Received: 30 January 2021 | Revised: 23 June 2021 | Accepted: 2 July 2021

DOI: 10.1096/fj.202100163RR

RESEARCH ARTICLE

THE FASEB JOURNAL

Motherhood-induced gene expression in the mouse medial amygdala: Changes induced by pregnancy and lactation but not by pup stimuli

María Abellán-Álvaro[1] | **Guillermo Ayala**[2] | **Manuela Barneo-Muñoz**[3] | **Fernando Martínez-García**[3] | **Carmen Agustín-Pavón**[1] | **Enrique Lanuza**[1]

[1]Unitat mixta UV-UJI de Neuroanatomia Funcional Comparada, Departament de Biologia Cel·lular, Biologia Funcional i Antropologia Física, Facultat de Ciències Biològiques, Universitat de València, València, Spain

[2]Department d'Estadística i Investigació Operativa, Facultat de Matemàtiques, Universitat de València, València, Spain

[3]Unitat mixta UV-UJI de Neuroanatomia Funcional Comparada, Unitat Predepartamental de Medicina, Fac. Ciències de la Salut, Universitat Jaume I, Castelló de la Plana, Spain

Correspondence
Enrique Lanuza, Departament de Biologia Cel·lular, Biologia Funcional i Antropologia

Abstract

During lactation, adult female mice display aggressive responses toward male intruders, triggered by male-derived chemosensory signals. This aggressive behavior is not shown by pup-sensitized virgin females sharing pup care with dams. The genetic mechanisms underlying the switch from attraction to aggression are unknown. In this work, we investigate the differential gene expression in lactating females expressing maternal aggression compared to pup-sensitized virgin females in the medial amygdala (Me), a key neural structure integrating chemosensory and hormonal information. The results showed 197 genes upregulated in dams, including genes encoding hormones such as prolactin, growth hormone, or follicle-stimulating hormone, neuropeptides such as galanin, oxytocin, and pro-opiomelanocortin, and genes related to catecholaminergic and cholinergic neurotransmission. In contrast, 99 genes were downregulated in dams, among which we find those encoding for inhibins and

Abbreviations: ACTH, adrenocorticotropic hormone; *Adcyap1*, adenylate cyclase activating polypeptide 1; *Adra2b*, alpha 2b adrenergic receptor; Arc, arcuate nucleus of the hypothalamus; *Atf3*, activating transcription factor 3; *Avp*/AVP, arginine vasopressin; *Avpr1a*, arginine vasopressin receptor 1A; BMA, anterior part of the basomedial amygdaloid nucleus; BST, bed nucleus of the stria terminalis; *Cga*, alpha subunit of glycoprotein hormones; *Chat*, choline acetyltransferase; *Chrm2*, cholinergic receptor, muscarinic 2, cardiac; *Chrna2*, cholinergic receptor, nicotinic, alpha polypeptide 2 (neuronal); *Chrna6*, cholinergic receptor, nicotinic, alpha polypeptide 6; *Chrnb3*, cholinergic receptor, nicotinic, beta polypeptide 3; *Cis*/CIS, cytokine-inducible SH2-containing protein; CNS, central nervous system; *Dlk1*, delta-like non-canonical Notch ligand 1; *Egr1*, early growth response 1; *Egr2*, early growth response 2; *Egr3*, early growth response 3; *Egr4*, early growth response 4; EGR, early growth response family; *Fos*/FOS, FBJ osteosarcoma oncogene; *Fosb*, FBJ osteosarcoma oncogene B; *Fshb*, follicle-stimulating hormone beta; FSH, follicle-stimulating hormone; *Gal*/GAL, galanin; *Gdpd2*, glycerophosphodiester phosphodiesterase domain containing 2; *Gh*/GH, growth hormone or somatotropin; *Ghrhr*, growth hormone-releasing hormone receptor; *Gnrh1*, gonadotropin-releasing hormone 1; GnRH, gonadotropin-releasing hormone; *Gnrhr*, gonadotropin-releasing hormone receptor; HPA, hypothalamic-pituitary-adrenal axis; *Inhbc*, inhibin beta-C; *Inhbe*, inhibin beta-E; *Isl1*, ISL1 transcription factor, LIM/homeodomain; *Itih3*, inter-alpha trypsin inhibitor, heavy chain 3; JAK/STAT, the Janus kinase/signal transducers and activators of transcription; *Junb*, Jun B proto-oncogene; LH, lateral hypothalamic area; Me, medial amygdala; MePD, posterodorsal part of medial amygdaloid nucleus; MPO/A, medial preoptic region; MSH, melanocyte-stimulating hormones; *Ngfr*, nerve growth factor receptor (TNFR superfamily, member 16); *Nkx2.1*, NK2 homeobox 1; *Nova1*, neuro-oncological ventral antigen 1; *Nrn1*, Neuritin 1; *opt*, optic tract; *Oxt*/OXT, oxytocin; *Pitx1*, paired-like homeodomain transcription factor 1; *Pmch*, pro-melanin-concentrating hormone; *Pomc*/POMC, pro-opiomelanocortin-alpha; *Pou1f1*, POU domain, class 1, transcription factor 1; *Prl*/PRL, prolactin; *Prl2c2*, prolactin family 2, subfamily c, member 2; *Prlr*/PRLR, prolactin receptor; pSTAT5, phosphorylation of STAT5; *Ptgs2*, prostaglandin D2 synthase; *Rln3*, relaxin 3; *Rxfp2*, relaxin/insulin-like family peptide receptor 2; *Siglece*, sialic acid-binding Ig-like lectin; *Stat5*/STAT5, signal transducer and activator of transcription 5; *Tbx19*, T-box 19; *Th*/TH, tyrosine hydroxylase; Trpv1/TRPV1, transient receptor potential cation channel, subfamily V, member 1; *Vmn2r-ps159*, Vomeronasal 2, receptor, pseudogene 159.

Física, Facultat de Ciències Biològiques, Universitat de València, C/ Dr. Moliner, 50. ES-46100, Burjassot, València, Spain.
Email: Enrique.Lanuza@uv.es

Funding information
This study was funded by the Spanish Ministry of Economy and Competitiveness-FEDER (BFU2016-77691-C2-2-P and C2-1-P), the Spanish Ministry of Science, Innovation and Universities (DPI2017-87333-R), the Generalitat Valenciana (PROMETEO/2016/076), and the Universitat Jaume I de Castelló (UJI-B2016-45). M.A-A. was a fellow of the "Programa de Empleo Joven" PEJ-2014-A-63220 of the Spanish Government

transcription factors of the Fos and early growth response families. The gene set analysis revealed numerous Gene Ontology functional groups with higher expression in dams than in pup-sensitized virgin females, including those related with the regulation of the Jak/Stat cascade. Of note, a number of olfactory and vomeronasal receptor genes was expressed in the Me, although without differences between dams and virgins. For prolactin and growth hormone, a qPCR experiment comparing dams, pup-sensitized, and pup-naïve virgin females showed that dams expressed higher levels of both hormones than pup-naïve virgins, with pup-sensitized virgins showing intermediate levels. Altogether, the results show important gene expression changes in the Me, which may underlie some of the behavioral responses characterizing maternal behavior.

KEYWORDS
aggression, prolactin, RNA-Seq, transcriptome, vomeronasal amygdala

1 | INTRODUCTION

Maternal aggression against intruders has an important adaptive value due to its direct relation to offspring survival.[1,2] In mice, this behavior is only displayed by lactating females, even though pup-sensitized virgin females display most of pup-directed maternal behaviors.[2-4] Thus, whereas dams display aggressive responses against intruder males, pup-sensitized females display sociosexual investigatory behavior toward males.[4] Both aggression and investigation are elicited by pheromones, which are chemosensory cues related with intraspecific communication.[5] One of these pheromones is a male-specific major urinary protein named darcin, which induces attraction in virgin females[6] and aggression in dams.[3] This suggests that the aggressive behavior of dams induced by darcin arises after hormonal changes linked to pregnancy and lactation.

The medial amygdala (Me) is the key node in the brain integrating chemosensory and hormonal information,[7,8] and therefore it plays a critical role in sociosexual behaviors in female mice.[9,10] This amygdaloid nucleus receives projections from both the main and accessory olfactory bulbs[11-13] and is enriched in neurons with hormonal receptors.[14,15] Therefore, we hypothesize that it may suffer key changes during pregnancy and/or lactation that underlie the switch from attraction to aggression.

The Me is implicated in the control of aggression in males,[16-19] and probably also in maternal aggression.[18] However, in spite of its central position in the chemosensory pathway and its hormonal inputs, little is known about the physiological changes happening in the Me during pregnancy and lactation, which may underlie the change in significance of male pheromones from attraction-inducing in virgin females to aggression-inducing in dams. Thus, in this work we analyze the changes induced by motherhood in the Me transcriptome by means of RNA-Seq technique, comparing lactating and pup-sensitized virgin females tested for maternal aggression against a male intruder. The use of pup-sensitized virgin females as controls allowed the comparison of two groups of females displaying similar pup-directed behaviors, but dramatically differing in their response toward intruder males. Thus, we expect that the observed changes in gene expression will be induced by the hormonal changes associated to pregnancy and lactation, and will include those mediating aggressive behavior against intruders.

