calcium signaling, and Regulation of the actin cytoskeleton. In the *V vs F* comparison group, the commonly recurring pathways

included: Protein processing in the endoplasmic reticulum, Regulation of the actin cytoskeleton, Ras signaling, Metabolic pathways, Axon guidance, Protein processing in the endoplasmic reticulum, Thyroid hormone synthesis, and Antigen processing and presentation. In the *P vs F* comparison group, the commonly recurring pathways included: Ras signaling, Rap1 signaling, Neuroactive ligand-receptor pathway, Calcium signaling, MAPK signaling, LTP, Glutamatergic pathways, Dopaminergic pathways, and Regulation of the actin cytoskeleton.

We next identified genes that were associated with multiple Kegg pathways. By excluding genes that were not associated with any Kegg pathways, or were associated with pathways that were not related to brain function, we further narrowed the range of genes of interest to 49 genes. Ultimately, in each comparison group we identified genes with differential expression across social experience and that were linked to biological pathways within the brain (Table 3). We standardized the expression of each gene relative to its expression in virgin males then grouped genes that were associated with nine commonly recurring Kegg pathways and compared the expression of those genes across groups. Since this was an exploratory study, we did not perform statistical tests, and instead used Cohen's *d* as a measure of effect size (Figure 4; Table 4). We found large effects of differential expression in genes that were associated with long term potentiation and long term depression (LTP and LTD) (d = 1.072), Neurotransmitters (d = 0.911), and Ca^{2+} Signaling (d = 0.877). We found medium effects of differential expression in genes that were associated with Oxytocin Signaling (d = 0.787), Protein Processing in the Endoplasmic Reticulum (d = 0.599), and Ras/Rap1 Signaling (0.578).

STRING Database Analysis:

We used the STRING database to assess the network connectivity between the genes in each comparison group that were identified as having a high degree of differential expression and functional significance. For each analysis, the STRING database constructed a network showing interactions between gene products, as well as the degree of enrichment. The STRING database also performed an enrichment analysis using both Gene Ontology Annotations and Kegg pathways, revealing statistically significant interactions between these gene products.

The 11 differentially expressed genes from the *V vs P* comparison group produced a network with 11 nodes and 11 edges, and a PPI enrichment p-value of 5.86×10^{-7} (Figure 5A). Thus, the proteins expressed by these genes have significantly more interactions than would be expected by chance, as defined as a random set of similarly sized proteins selected from the genome. There was one cluster of 7 interacting proteins, and the functions of these gene products were primarily related to functions of the endoplasmic reticulum, as well as the cellular response to stimulation.

The 33 differentially expressed genes from the *V vs F* comparison group produced a network with 32 nodes and 29 edges, and a PPI enrichment p-value of 6.99×10^{-15} (Figure 5B), indicating that the proteins expressed by these genes have significantly more interactions than would be expected by chance. These gene products produced one large cluster of 9 interacting proteins, one medium cluster of 5 interacting proteins, and three separate small clusters of 2 interacting proteins. The large cluster was predominantly involved with the function of the endoplasmic reticulum. The medium cluster was involved with process of neural plasticity, including signaling pathways and modification

of the actin cytoskeleton. The three small clusters were involved with the elongation of fatty acid chains, the formation of cholinergic receptors, and GPI-anchor synthesis.

The 31 differentially expressed genes from the *P vs F* comparison group produced a network with 31 nodes and 27 edges, and a PPI enrichment p-value of 1.36×10^{-12} (Figure 5C), indicating that the proteins expressed by these genes have significantly more interactions than would be expected by chance. These gene products produced one large network consisting of 20 interacting proteins. The genes in this network were involved in a variety of functions, including synaptic plasticity and neural transmission, ion transmembrane transport, the cellular response to stimulus, and the structure of the synapse and dendrite.

NanoString Analysis

A total of 33 genes (30 target genes and 3 housekeeping genes) were selected for quantitative analysis using NanoString. The housekeeping genes (*Gusb*, *Pgk1*, and *Eif4a2*) did not show differential levels of expression across conditions, confirming that these genes can serve as a good baseline in prairie voles. A heat map analysis revealed that 23 of our 30 target genes had lower expression levels in fathers than in either virgins or paired males (Figure 6). Six genes had lower expression levels in virgins, and no gene 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 7A).

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 7B-O; Table 5). We also calculated the effect sizes using Cohen's d, examining differential expression of each gene across groups (Figure7B-O; Table 6). 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*.

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 brain tissue. As such, we faced several technical challenges over the course of this study. For instance, while the prairie vole genome has been sequenced (36, 37), its annotation is incomplete, leaving us to rely on the annotated mouse genome (*Mus musculus*) for many of our analyses. In addition, it is important to consider the consequences associated with working in an outbred rodent like prairie voles. The individual differences associated

with an outbred population may have masked additional target genes associated with the onset of paternity. Regardless, we still observed significantly altered expression on both the individual gene and system level. These results suggest that paternity engages similar physiological mechanisms across males despite genetic diversity.