2 | MATERIALS AND METHODS

2.1 | Animals

For this experiment, we used 34 adult mice (*Mus musculus*) from the CD1 strain (females, n = 26; males, n = 8, Janvier; Le Genest Saint-Isle, France). Mice were treated throughout according to the guidelines of the European Union Council Directive of September 22, 2010 (2010/63/UE). The Ethics Committee on Animal Experimentation of the University of Valencia approved all of the experimental procedures.

The animals were 8-12 weeks old and weighed between 27.2 and 49.1 g at the beginning of the experiments. Females for the RNA-Seq experiment were randomly assigned to two groups: dams (n = 6) and pup-sensitized virgin females (n = 6). The group of dams were housed individually with a stud male during 4 days for mating. The day of birth was considered as postpartum day 0, and in PPD2 litters were culled to eight pups. Pup-sensitized virgin females were pair-housed with dams after mating, and therefore were continuously exposed to pups and shared maternal caregiving with

the accompanying dams.[4] For the qPCR experiments, females were randomly assigned to a group of dams (n = 7) or pup-sensitized virgin females (n = 7) treated as described for the RNA-Seq experiments and a group of pup-naïve virgin females (n = 8) (not exposed to pups).

2.2 | Behavioral tests and Me microdissection

Females used for the RNA-Seq were exposed at PPD4 or PPD5 during 5 minutes to an intruder male in their home cage. Pups were separated during behavioral testing to avoid possible damage. Tests were video-recorded and a researcher blind to the experimental groups analyzed the number and duration of attacks, and body and anogenital approaches that females directed toward males, using the SMART 3.0 video tracking system (Panlab, Cornellà, Barcelona, Spain).

Immediately after the behavioral test, animals were sacrificed by cervical dislocation, between 11:00 and 12:00, and the brain was removed for Me microdissection (Figure 1A). Tissue samples were dissected rapidly in ice-cold Dulbecco's phosphate-buffered saline (PBS; Cat # L0615, Biowest, Nuaillé, Pays De La Loire, France), flash-frozen in liquid nitrogen, and maintained on dry ice until storage at –80°C. The micro dissection of the Me of both hemispheres was done following histological landmarks in a 1-mm thick slice obtained by positioning the brain in a stainless-steel brain matrix with a coronal cut of 1 mm. Then, under a stereoscopic microscope, two incisions were made, one following the optic tract (*opt*) and the other tracing an approximately dorso-ventral straight line (Figure 1A). The approximate stereotaxic coordinates of the microdissected tissue (relative to Bregma, from the mouse brain atlas of Paxinos and Franklin[20]) were: A-P: −0.7 to −2.06 mm, M-L: −2 to −2.2 mm, and D-V: −4.2 mm to the ventral surface of the brain. After microdissection, the 1-mm thick brain slice was post-fixed overnight in 4% paraformaldehyde, embedded in 15% gelatin in phosphate buffer, and cut in the freezing microtome in 40-micrometer thick sections. The resulting sections were Nissl-stained for histological validation (Figure 1B-E).

2.3 | RNA extraction

Total RNA from dissected Me was isolated using the RNeasy Mini kit (Cat # 74104, Qiagen, Hilden, Germany). Frozen brain tissue was homogenized in β-mercaptoethanol (Cat # M3148, SIGMA, St. Louis, Missouri, USA) with RLT buffer (RNeasy Mini kit) according to the manufacturer's protocol (10 μL β- mercaptoethanol per 1 mL Buffer RLT). Genomic DNA contamination was eliminated following the RNase-Free DNase Set steps (Cat # 79254, Qiagen, Hilden, Germany). Total RNA was eluted from the RNeasy Mini columns with 40 μL of RNase-free water (Cat # 129112, Qiagen, Hilden, Germany). The amount of total or mRNA isolated from the tissue was assessed for quality and quantified using Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Eight females (dams n = 4; pup-sensitized virgin females n = 4) with the highest levels of aggression (dams) and RNA integrity number (RIN)[21] were selected for RNA-Seq analysis.

2.4 | Illumina sequence read mapping and analyses

RNA-Seq data were obtained at the Genomic core facility of the Universitat de València (Secció de Genòmica-SCSIE, Burjassot, Valencia, Spain). Libraries were obtained using the Illumina TruSeq Stranded mRNA Sample Preparation Kit (San Diego, CA, USA). Cluster generation and sequencing were done in a NextSeq 500 instrument using a High Output kit 1 × 75 bp. Initial quality checks on the Illumina sequencing data were done using FastQC v0.11.3 (http://www.bioinformatics.babraham.ac.uk). Adaptor sequences were trimmed with Cutadapt software (version 1.8.3[22]). Sequencing data were filtered by discarding reads of fewer than 20 nucleotides and more than 30 undetermined nucleotides.

The *M musculus* reference genome was obtained from Ensembl.[23] A total of 375 237 009 bp single-end reads were generated from the 8 RNA samples (average of 46 904 626 reads per sample). The processed Illumina reads were mapped to the reference genome using the Tophat alignment tool,[24,25] version 2.0.8, which uses Bowtie2 as its underlying alignment algorithm.[26] The obtained SAM files were compressed into the BAM files using samtools.[27] The count matrix was calculated using Rsamtools, GenomicFeatures, and GenomicAlignments[28] (Supporting Information I).

The parameters of Tophat were set to their default values. The GTF option was used to provide Tophat with a set of gene model annotations (note that we are using Rsamtools). Genes with a positive count for any sample were included in the study. To analyze differential gene expression, the Bioconductor package edgeR was used (Bioconductor version 3.0.2).[29] To determine whether a gene was differentially expressed in dams versus pup-sensitized virgin females, a common dispersion parameter was assumed and estimated. The raw *P* values obtained with the edgeR method were transformed to adjusted *P* values (adjp) using the Benjamini-Hochberg correction in order to control the false discovery rate (FDR).[30] Genes with significant differential expression using a FDR equal or below to 0.05 are listed in Supporting Information II, sections A and B. Genes without significant differential expression but adjusted *P* values <1 are listed in Supporting Information II, section C.

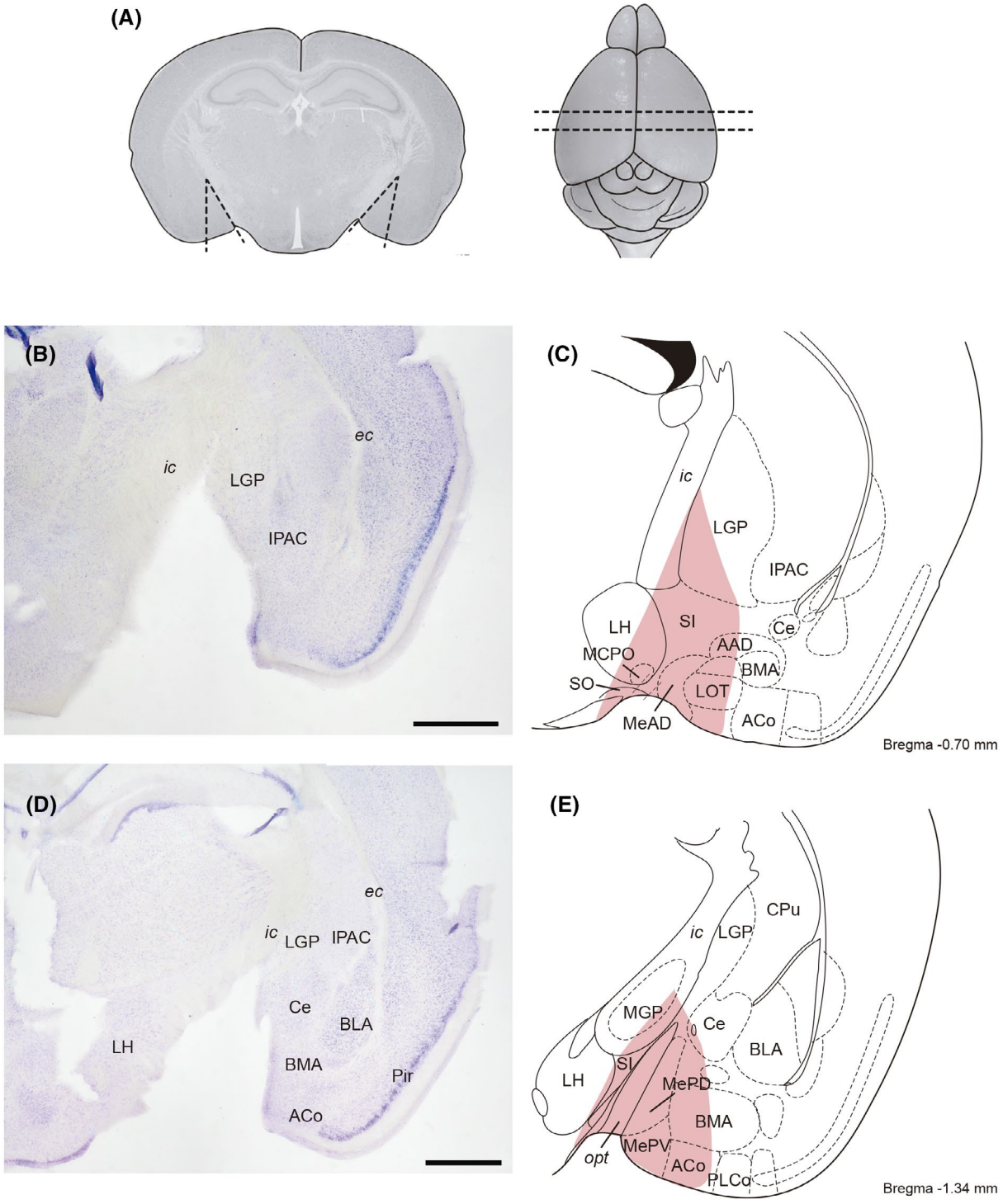


FIGURE 1 Histological validation of Me microextraction, showing the anatomical position of the slice taken to perform the microdissection (A). Photomicrographs of Nissl-stained sections showing the rostral (B) and caudal (D) boundaries of the tissue included in the study in a representative case. C and E, depict schematic reconstructions of coronal brain sections corresponding to photomicrographs B and D, showing the structures included. The colored outline represents the extent of Me extraction. Scale bar in B and D: 1 mm. AAD, Anterior amygdaloid area, dorsal part; ACo, anterior cortical amygdaloid nucleus; BLA, basolateral amygdaloid nucleus, anterior part; BMA, basomedial amygdaloid nucleus, anterior part; Ce, central amygdaloid nucleus; CPu, caudate putamen (striatum); *ic*, internal capsule; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; LGP, lateral globus pallidus; LH, lateral hypothalamic area; LOT, nucleus of the lateral olfactory tract; MCPO, magnocellular preoptic nucleus; MeAD, medial amygdaloid nucleus, anterodorsal; MePD, medial amygdaloid nucleus, posterodorsal part; MePV, medial amygdaloid nucleus, posteroventral part; MGP, medial globus pallidus; *opt*, optic tract; PLCo, posterolateral cortical amygdaloid nucleus; SI, substantia innominate; SO, supraoptic nucleus

Gene set analysis includes evaluation of the expression of genes that are grouped based on their interacting role in biological pathways (biological pathway analysis) and genes that share similar cellular functions (functional gene sets). Gene Ontology was used to obtain the gene set collection. The self-contained null hypothesis[31] was tested using permutation tests. This null hypothesis establishes that the gene set considered does not contain any genes which expression levels are associated with the phenotype of interest. Statistical significance was calculated using permutation tests. The significant groups, using a FDR equal to 0.05, are shown in Supporting Information III. The representation of gene interactions shown in Figures 4 and 5 is generated using GeneMANIA.[32]

2.5 | Validation of the dissection

First, we performed a histological validation of the dissections by means of the analysis of the Nissl-stained sections obtained after the Me extraction (Figure 1B-E). In addition, we performed a literature survey to identify genes specifically expressed in the Me.[33-35] The same strategy was carried out with the Allen Brain Atlas and the Gene Help of NCBI's databases.[33] The genes expressed in Me that are gathered from the literature search and from the Allen Brain Atlas were compared with the list obtained in the RNA-seq (Supporting Information IV).

2.6 | Quantitative PCR experiments

The procedures for Me microdissection and the subsequent RNA isolation were the same as those used for the RNA-Seq experiments. The RNA concentrations were quantified with Qubit 3.0 Quantitation Starter Kit (Invitrogen, Cat # Q33217, Thermo Fisher Scientific, Waltham, MA, USA). cDNA was produced from each sample using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Cat # 1808093) according to the manufacturer's instructions. For each sample, 132 ng of total RNA was copied. The primers used in qPCR reactions were designed using Primer3Plus software.[36] Primers for Prl and Actinβ (*Actb*) (Table 1) were designed to span an exon-exon boundary (to exclude genomic amplification) and to amplify a product between 50 and 150 bp. Regarding *Gh* and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), the primers were the same as those used in Daude et al.[37] *Actb* and *Gapdh* were employed as housekeeping controls. All primers used were tested in a nucleotide Basic Local Alignment Search Tool (BlastN; BLAST: Basic Local Alignment Search Tool [nih.gov]) specific for mouse (taxid: 10090). GenBank IDs (see Table 1) corroborate mRNA specificity of each pair of primers.

Quantitative PCR was performed using TB Green Premix Ex Taq (Tli RNase H Plus, Cat # RR420A, Takara Bio, Shiga, Japan) in a 10 μL reaction volume, using 2 μL cDNA reaction and 0.4 μL of primers (5 μM), following the manufacturer's instructions. Master mixes were made for each pair of primers and cDNAs were added independently for the genes of interest and the housekeepings. Same master mixes were used for a non-template control (NTC) adding water instead cDNA and they were loaded at same plate. Briefly, following an enzyme activation step of 30 s at 95°C, we performed 40 cycles of 15 s denaturation step at 95°C, 34 s annealing at 56°C, and 15 s extension at 95°C, and fluorescence data collection at the end of the extension step. Melt-curve analysis followed each run with ramping from 50° to 95°C, with fluorescence data collection in 0.5°C increments. Each sample, comprising cDNA from one animal, was run in triplicate.

Quantification was based on the number of PCR cycles required to cross a threshold (Ct) of fluorescence intensity. NTC was undetermined in 71% of the cases, and fluorescent readings in the rest of cases showed different melting temperature (Tm) than the Tm of the experimental wells. The mean Ct value of the NTC showing fluorescence was 36.004 ± 0.669 (range 30.124-39.723). These Ct and Tm values are consistent with primer hybridization. A few reads of the genes of interest showed two (or three) Tm values. Those readings (17 out of 325) were excluded of the analysis, to avoid any fluorescence due to primer

TABLE 1 Sequences of primers used for qPCR and GenBank Accession Number for each gene analyzed

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)	GenBank ID: Accession Number
Prl	atcaatgactgccccacttc	ctgcaccaaactgaggatca	NM_011164.2; NM_001163530.1
Gh	aagagttcgagcgtgcctac	ggatggtctctgagaagcaga	NM_008117.3
Actb	gctgtattcccctccatcgt	gacccattcccaccatcac	NM_007393.5
Gapdh	aagagttcgagcgtgcctac	ggatggtctctgagaagcaga	NM_001289726.1; NM_008084.3

Note: The alignment specificity of each pair of primers to the target genes was checked using basic local alignment search tool (BLAST).

hybridization to be included in the experimental data. Data point was calculated for each animal as the average of the three replicates (after excluding the replicates with several melting temperatures [Tm]). The Ct data for each studied gene was normalized to the geometric mean of the Ct values of the housekeeping genes (*Actb* and *Gadph*) to obtain the ΔCt values for each animal. The ΔCt values of the three experimental groups were normalized to the ΔCt mean of the pup-naïve virgins, giving a ΔΔCt value. This was converted into expression fold change using the $2^{\Delta\Delta Ct}$, called relative quantification (RQ).[38-40]

Statistical analyses were performed using IBM SPSS Statistics 26.0. First, we checked if the data fulfilled the conditions of the ANOVA: normality (Kolmogorov-Smirnov test with Lilliefors' correction) and homoscedasticity (Levene's test). In the case of lack of normality, data were analyzed using Mann-Whitney *U* or Kruskal-Wallis nonparametric tests. Statistically significant differences ($P < .05$) were further explored by means of post hoc pairwise comparisons with Bonferroni's correction.

Finally, the data obtained in the results of both qPCR (Prl and Gh) of Me microdissections from the same animals were analyzed with a Spearman correlation for nonparametric measures.

3 | RESULTS

3.1 | Behavioral tests

This experiment was carried out to select the dams with higher levels of maternal aggression for RNA-seq studies. The comparison of the total duration of attacks showed that four of the females displayed maternal aggression (Figure 2A), whereas the other two animals showed low levels of aggressive behavior (less than 2 seconds, not shown). Therefore, the four dams with higher levels of aggression and the pup-sensitized virgin females which shared the home cage with them were selected for further RNA-Seq studies. As expected, pup-sensitized virgin females did not display aggression toward males, and thus the comparison of the time spent attacking the intruder males showed significant differences (Mann-Whitney *U* test, $P < .05$). Pup-sensitized virgin females showed chemoinvestigatory behaviors (body approaches and anogenital sniffing toward males), but in this case the time spent in these sociosexual behaviors did not differ between virgins and dams (Mann-Whitney *U* test, $P > .05$) (Figure 2B).