Biparental care is rare in mammals, but prairie voles are not the only rodents who exhibit this behavior. The males of several species of *Peromyscus*, including *Peromyscus californicus* and *Peromyscus polionotus*, exhibit paternal care, while other species, including *Peromyscus maniculatus*, do not. This behavioral distinction allowed Bendesky and colleagues to investigate genetic differences between *P. polionotus* and *P. maniculatus* that are linked to parenting behavior (38). In a series of experiments, they identified several quantitative trait loci that were linked to specific behaviors of interest, including nest building. Further analysis revealed that the gene for arginine vasopression (AVP) was directly related to nest building, and when AVP was administered intracerebroventricularly there was a significant decrease in the quality of nest building (38). Unlike the study by Bendesky and colleagues, we did not find changes implicating AVP. However, there are several differences between the two experiments. In this study, we specifically examined gene expression within one hypothalamic nucleus, the MPOA. Our study was in a different species and used males that had very specific social experiences: virgin males, pair bonded males, and males with fathering experience.

We targeted the MPOA specifically because it has long been understood to play a role in maternal behavior, and is also believed to be involved in paternal behavior. Lesions to the MPOA disrupt parental behavior in both male and female California mice (39). In California mouse males, testosterone levels within the MPOA vary in response to

parental status (40). Likewise, male California mice with fathering experience show increased Fos-like immunoreactivity in the MPOA following pup exposure (41). To our knowledge, this is the first time that gene expression in the MPOA has been analyzed in the context of fathering behavior.

RNA sequencing is a powerful technique that allows us to identify alterations in gene expression that are associated with behavioral and other phenotypic changes (42). The greatest challenge with this technique, however, is the large amount of data it produces. There is no one agreed upon analysis that most effectively identifies specific genes of interest (43, 44). Thus, in this study we used several techniques to reveal novel gene targets to further our understanding of paternal behavior. We believe that this is a strength rather than a weakness. All of the target genes identified in this experiment are associated with the experimental differences in social experience. The ultimate goal of this experiment was to increase our understanding of the alterations that occur within the MPOA following exposure to different social contexts in male prairie voles. As such, we have identified a set of genes and their associated pathways that we can use to further explore male parenting behavior.

Our quantitative assessment of gene expression revealed an overall decrease in the expression of many genes in fathers relative to both virgins and pair-bonded males. The specific genes of interest that we identified were involved in a range of physiological processes, including metabolism, stress responsiveness, and plasticity. However, most of the genes that showed significant differential expression, and specifically decreased expression, were associated with synaptic transmission and dendritic spine motility (Table 7). For example, several genes involved in the production and maintenance of

receptors (including *Cckbr*, *Chrm1*, *Gabrd*, *Grin2b*, and *Itpr1*) and ion channels (including *Cacna2d3*, *Kcnj4*, and *P2rx3*) were significantly downregulated. These results suggest that GABA, glutamate, and cholinergic systems are all affected by fathering experience, as are calcium and potassium channels. Other genes that exhibited significant downregulation in fathers were involved with the actin cytoskeleton, dendritic spine motility, and other components of the physical plasticity of dendrites. We emphasize that this is not an exhaustive list of differentially expressed genes, however, these results suggest that synaptic plasticity may be diminished in the MPOA of male prairie voles with fathering experience.

Fatherhood also seems to be associated with structural alterations in neural plasticity, as measured by changes in the number and density of dendritic spines. Mice with fathering experience show increased survival of newborn neurons and increased dendritic spine density within the hippocampus (45, 46). Male marmosets show an increase in dendritic spine density in the prefrontal cortex after fathering experience (47). However, other studies have shown reductions in the survival of adult-generated neurons in the amygdala and hippocampus (48, 49). It seems clear that the effects of fatherhood vary across brain regions, but we do not yet know what is causing these changes in neural plasticity.

This lower gene expression related to dendritic spines, shown to be associated with fatherhood in the present study, is evocative of similar changes seen in a recent study of the MPOA of mother rats (50). *Rem2*, a gene associated with reduction of dendritic branching but increases in spine density (51, 52) was increased in the MPOA of high licking/grooming rats, but only in lactating mothers (not in virgins). This increase

was accompanied by decreased dendritic complexity. *Rem2* is involved with GTPase activity and GTP binding. While we did not see alterations in *Rem2* expression in this study, we found differential expression of several genes that are involved in Ras and Rap1 signaling. Both Ras and Rap1 are GTPases that play an important role in the structural and functional plasticity of cells (53, 54).

The down-regulation of genes associated with dendritic complexity in the present study, as well as the study by Parent and colleagues, is similar to what one would expect in an animal that had experienced high amounts of stress. It is well established that stress, mediated by corticotropin-releasing hormone, results in a loss of dendritic spines (55-59). Interestingly, rat mothers show a decrease in the number and density of dendritic spines in the amygdala and stria terminalis four days after birth (60), and an increase in dendritic spine density in the hippocampus during the postpartum period (61). This suggests that alterations in dendritic spine density in mothers are both transient and region specific. More studies must be done to determine if the same holds true for vole fathers.

In many species, the transition to fatherhood is associated with a suite of behavioral and hormonal changes, including those indicative of stress. In California mice (*Peromyscus californicus*), fathers exhibit attenuated anxiety-like behavior approximately two weeks after pups are born (45, 46). Human males show a peak in cortisol levels during the transition to fatherhood (62), but this is highly variable across studies (63). Prairie vole fathers show increased anxiety-like behavior, and chronic pup exposure results in an increase in basal CORT levels (48). In an open field test, fathers spent more time in corner squares, and in an elevated plus maze, fathers spent less time in open arms. In forced swim tests, fatherhood decreased the latency to immobility, and increased the

number and duration of immobility bouts (48). Additionally, males with fathering experience showed fewer BrdU-labeled cells in the amygdala, hippocampus, and ventromedial hypothalamus than virgin males (48). In the long term, fatherhood may be beneficial for male health, but the transition to fatherhood is a tremendously stressful period (64).