In addition to behavior, after the RNA extraction, RIN value of these highly aggressive dams was checked to discard animals with RIN lower than 8. The results showed that all the highly aggressive dams and their respective pup-sensitized virgin females presented RIN values above 8 (data not shown).

3.2 | Histology

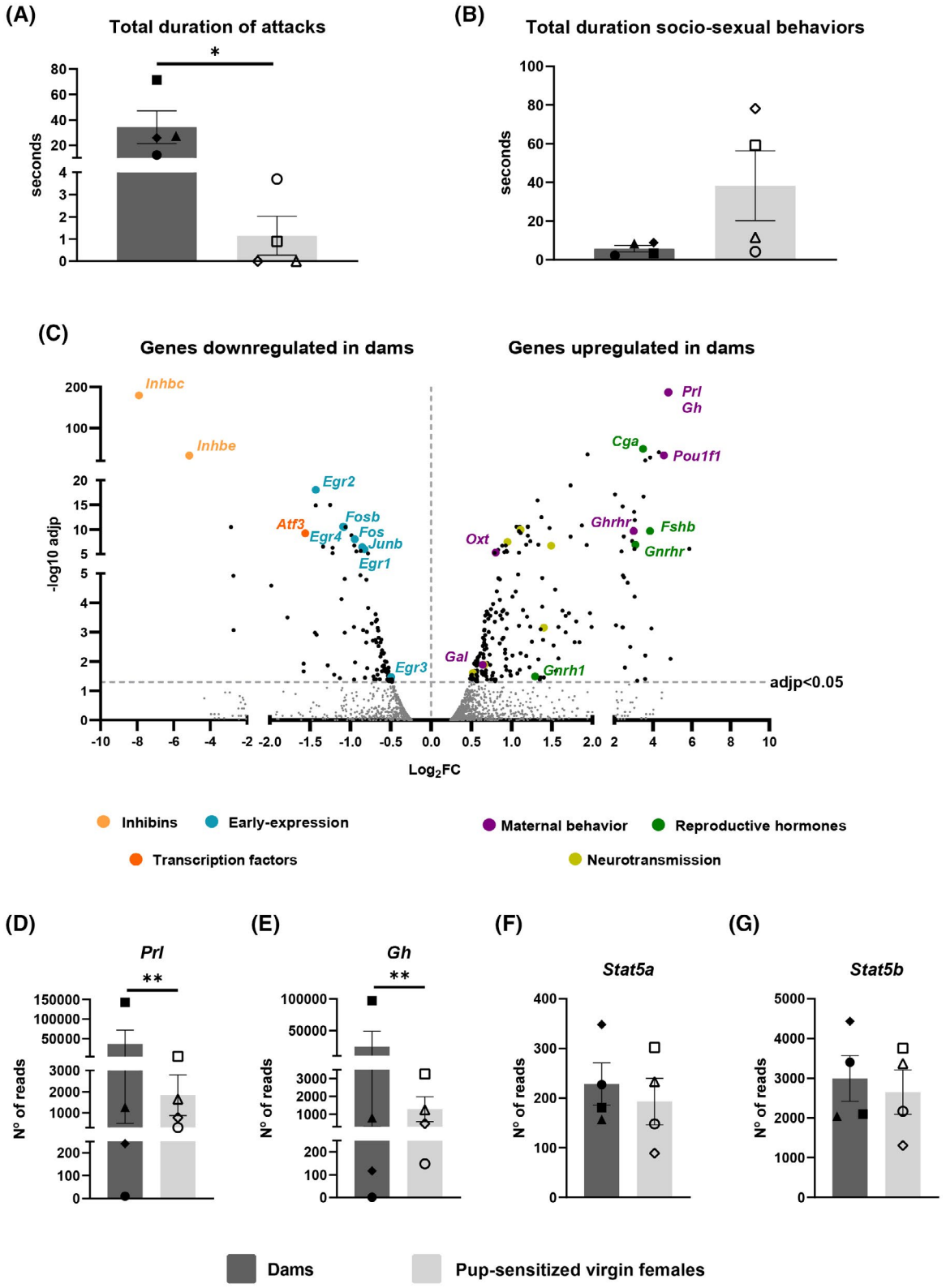
The Nissl preparations of the tissue showed that the Me was completely dissected in all the studied cases, including the three Me subdivisions (anterior, posterodorsal, and posteroventral parts) (Figure 1). It is important to remark that small portions of the structures adjacent to Me were included in the microdissection in most cases. At rostral levels, the nearest aspects of the substantia innominata, lateral globus pallidus, and internal capsule were affected by the incisions. In the rostral amygdala, parts of the anterior amygdaloid area, the nucleus of the lateral olfactory tract, the bed nucleus of the accessory olfactory tract, and the anterior cortical amygdaloid nucleus were encompassed, to some extent, by the microdissected tissue. More caudally, parts of the anterior basomedial amygdaloid nucleus (BMA), the central amygdala (Ce), the intra-amygdaloid division of the bed nucleus of the stria terminalis, the posteromedial cortical amygdaloid nucleus, and the amygdalohippocampal area were also included. In addition, extra-amygdaloid structures located medial to the microdissection, such as the supraoptic nucleus (SO), the *opt*, the lateral hypothalamic area, and the magnocellular preoptic nucleus were pulled out partially in some animals.

3.3 | Differential gene expression analysis in Me

The differential gene expression analyzed using the method proposed in Robinson and Smyth[41,42] and implemented in the R package edgeR, with an adjusted *P* value <.05 (ie, a FDR = 0.05), yielded a total of 296 differentially expressed genes in the Me. From these, 197 genes were upregulated in dams as compared to pup-sensitized virgin females, and 99 were downregulated. The full list of genes with significant differential expression is given in sections A and B of Supporting Information II. In these tables, genes were classified depending on their fold change (FC), in other words, depending on how much they were upregulated or downregulated in dams with respect to pup-sensitized virgin females. The number of reads per animal is also provided in these tables.

3.4 | Genes upregulated in dams

Some of the most upregulated genes, according to the FC, were related to maternal behavior and lactation (Figure 2C). Specifically, within those genes related with maternal behavior, prolactin (*Prl*) and growth hormone (*Gh*) were the ones with more upregulated mean expression in dams respect to pup-sensitized virgin females. Genes coding for peptides



previously reported to be involved in maternal behavior were also upregulated, including oxytocin (*Oxt*) and galanin (*Gal*). In addition, other genes related to *Gh* and/or *Prl* were also more expressed in dams than in pup-sensitized virgin females, such as the growth hormone-releasing hormone receptor (*Ghrhr*), the transcription factor POU domain, class 1, transcription factor 1 (*Pit-1* or *Pou1f1*), the paired-like homeodomain transcription factor 1 (*Pitx1*), which encodes a transcriptional regulator involved in basal and hormone-regulated activity of prolactin, and the cytokine-inducible SH2-containing protein (*Cis* or *Cish*) which play a role in the regulation of signal transducer and activator of transcription protein-5 (STAT5)

FIGURE 2 A, Maternal aggression test. Scatter plot showing the individual levels of aggression of dams when exposed to an intruder male. Pup-sensitized virgin females displayed almost no aggressive behavior. Individual animals are identified by the geometric shape representing the data point. The same geometric shape identifies co-housed dams and pup-sensitized females. *$P < .05$ (Mann-Whitney *U* test). B, Time spent performing non-aggressive body and anogenital exploration (sociosexual behaviors). No significant difference in this variable was observed. Data points of individual animals coded as in panel A. C, Volcano plot showing the differential gene expression analysis between dams and pup-sensitized virgin females. The graph displays the expression FC (Log_2FC) for each gene and its associated adjusted *P* value ($-Log_{10}$ adjp). The gray dots below the dotted line show genes with non-significant differences in expression between dams and pup-sensitized virgin females. The right side of the graph (positive fold change values) shows genes significantly upregulated in dams, with those related with maternal behavior (purple), reproductive hormones (dark green), and neurotransmission (light green) highlighted in color. The left side of the graph (negative fold change values) shows significantly downregulated genes in dams, highlighting in color those used as neural activity markers (blue), inhibins (light orange), and transcription factors (dark orange). D-G, Scatter plot showing the data of the number of reads obtained in the RNA-Seq for the *Prl*, *Gh*, *Stat5a*, and *Stat5b* genes. Data points of individual animals coded as in panel A. *adjp <.01 (edgeR analysis, see text and Table 2). *Atf3*, Activating transcription factor 3; *Egr1*, early growth response 1; *Egr2*, early growth response 2; *Egr3*, early growth response 3; *Egr4*, early growth response 4; *Fos*, FBJ osteosarcoma oncogene; *Fosb*, FBJ osteosarcoma oncogene B; *Fshb*; follicle-stimulating hormone beta; *Gal*, galanin; *Cga*, glycoprotein hormones, alpha subunit; *Gnrh1*, gonadotropin-releasing hormone 1; *Gnrhr*, gonadotropin-releasing hormone receptor; *Gh*, growth hormone; *Ghrhr*, growth hormone-releasing hormone receptor; *Inhbc*, inhibin beta-C; *Inhbe*, inhibin beta-E; *Junb*, jun B proto-oncogene; *Ngfr*, nerve growth factor receptor (TNFR superfamily, member 16); *Oxt*, oxytocin; *Pou1f1*, POU domain, class 1, transcription factor 1; *Prl*, prolactin; *Stat5a* and *Stat5b*, signal transducer and activator of transcription 5a and b

signaling (Table 2). In contrast, other genes important to social and maternal behavior such as prolactin receptor (*Prlr*), signal transducer and activator of transcription 5 (*Stat5a/b*), arginine vasopressin receptor 1A (*Avpr1a*), and arginine vasopressin (*Avp*) did not show significant adjusted *P* values using a FDR= 0.05 (Supporting Information IIC).

Since it has been previously reported that the expression of *Prl* and *Gh* in the brain is coordinated, although superimposed to a large interindividual variation,[37] we analyzed the intragroup variability (Figure 2D-G) of the reads of these genes, and also those of the *Stat5a/b*, which are involved in the common signaling pathway of *Prl* and *Gh*, as well as the possible correlation between the expression levels of these genes and the aggressive behavior displayed in the maternal aggression test, using the nonparametric Spearman tests. The results confirm the previously reported large interindividual variation, and also show that the number of reads of *Prl* and *Gh* is significantly correlated within dams ($P < .01$). In addition, the levels of *Prl* and *Gh* show a significant correlation with the aggression levels ($P < .01$). In contrast, in pup-sensitized virgin females *Prl* and *Gh* are significantly correlated ($P < .01$) but no correlation is present with the aggression levels ($P > .6$ in both cases). On the other hand, the levels of expression of *Stat5a* and *Stat5b* are significantly correlated both in dams and pup-sensitized females ($P < .01$ in both groups), but in no case show correlation with *Prl* and *Gh* of aggressive responses ($P > .4$ in all cases).

Other genes related with reproductive hormones or involved in the reproductive cycle were also significantly upregulated in dams (Figure 2C). These genes included the follicle-stimulating hormone beta (*Fshb*), the alpha subunit of glycoprotein hormones (*Cga*), which encodes the follicle-stimulating hormone (FSH) alpha subunit, the gonadotropin-releasing hormone receptor (*Gnrhr*), and the gonadotropin-releasing hormone 1 (*Gnrh1*) (Table 2).

A third group of genes overexpressed in dams were related with different kinds of neurotransmission (Table 2). Among them, we found several genes related with cholinergic neurotransmission, such as the choline acetyltransferase (*Chat*), several subunits of the nicotinic receptor, including the beta polypeptide 3 (*Chrnb3*), the alpha polypeptide 6 (*Chrna6*), and the alpha polypeptide 2 (neuronal) (*Chrna2*), and the cardiac muscarinic 2 subunit (*Chrm2*). In addition, the nerve growth factor receptor (TNFR superfamily, member 16) (*Ngfr*) a p75 neurotrophin receptor, which is expressed in the cholinergic neurons of the basal telencephalon, was upregulated in dams (Table 2).

Another group of genes with significantly increased mean expression in the Me of dams was related with catecholaminergic pathways. Within these genes, the alpha 2b adrenergic receptor (*Adra2b*) and the tyrosine hydroxylase (*Th*) were upregulated in dams respect to pup-sensitized virgin females (Table 2).

The Me of dams also showed significant differences in the regulation of genes involved in peptidergic neurotransmission. One of these overexpressed genes in dams is the pro-opiomelanocortin-alpha (*Pomc*). This gene codifies for pro-opiomelanocortin (POMC), which is cleaved in different peptides as adrenocorticotropic hormone (ACTH), the alpha-, beta-, and gamma-melanocyte-stimulating hormones (α-, β-, and γ-MSH) and β-endorphin. A related overexpressed gene is the T-box 19 (Tbx19), which appears to be selectively expressed in precursors of the corticotrope/melanotrope lineages and in the rostral ventral diencephalon, and may act as a component of the POMC gene activation program. Also, the pro-melanin-concentrating hormone (*Pmch*) was upregulated in the Me of dams. On the other hand, the gene encoding the relaxin/insulin-like family peptide receptor 2 (*Rxfp2*) was expressed more in dams than in pup-sensitized virgin females (Table 2). However, the relaxin 3 gene (*Rln3*) did not

TABLE 2 Summary of significant upregulated genes in dams with respect to pup-sensitized virgin females

Group	Gene	FC	Adjp
Maternal behavior	*Prl*	27.8	2.66×10^{-188}
	Gh	27.47	1.15×10^{-187}
	Ghrhr	23.59	3.77×10^{-34}
	Pou1f1	8	2.21×10^{-10}
	Oxt	1.75	4.82×10^{-06}
	Gal	1.56	1.31×10^{-02}
Reproductive hormones	*Fshb*	14.32	2.21×10^{-10}
	Cga	11.16	4.04×10^{-50}
	Gnrhr	8.51	1.30×10^{-07}
	Gnrh1	2.45	3.24×10^{-02}
Neurotransmission			
Cholinergic	*Chrnb3*	2.81	1.99×10^{-07}
	Chrna6	2.64	7.03×10^{-04}
	Chat	2.16	1.13×10^{-10}
	Ngfr	1.93	3.45×10^{-08}
	Chrna2	1.58	1.30×10^{-02}
	Chrm2	1,43	2.47×10^{-02}
Catecholaminergic	*Adra2b*	1.90	1.47×10^{-02}
	Th	1.58	3.94×10^{-03}
	Pomc	2.30	2.43×10^{-11}
	Pmch	1.86	2.43×10^{-11}
	Rxfp2	1.74	4.16×10^{-02}
Glutamatergic	*Trpv1*	8.34	1.29×10^{-12}
Cell adhesion	*Epcam*	4.11	7.57×10^{-18}
	Siglece	3.10	1.87×10^{-04}
Others	*Tbx19*	12.13	6.24×10^{-03}
	Pitx1	8.11	2.73×10^{-14}
	Vmn2r-ps159	7.06	3.17×10^{-03}
	Cis	1.47	4.47×10^{-02}

Note: Genes are ordered according to their fold change (fc) in each group. Raw *P* values (Supporting Information IIA) were transformed to adjusted *P* values (adjp).

Abbreviations: *Adra2b*, Adrenergic receptor, alpha 2b; *Cga*, glycoprotein hormones, alpha subunit; *Chat*, choline acetyltransferase; *Chrm2*, cholinergic receptor, muscarinic 2, cardiac; *Chrna2*, cholinergic receptor, nicotinic, alpha polypeptide 2 (neuronal); *Chrna6*, cholinergic receptor, nicotinic, alpha polypeptide 6; *Chrnb3*, cholinergic receptor, nicotinic, beta polypeptide 3; *Cis*, cytokine-inducible SH2-containing protein; *Epcam*, epithelial cell adhesion molecule; *Fshb*, follicle-stimulating hormone beta; *Gal*, galanin; *Gh*, growth hormone; *Ghrhr*, growth hormone-releasing hormone receptor; *Gnrh1*, gonadotropin-releasing hormone 1; *Gnrhr*, gonadotropin-releasing hormone receptor; *Ngfr*, nerve growth factor receptor (TNFR superfamily, member 16); *Oxt*, oxytocin; *Pitx1*, paired-like homeodomain transcription factor 1; *Pmch*, pro-melanin-concentrating hormone; *Pomc*, pro-opiomelanocortin-alpha; *Pou1f1*, POU domain, class 1, transcription factor 1; *Prl*, prolactin; *Siglece*, sialic acid-binding Ig-like lectin E; *Tbx19*, T-box 19; *Th*, tyrosine hydroxylase; *Trpv1*, transient receptor potential cation channel, subfamily V, member 1; *Vmn2r-ps159*, vomeronasal 2, receptor, pseudogene 159.

TABLE 3 Genes most downregulated in dams compared with pup-sensitized virgin females

Group	Gene	−FC	Adjp
Inhibins	*Inhbc*	238.86	2.06×10^{-180}
	Inhbe	35.51	1.02×10^{-33}
Transcription factors	*Atf3*	2.95	6.01×10^{-10}
Early expression genes	*Egr2*	2.69	8.89×10^{-19}
	Fos	2.13	9.33×10^{-09}
	Egr4	2.11	3.06×10^{-11}
	Fosb	1.93	2.67×10^{-11}
	Junb	1.81	4.07×10^{-7}
	Egr1	1.77	1.13×10^{-06}
	Egr3	1.41	3.42×10^{-02}

Note: Genes are ordered in each group according to their negative fold change (−FC). Raw *P* values (Supporting Information IIB) were transformed to adjusted *P* values (adjp).

Abbreviations: *Atf3*, activating transcription factor 3; *Egr1*, early growth response 1; *Egr2*, early growth response 2; *Egr3*, early growth response 3; *Egr4*, early growth response 4; *Fos*, FBJ osteosarcoma oncogene; *Fosb*, FBJ osteosarcoma oncogene B; *Inhbc*, inhibin beta-C; *Inhbe*, inhibin beta-E; *Junb*, jun B proto-oncogene.

show significant differences in expression (adjusted *P* value >.05; Supporting Information IIC).