In male voles with fathering experience we also see the upregulation of genes related to protein processing in the endoplasmic reticulum. The endoplasmic reticulum is instrumental in managing the protein folding process, including disposing of misfolded proteins (65). Homeostatic imbalances, including stress, can alter the functioning of the endoplasmic reticulum, leading to the initiation of the unfolded-protein response, which can in turn lead to apoptosis (66, 67). This may be one mechanism by which physiological stress can result in homeostatic perturbations (68, 69), including some of the changes that are evident in vole fathers, such as weight loss (70, 71).

While many of the changes we saw in gene expression may be partially attributable to stress, there are likely many other additional factors at play. Fathers in many species show systematic endocrine changes (32). Environmental factors, including changes in the types and amount of sensory stimulation, or the amount of parental care they received, may play a role as well (72, 73). Much more work must be done to tease apart these many factors.

In this study we saw the most varied and interesting differences between the paired males and males with fathering experience. This was surprising, as we expected that the greatest differences would be between the virgin males and fathers. However, examination of the quantitative results begins to clarify these findings (Figs 4 and 7). The

expression of genes of interest is slightly elevated in paired animals relative to virgins, but the expression in fathers is decreased relative to virgins. Thus, while the expression levels of some genes do not significantly differ between virgins and paired males, and virgins and fathers, we found significant differences between paired males and fathers. This may suggest that the experience of fathering is functionally distinct from any other type of social interactions that these animals have encountered.

Conclusions

The purpose of this study was to explore how gene expression changed across the transition to fatherhood, and to identify novel targets to allow for deeper investigation of male parenting behavior. The use of RNA sequencing confirmed that there are differences in gene expression between voles that had different social experiences, including virgin males, males that had formed a pair bond with a female, and males with parenting experience. The genes identified in this study suggest novel mechanisms that underlie paternal behavior and offer novel targets for the further exploration of fathering behavior.

Materials and Methods:

Subjects:

Subjects were 18 adult male prairie voles (Table 1). Animals were born and housed in the Psychology Department Vivarium at the University of California, Davis. These animals were descendants of a wild stock originally caught near Champaign, Illinois. The animals were weaned at 20 days of age and pair housed with an animal of the same sex (sibling if

available, similarly aged non-sibling if not) in small laboratory cages (27 x 16 x 13 cm) in which food and water were available *ad libitum*. All animals were maintained at approximately 70°F (21°C) on a 14:10 light/dark cycle with the lights on at 6 a.m. All experiments were performed under National Institutes of Health guidelines for the care of animals in research and were approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

At postnatal day (P) 42-45 subjects were placed in one of three groups of age-matched males: 1) virgin males, 2) sexually experienced, "pair-bonded" males, or 3) males with fathering experience (Figure 1). This was designed to dissociate alterations in gene expression that were related to pair bonding from alterations related to paternal behavior. Virgin males were housed with a male same-age conspecific, and they were euthanized without engaging in sexual contact with females. Pair-bonded males were housed with a same-age female conspecific for ~20 days, after which the males were euthanized. Because mating and pregnancy strengthens pair bonds in prairie voles, we confirmed that females were pregnant. Pair-bonded males were euthanized before females gave birth, ensuring they had no contact with pups. The third group consisted of males which had ~3 days of paternal experience. These males were also housed with female pair-mates with whom they formed a pair-bond. The females gave birth, and the males were permitted up to three days of contact with pups before they were euthanized.

Upon euthanasia, brains were removed and flash frozen. The brains were sliced on a cryostat into 120 µm sections and mounted on slides. Punches were taken from the MPOA using a 15.5-gauge blunt needle (Figure 2), and were stored in a -80 freezer until RNA extraction. The sequence of analyses for this study is outlined in Figure 3.

RNA Extraction:

Total RNA was isolated with Qiazol reagent (Qiagen) and purified with an RNeasy® Plus Micro Kit (74004; Qiagen, Valencia, CA) as well as the optional DNase digestion (Qiagen 129046). A Nanodrop™ Spectrophotometer was used to determine the quality and quantity of the RNA. All samples had a 260/280 ratio > 1.8.

RNA Sequencing:

A total of 18 RNA-seq libraries were prepared from the RNA of the 18 male prairie voles (Table 2). RNA sequencing and library preparation was performed by the DNA Technologies and Expression Analysis Core at the Genome center of the University of California, Davis. Barcode indexed RNA-seq libraries were generated from 1 ug total RNA each after poly-A enrichment using the Kapa Stranded RNA-seq kit (KapaBiosystems, Cape Town, South Africa) following the instructions of the manufacturer. The libraries were generated on a Sciclone G3 liquid handler (Caliper Life Sciences, Alameda, CA). Libraries were analyzed with a Bioanalyzer 2100 instrument (Agilent, Santa Clara, CA) quantified by fluorometry on a Qubit instrument (LifeTechnologies, Carlsbad, CA) and pooled in equimolar ratios. The pool was quantified by qPCR with a Kapa Library Quant kit (KapaBiosystems) and sequenced on 1 lane of an Illumina HiSeq 4000 (Illumina, San Diego, CA) with paired-end 150bp reads.