In addition, the transient receptor potential cation channel, subfamily V, member 1 (*Trpv1*), which is involved in glutamatergic neurotransmission, was also overexpressed in dams (Table 2).

Two genes related with cell adhesion showed significant mean expression differences between dams and pup-sensitized virgin females in the Me: the epithelial cell adhesion molecule (*Epcam*) and the sialic acid-binding Ig-like lectin E (*Siglece*) (Table 2).

Finally, a non-expected finding within the genes showing overexpression in dams was the vomeronasal type 2 receptor, pseudogene 159 (*Vmn2r-ps159*) (Table 2).

3.5 | Genes downregulated in dams

The most downregulated genes in dams compared with pup-sensitized virgin females were inhibin Beta-C (*Inhbc*) and E (*Inhbe*) (Figure 2C; Table 3).

In addition, the activating transcription factor 3 (*Atf3*) was significantly downregulated in dams. *Atf3* is a member of the mammalian activation transcription factor/cAMP responsive element-binding (CREB) protein family of transcription factors (Figure 2C). Some other genes related with cell activation were also significantly downregulated in dams (Figure 2C), such as the FBJ osteosarcoma oncogene (*Fos*), the FBJ osteosarcoma oncogene B (*Fosb*) (Table 3), and several members of the early growth response family, such as

the early growth response 1 (*Egr1*), early growth response 2 (*Egr2*), early growth response 3 (*Egr3*), and early growth response 4 (*Egr4*) (Table 3).

3.6 | Genes with no differential expression

Finally, our data revealed the expression of a number of genes encoding olfactory (OR558, OR690, OR691, OR1349, OR1392, OR1417, and OR1564) and vomeronasal receptors (type 1, V1R32, and type 2, V2R29, V2R54, V2R57, and V2R87), which were not differentially expressed in the Me of virgins and dams (Supporting Information IIC).

3.7 | Bibliographic survey

The genes obtained in the RNA-Seq were compared with those previously reported to be expressed in the Me (according to the literature and database survey described in the Methods). Some of the genes that matched with the literature were overexpressed in dams respect to pup-sensitized virgin females, including: *Oxt*,[33] ISL1 transcription factor LIM/homeodomain (*Isl1*),[34] inter-alpha trypsin inhibitor, heavy chain 3 (*Itih3*),[43] and NK2 homeobox 1 (*Nkx2.1*).[34]

On the other hand, aromatase (*Cyp19a1*),[18] vasopressin (*Avp*),[33] adenylate cyclase activating polypeptide 1 (*Adcyap1*),[33] neuritin 1 (*Nrn1*),[43] prostaglandin D2 synthase (*Ptgs2*),[35] glycerophosphodiester phosphodiesterase domain containing 2 (*Gdpd2*), delta-like non-canonical Notch ligand 1 (*Dlk1*), and neuro-oncological ventral antigen 1 (*Nova1*)[43] concur with the bibliography as genes expressed in Me. However, these genes did not show significant differences in expression between dams and pup-sensitized virgin females (adjusted *P* value >.05) (Table 4).

3.8 | Gene set analysis

We have tested the self-contained null hypothesis of no association between gene set and phenotype using a gene enrichment set analysis where the per-gene statistic has been the *P* value previously considered. The enrichment measure has been the mean of the *P* values per group. The randomization null distribution of the enrichment measure has been used. The gene set collection was obtained from Gene Ontology.[44] The analysis of these functional groups (Supporting Information III) yields several groups of genes with significant differential expression, among which it is important to remark the positive regulation of Janus kinase/signal transducer and the activator of transcription protein (JAK-STAT) cascade and the negative regulation of extracellular signal-regulated kinases 1 and 2 cascade, because of the relation with the study ($P < .01$).

TABLE 4 Genes in the present results already reported to be characteristically expressed in the Me.

Group	Gene	FC	Adjp
With differential expression between groups	*Oxt*	1.75	3.6×10^{-03}
	Nkx2.1	1.61	3.6×10^{-03}
	Isl1	1.57	7.41×10^{-03}
	Itih3	1.40	3.89×10^{-02}
Without differential expression between groups	*Dlk1*	1.26	4.51×10^{-01}
	Avp	1.25	5.16×10^{-01}
	Nova1	1.22	7.13×10^{-01}
	Gdpd2	1.21	8.30×10^{-01}
	Adcyap1	0.84	9.05×10^{-01}
	Nrn1	0.82	6.51×10^{-01}
	Ptgs2	0.79	3.73×10^{-01}
	Cyp19a1	0.78	9.17×10^{-01}

Note: Genes are ordered in each group according to their fold change (FC). Raw *P* values (Supporting Information IV) were transformed to adjusted *P* values (adjp).

Abbreviations: *Adcyap1*, adenylate cyclase activating polypeptide 1; *Avp*, arginine vasopressin; *Cyp19a1*, cytochrome P450, family 19, subfamily a, polypeptide 1; *Dlk1*, delta-like non-canonical Notch ligand 1; *Gdpd2*, glycerophosphodiester phosphodiesterase domain containing 2; *Isl1*, ISL1 transcription factor, LIM/homeodomain; *Nkx2.1*, inter-alpha trypsin inhibitor, heavy chain 3 NKX2-1 antisense RNA 1; *Nova1*, NOVA alternative splicing regulator 1; *Nrn1*, neuritin 1; *Oxt*, oxytocin; *Ptgs2*, prostaglandin-endoperoxide synthase 2.

3.9 | Validation of the differential expression of Prl and Gh by means of qPCR

To validate and extend the results of the RNA-Seq study on the expression levels of Prl and Gh, we performed a qPCR experiment of the Me in dams, pup-sensitized virgins, and also pup-naïve virgin females. The housekeeping genes *Actb* and *Gapdh* showed stable expression levels among the three studied groups (One-way ANOVA of the geometric mean values, $F_{2,21} = 3.04$; $P > .05$). Since RQ values of *Prl* and *Gh* did not follow a normal distribution, we used the nonparametric Kruskal-Wallis test to analyze these data. The results showed significant differences in *Prl* and *Gh* expression levels between the studied groups (*Gh* $\chi^2(2) = 8.065$, $P < .05$; *Prl* $\chi^2(2) = 9.08$, $P < .05$). Post hoc pairwise comparisons with Bonferroni's correction revealed significant differences between gene expression levels in the group of lactating females and pup-naïve virgins in both studied genes (*Prl* $P < .01$; *Gh* $P < .05$) (Figure 3A,B). There was no significant difference in gene expression levels between pup-sensitized and pup-naïve virgin females (*Prl* $P > .2$; *Gh* $P > .15$) and between

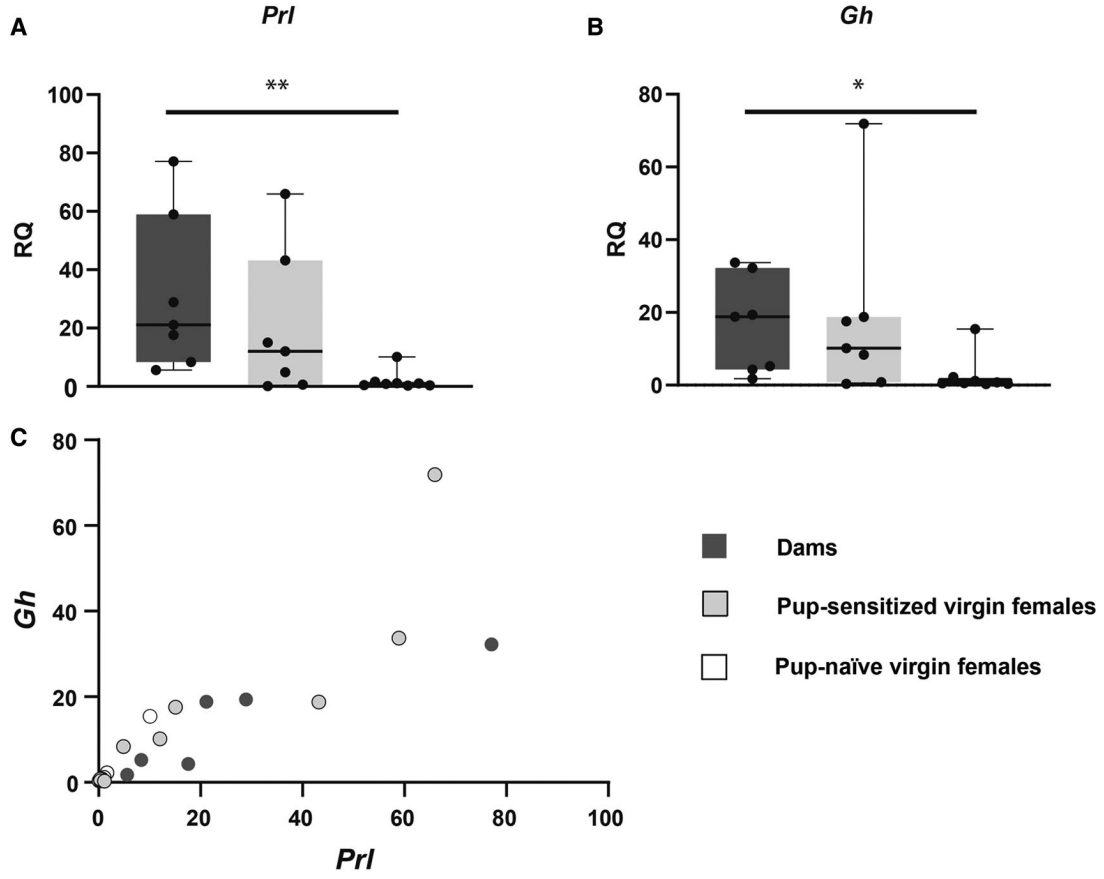


FIGURE 3 The analysis of *Prl* and *Gh* expression levels (RQ) using qPCR showed significant differences among dams, pup-sensitized virgin females, and pup-naïve virgin females. *Prl* (A) and *Gh* (B) expression was significantly higher in dams than in pup-naïve virgin females, with the levels of expression of both genes being intermediate in pup-sensitized virgin females. Data are represented as box and whiskers plot (box: median and 25th to 75th percentiles; whiskers: maximum and minimum values) and analyzed by Kruskal-Willis tests and post hoc pairwise comparisons with Bonferroni corrections; $^{**}P \leq .01$, $^{*}P \leq .05$. C, Positive correlation between the *Prl* and *Gh* expression levels obtained by qPCR in the same animals (Spearman test, $r = .925$, n = 22, $P < .01$)

lactating females and pup-sensitized virgin females (*Prl* $P > .6$; *Gh* $P = 1$) (Figure 3A,B).

As performed above for the RNA-Seq data, a Spearman correlation test was run with the data of the qPCR of *Prl* and *Gh* of the same animals. This analysis showed a strong, positive, and significant correlation ($r = .925$, n = 22, $P < .01$), between the expression levels of both genes (Figure 3C). The correlation was also significant if dams and pup-sensitized virgin females were analyzed separately (dams: $r = .929$, $P < .01$; pup-sensitized females, $r = 1$, $P < .001$). However, no correlation was observed in the pup-naïve virgin females when this group was analyzed separately ($r = .476$, $P > .2$).

4 | DISCUSSION

The goal of this study was to determine the changes in gene expression in the medial amygdaloid region of the female mouse brain that result from pregnancy, parturition, and lactation, specifically in dams displaying maternal aggression. The use of pup-sensitized virgin females as controls allows highlighting those differences between dams and virgin females not due to exposure to pup-derived stimuli, but due to the effects of motherhood-associated hormones. The results show 197 genes upregulated in dams respect of pup-sensitized virgin females in Me and other 99 genes with higher expression in the group of pup-sensitized virgin females compared with lactating females (summarized in Tables 2 and 3). Thus, there are important changes in gene expression in Me during the lactation period. The differentially expressed genes in Me may have an important functional role in hormonally induced maternal behavior, and probably include those relevant for maternal aggression, since pup-sensitized virgin females express maternal care but not maternal aggression.[2] Of note, an important variation is present in the RNA-Seq results, especially in dams, and therefore the present findings should be confirmed by future investigations on the role that the different genes may play in maternal behavior.

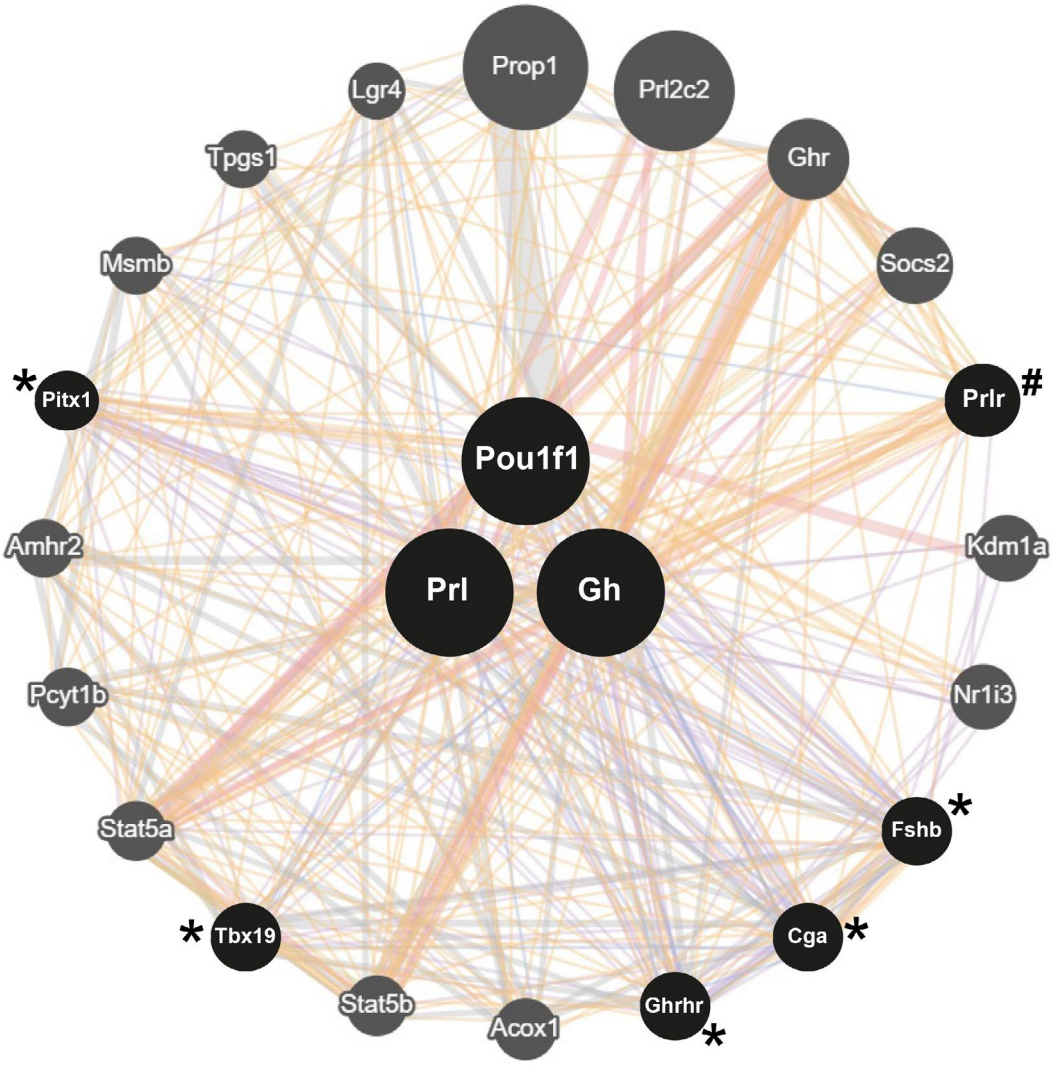


FIGURE 4 Overview of *Pou1f1*, *Prl*, and *Gh* signaling interactions with other genes in *M musculus*, according to GeneMANIA database. The grapho shows in black circles the genes obtained in our RNA-seq study, and in gray those not present in the RNAseq results. The significantly upregulated genes obtained in mothers with respect to pup-sensitized virgin females are indicated with an asterisk (*) and the non-significant expressed genes appearing in the RNA-seq list (see non-significant genes in Supporting Information II, section C) are depicted with a hash symbol (#). For GeneMANIA network color categories, see http://pages.genemania.org/help/. *Acox1*, Acyl-Coenzyme A oxidase 1, palmitoyl; *Amhr2*, anti-Mullerian hormone type 2 receptor; *Cga*, glycoprotein hormones, alpha subunit; *Fshb*, follicle-stimulating hormone beta; *Gh*, growth hormone; *Ghr*, growth hormone receptor; *Ghrhr*, growth hormone-releasing hormone receptor; *Kdm1a*, lysine (K)-specific demethylase 1A; *Lgr4*, leucine-rich repeat-containing G protein-coupled receptor 4; *Msmb*, beta-microseminoprotein; *Nr1i3*, nuclear receptor subfamily 1, group I, member 3; *Pcyt1b*, phosphate cytidylyltransferase 1, choline, beta isoform; *Pitx1*, paired-like homeodomain transcription factor 1; *Pou1f1*, POU domain, class 1, transcription factor 1; *Prl*, prolactin; *Prl2c2*, prolactin family 2, subfamily c, member 2; *Prlr*, prolactin receptor; *Prop1*, paired-like homeodomain factor 1; *Socs2*, suppressor of cytokine signaling 2; *Stat5a*, signal transducer and activator of transcription 5A; *Stat5b*, signal transducer and activator of transcription 5B; *Tbx19*, T-box 19; *Tpgs1*, tubulin polyglutamylase complex subunit 1

The females in the group of dams also differed from the pup-sensitized virgin females in the mating experience. Mating took place about 25 days before the sacrifice of the animals, and thus long-lasting changes in gene expression would also be observed in the present experimental design. This type of long-lasting changes has been described (mainly in the hypothalamus) in the female brain 4 weeks after weaning.[45] No changes were found in the amygdala in that case. Mating is known to induce long-lasting plastic changes in the activity of the Me neurons,[46] but the genetic changes underlying this plasticity have not been described. Certainly, these changes are probably also included in our results.