Raw sequencing data have been deposited at NCBI's Sequence Read Archive (SRA) under study accession number SRP128134.

Bioinformatic analysis:

Bioinformatic analysis was performed at the UC Davis Bioinformatics Core Facility. The reads were trimmed for adapter contamination and quality using scythe (version c128b19) and sickle (version 7667f147e6) respectively. The reads were then aligned to the prairie vole genome (MicOch1.0) using bwa (version 0.7.13), after which featureCounts (version 1.5.0-p1) was used to create the raw expressions counts. Finally, R (version 3.3.2) with the edgeR and limma/voom libraries was used to fit the model and calculate the differential expression for all the genes for each of the contrasts.

Gene Ontology Analysis

Differential gene expression was directly compared between each group, resulting in three comparisons: Virgin males vs Paired males (V vs P), Virgin males vs Fathers (V vs F), and Paired males vs Fathers (P vs F). Each comparison returned over 16,000 differentially expressed genes which were ranked by their relative difference in expression.

None of the differentially expressed genes reached the level of statistical significance. In order to capture the genes that were most likely to show functional differentiation between comparison groups we performed an enrichment analysis on the top 500 differentially expressed genes from each comparison using the Gene Ontology database (http://www.geneontology.org/). This number was chosen following advice from the UC Davis Bioinformatics Core Facility. The gene enrichment analysis annotated

the differentially expressed genes using one of three data sets: cellular component, molecular function, or biological process.

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.

Using the qualitative procedures described previously, we identified 49 candidate genes which display differential expression. We then averaged gene expression across animals within each condition and transformed the data into ratios; the values we used for all analyses were the ratios of gene expression in each condition relative to the virgin condition. The expression ratio of genes in virgin animals was set at 1, a value >1 indicated that genes were more expressed relative to virgins, and a value <1 indicated that genes were less expressed relative to virgins. Thus, we analyzed whether gene expression

for each gene varied between paired animals and fathers. Effect size was measured using Cohen's *d*.

Assessment of gene interaction networks

After identifying each set of differentially expressed genes, we analyzed the connectivity of the gene network using the STRING Database (string-db.org) (74). The STRING database identifies protein-protein interactions between members of a gene set, which allows the user to build a network of functional gene interactions. STRING also measures the functional and interaction enrichments of the gene network, calling upon GO Annotations, Kegg pathways, and connections between nodes.

NanoString Analysis

Following the identification of differentially expressed candidate genes, we performed a quantitative analysis of the expression of 33 genes (30 target genes and 3 housekeeping genes) using the nCounter SPRINT profiler (NanoString Technologies, Seattle, WA). Genes were chosen to be included in the NanoString analysis based on their differential expression values as determined by the raw expression data, as well as their functional significance. One additional gene, *Bdnf*, was chosen due to previous studies indicating that it plays a significant role in plasticity and parenting (i.e., *Bdnf*) (75, 76).

The nCounter analysis assay was conducted using RNA that remained after the completion of the sequencing experiment.

Briefly, NanoString is a medium-throughput method that can analyze many genes within a single sample with comparable sensitivity and accuracy to quantitative real-time

RT-PCR (77). NanoString designed and manufactured custom probes corresponding to 33 genes we identified for quantitative analysis, consisting of 30 target genes and 3 housekeeping genes (*Gusb*, *Pgk1*, and *Eif4a2*). A codeset specific to a 100-base region of the target mRNA was designed using a 3' biotinylated capture probe and a 5' reporter probe tagged with a specific fluorescent barcode. Data were collected using the nCounter Digital Analyzer by counting the number of individual barcodes.

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

Acknowledgments:

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Figure Legends:

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

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

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

then analyzed the interrelatedness of these groups of genes using STRING Database analysis (string-db.org). Finally, the expression of 30 genes of interest was quantitatively expressed using the NanoString Analysis.

Figure 4: Gene expression in paired males and fathers relative to virgin males. Using the Kegg Pathways analysis, we identified nine pathways of biological or behavioral significance and their associated differentially expressed genes. The mean expression of genes associated with each pathway in fathers was averaged and compared against expression in virgin males. On the whole, gene expression was decreased in fathers relative to both virgins and paired males. Of the nine pathways, only one showed an increase in gene expression in fathers (protein processing in the endoplasmic reticulum), while five showed decreases in gene expression in fathers (Ras/Rap1 signaling, Oxytocin signaling, Neurotransmitters, Calcium signaling, and LTP/LTD). The overall average gene expression is indicated by black stars. Values that exhibited medium effect sizes ($0.5 < \text{Cohen's } d < 0.8$) are indicated by blue squares and values that exhibited large effect sizes ($\text{Cohen's } d > 0.8$) are indicated by red triangles).

Figure 5: STRING Database analysis of gene product interaction networks. Selected differentially expressed genes were run through the STRING database of gene product interactions, and networks were generated for each comparison. A) Paired vs Virgin network. B) Virgin vs Father. C) Paired vs Father.

Figure 6: Heat map representing the relative expression of individual genes in virgin males, paired males, and males with fathering experience. Gene enrichment is encoded in the heat map ranging from low (green) to high (red). Genes that show similar expression patterns are clustered together, as indicated by the dendrogram to the left of the heat map.

Figure 7: Quantitative analysis of differential gene expression. A) A scatterplot showing B-O) Box and whisker plots showing the expression of genes in virgins, paired males, and fathers. The whiskers represent 1.5x the interquartile range. While we quantified 30 genes, here we show the 14 genes that exhibited significantly different levels of expression across groups or exhibited large effect size. In each gene of these genes, expression in fathers was lower than expression in paired males, and in three cases (Rgs14, Prkcg, and Cacna2d3) expression in fathers was also significantly lower than in virgin males. * - Significantly differs from fathers ($p < 0.05$). † - Large effect size compared to fathers (Cohen's $d > 0.8$).

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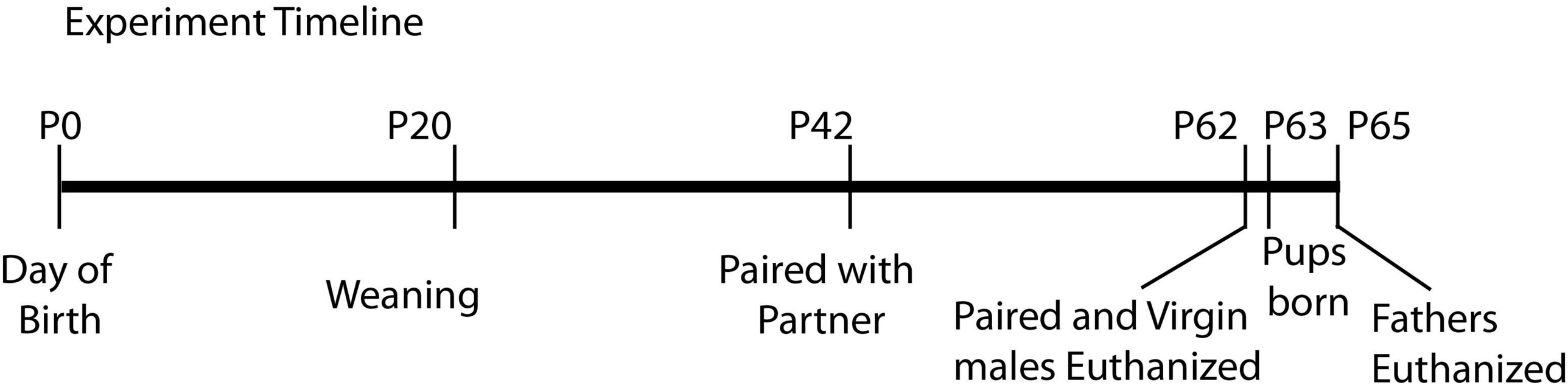
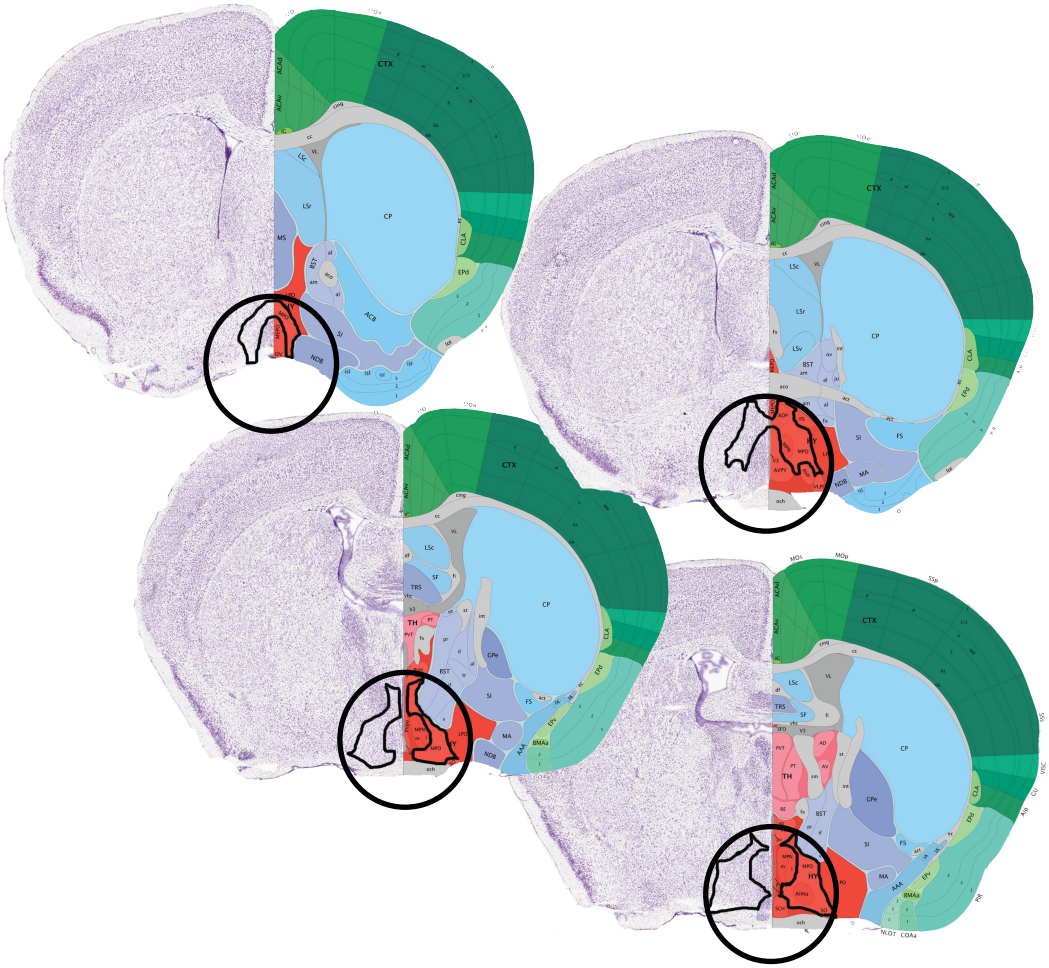

Experiment Timeline
P0
P20
P42
P62
P63
P65
Day of Birth
Weaning
Paired with Partner
Paired and Virgin males Euthanized
Pups born
Fathers Euthanized


CTX
CP
LSc
LSr
MS
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GPe
TH
HY
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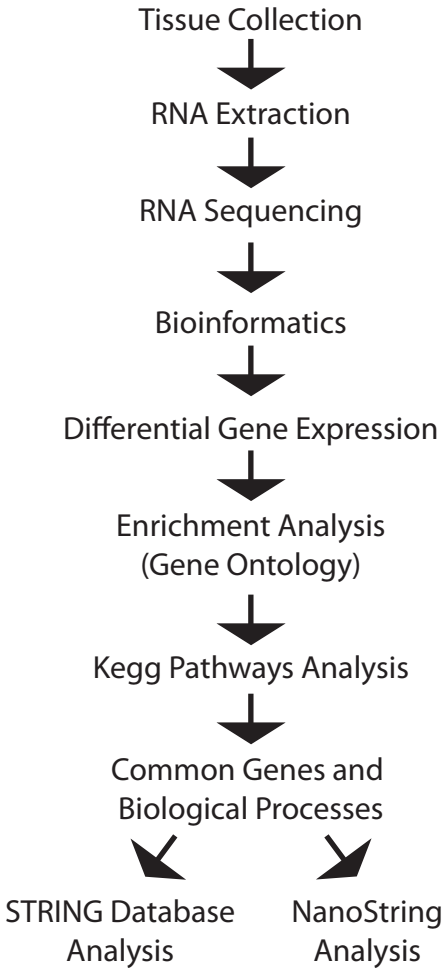
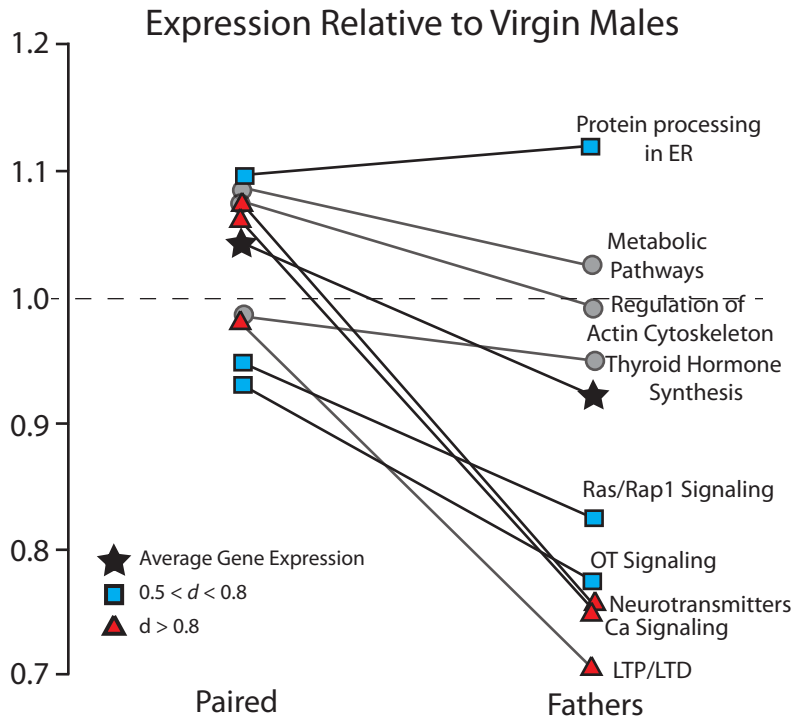
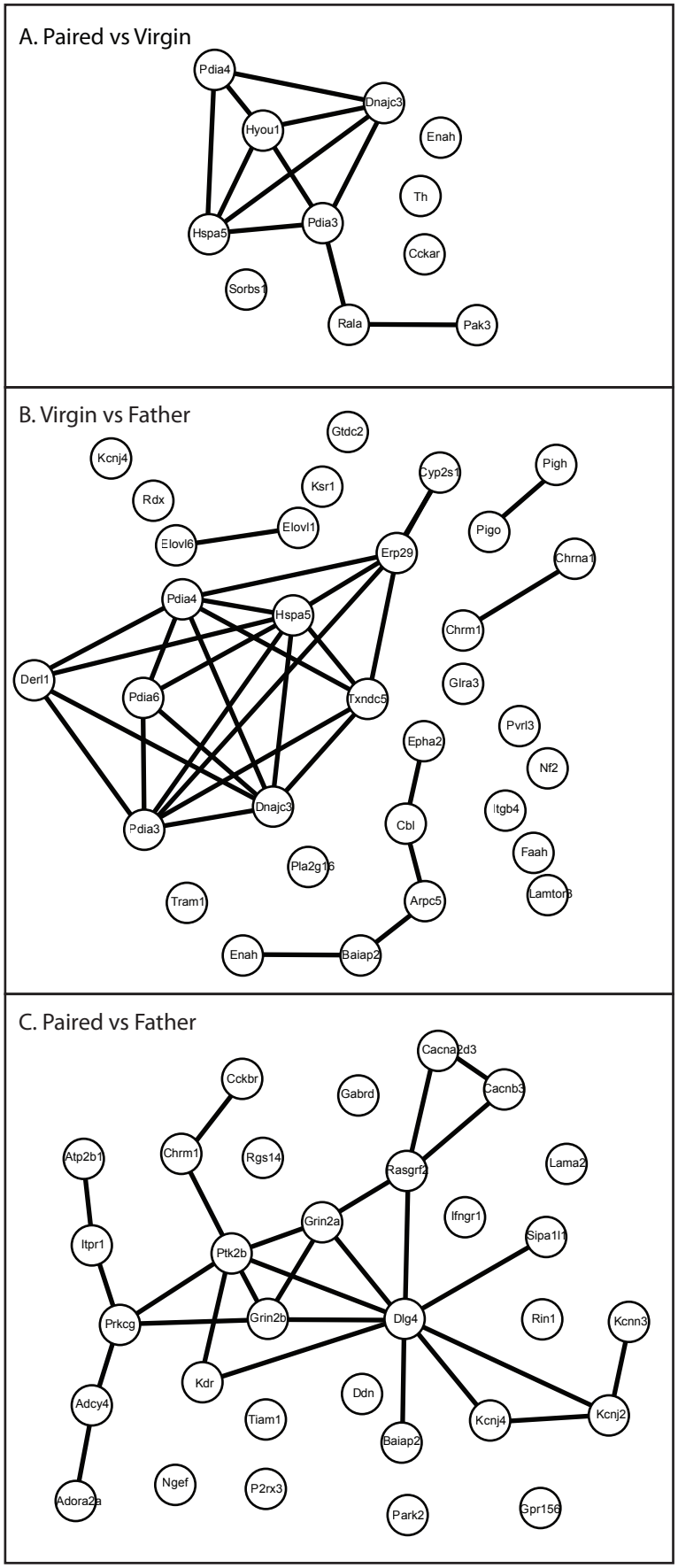
AAA
NLOT
COAa


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


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
Pdia4
Dnajc3
Hyou1
Enah
Th
Hspa5
Pdia3
Cckar
Sorbs1
Rala
Pak3
B. Virgin vs Father
Gtdc2
Kcnj4
Cyp2s1
Pigh
Ksr1
Rdx
Elovl1
Pigo
Elovl6
Erp29
Chrna1
Pdia4
Hspa5
Chrm1
Derl1
Glra3
Pdia6
Txndc5
Pvrl3
Epha2
Nf2
Dnajc3
Itgb4
Pdia3
Cbl
Faah
Pla2g16
Lamtor3
Tram1
Arpc5
Enah
Baiap2
C. Paired vs Father
Cacna2d3
Cckbr
Gabrd
Cacnb3
Atp2b1
Chrm1
Rgs14
Rasgrf2
Lama2
Grin2a
Ifngr1
Itpr1
Ptk2b
Sipa1l1
Prkcg
Grin2b
Dlg4
Rin1
Kcnn3
Kdr
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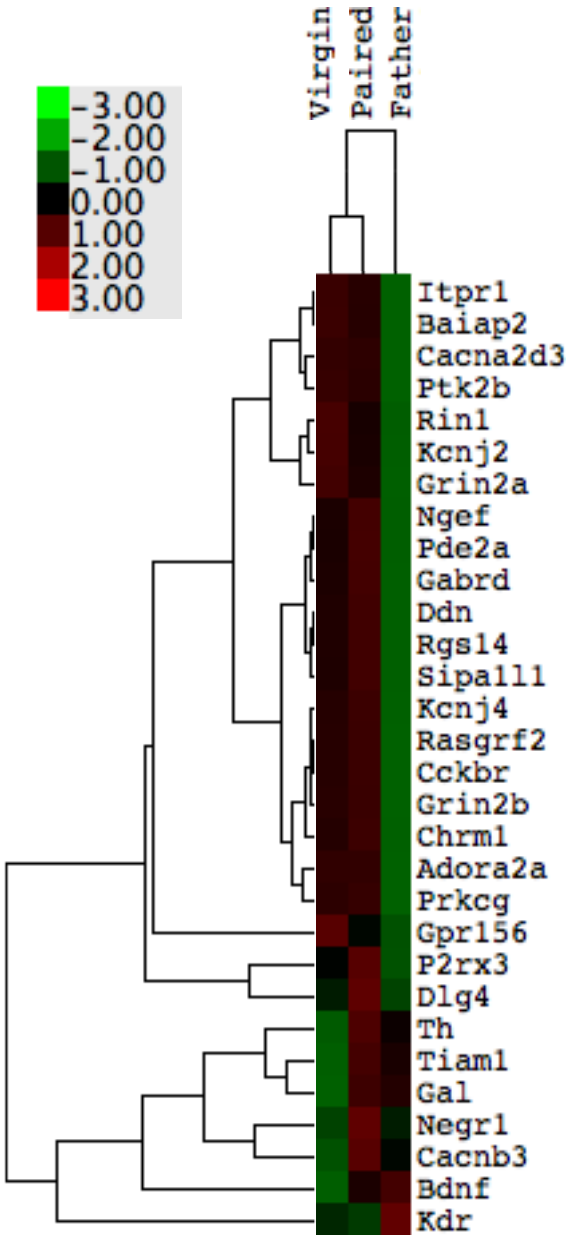
Adora2a
Park2
Gpr156


-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
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

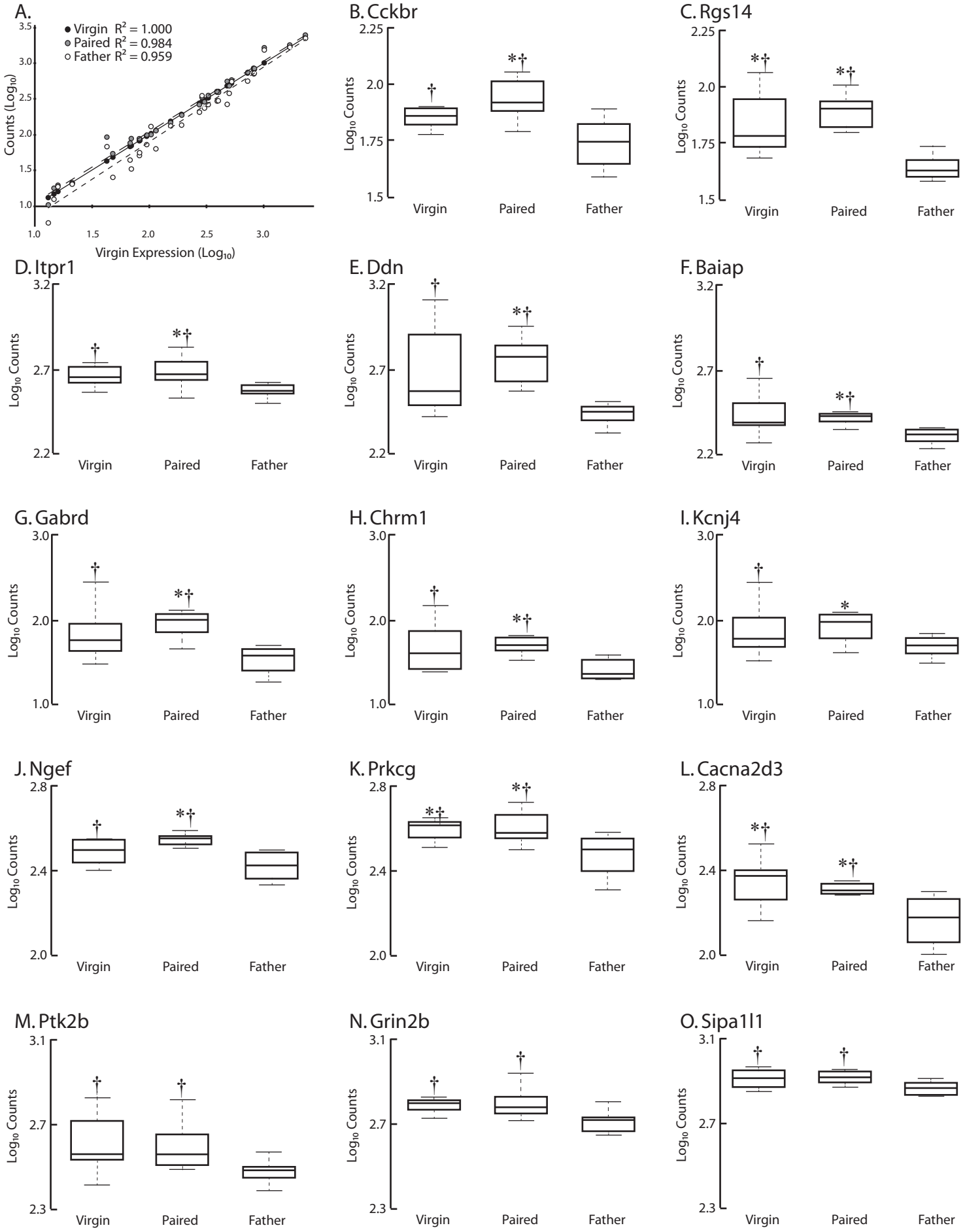

A.
Virgin R^2 = 1.000
Paired R^2 = 0.984
Father R^2 = 0.959
Counts (Log_{10})
Virgin Expression (Log_{10})
B. Cckbr
C. Rgs14
D. Itpr1
E. Ddn
F. Baiap
G. Gabrd
H. Chrm1
I. Kcnj4
J. Ngef
K. Prkcg
L. Cacna2d3
M. Ptk2b
N. Grin2b
O. Sipa1l1
Log_{10} Counts
Virgin
Paired
Father

Table 1: Subjects

ID	condition	paired	pregnant partner	pups born
V1	virgin	no	- -	- -
V2	virgin	no	- -	- -
V3	virgin	no	- -	- -
V4	virgin	no	- -	- -
V5	virgin	no	- -	- -
V6	virgin	no	- -	- -
P1	paired	yes	yes	- -
P2	paired	yes	yes	- -
P3	paired	yes	yes	- -
P4	paired	yes	yes	- -
P5	paired	yes	yes	- -
P6	paired	yes	yes	- -
F1	father	yes	yes	yes
F2	father	yes	yes	yes
F3	father	yes	yes	yes
F4	father	yes	yes	yes
F5	father	yes	yes	yes
F6	father	yes	yes	yes

Table 2: RNA data

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
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

Table 3: Differentially expressed genes

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

Table 4: Pathways associated with differentially expressed genes

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

Table 5: P-values

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

Table 6: Cohen's d values

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**

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