4.1 | Overexpressed genes

Several of the most upregulated genes in the Me of dams are related with maternal behaviors. In this group, we find

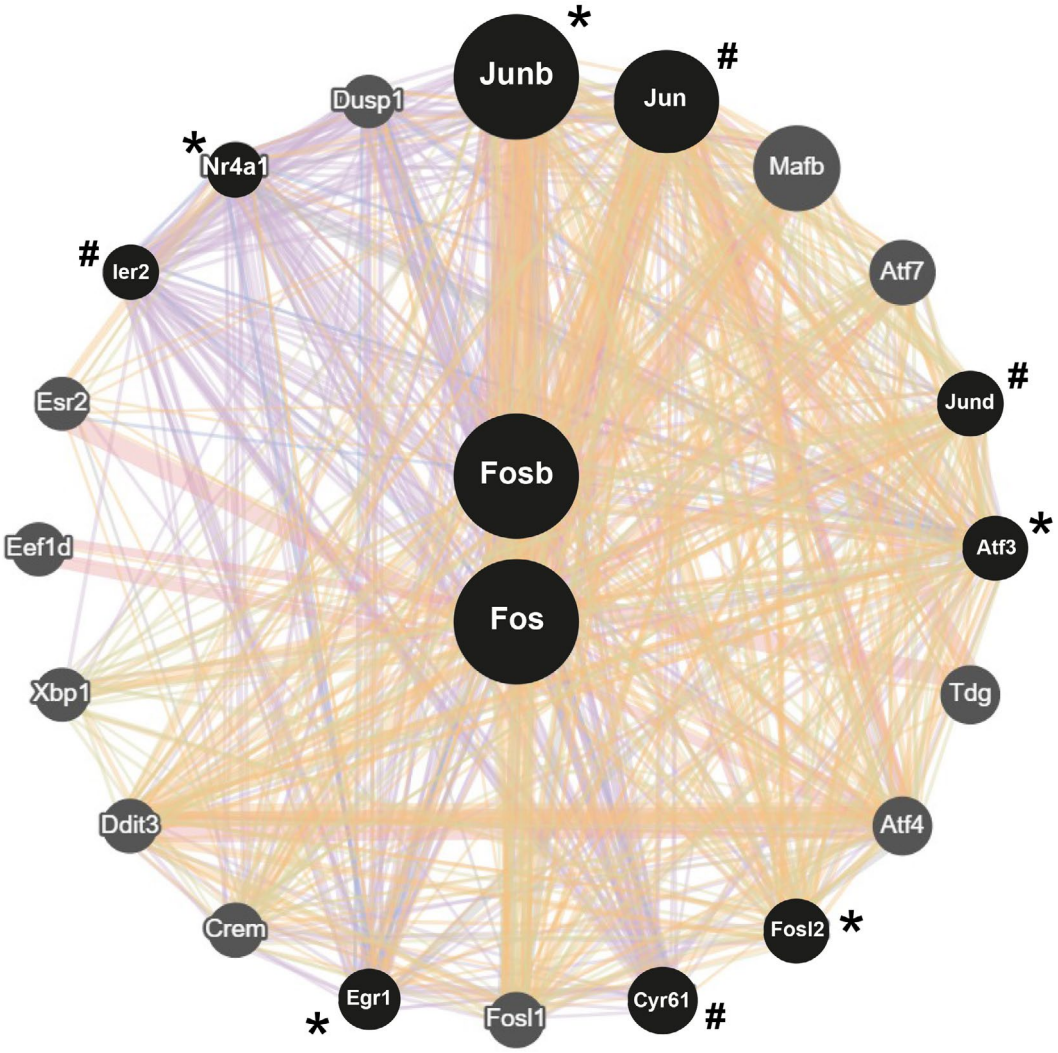


FIGURE 5 Summary of *Fos and Fosb* signaling interactions with other genes in *M musculus*, according to GeneMANIA database. Black circles depict the genes obtained in our RNA-seq study. The asterisk (*) shows the significant upregulated genes in pup-sensitized virgin females (section B of Supporting Information II) and the hash symbol (#) remark the genes with no significant differential expression obtained in our study but adjp <1 (Supporting Information IIC). For GeneMANIA network color categories and genes abbreviations see http://pages.genemania.org/help/. *Atf3*, Activating transcription factor 3; *Atf4*, activating transcription factor 4; *Atf7*, activating transcription factor 7; *Crem*, cAMP responsive element modulator; *Cyr61*, cysteine-rich protein 61; *Ddit3*, DNA damage-inducible transcript 3; *Dusp1*, dual specificity phosphatase 1; *Eef1d*, eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein); *Egr1*, early growth response 1; *Esr2*, estrogen receptor 2 (beta); *Fos*, FBJ osteosarcoma oncogene; *Fosb*, FBJ osteosarcoma oncogene B; *Fosl1*, fos-like antigen 1; *Fosl2*, fos-like antigen 2; *Ier2*, immediate early response 2; *Jun*, jun proto-oncogene; *Junb*, jun B proto-oncogene; *Jund*, jun D proto-oncogene; *Mafb*, v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian); *Nr4a1*, nuclear receptor subfamily 4, group A, member 1; *Tdg*, thymine DNA glycosylase; *Xbp1*, X-box binding protein 1

the maternal hormone *Prl*. In addition to its well-known expression as a hypophyseal hormone, *Prl* expression was observed previously in the hypothalamus of male and female rodents,[47-51] as well as in other brain structures as the cerebellum, caudate, brain stem, amygdala, thalamus, cortex, and hippocampus.[48] Notably, the sequence of the *Prl* mRNA expressed in the brain seems to be the same as the one expressed in the anterior pituitary gland, according to a study carried out in male rats.[52] In a different group of females, the expression of *Prl* in the Me of dams was compared, by means of qPCR, with that present in pup-sensitized and pup-naïve virgin females. The results showed that dams expressed higher levels of *Prl* in the Me than pup-naïve females, with pup-sensitized virgin females displaying intermediate levels between dams and naïve virgins. Thus, the results suggest that *Prl* expression in the Me may be involved not only on maternal behavior in dams but also in the sensitization process that induced pup care in virgin females.

Previous studies have observed an increase in *Prl* mRNA expression in the hypothalamus of pregnant and lactating rats

in comparison to virgin females,[53,54] consistent with our observation of a significant increase in *Prl* mRNA in the Me. However, the existence of neural *Prl* has been a controversial issue for more than 30 years[55] since the presence of *Prl* mRNA may also be due to circulating immune cells[56] or contamination from the pituitary stalk.[55] Concerning *Prl* expression in immune cells, its regulation seems to be different from that of hypophyseal *Prl*, and in particular seems to be independent of estrogens (at least in humans, see Reference 57), thus suggesting that the upregulation observed in lactating females is not due to *Prl* of immune origin. Regarding the possibility of contamination from the pituitary stalk, although we cannot discard it with absolute certainty, observation of histological sections of our dissections of the Me show that the tuberal hypothalamus was not affected (Figure 1).

In vitro studies suggest that the estrogen response of the *Prl* gene requires a functional "unit" involving several factor-binding sites, the estrogen receptor, and the tissue-specific transcription factor, *Pit-1*.[58] Expression of *Pit-1* mRNA was detected previously by qPCR in the Pa of estrous rats, but not of other cycling females or male rats.[59] Detection of *Pit-1* mRNA in the Pa during estrus coincides with previous suggestion that both *Pit-1* and the estrogen receptor are required for the effect of estrogens on *Prl* gene expression.[58,59] Our study is the first one describing the expression of *Pit-1* in Me (Table 2), which is upregulated in dams.

In addition, the transcription of the pituitary *Gh* gene is also dependent upon Pit-1, since GH synthesis fails to occur in its absence.[60,61] The presence of GH in the brain was previously studied by radioimmunoassay in male and female rats and macaques,[62] observing the presence of this protein in the amygdala. The expression of *Gh* in the Me was confirmed using qPCR. As in the case of *Prl*, the qPCR results showed a higher level of *Gh* expression in dams compared to pup-naïve virgins, with pup-sensitized females showing intermediate levels. This suggests that the sensitization process may induce *Gh* expression in Me, in a coordinated way with that of *Prl*. Further studies are needed to explore this possibility and also to clarify whether the expression of these two genes is increased in dams relative to pup-sensitized females.

Importantly, the expression of GH and PRL in the adult brain has been shown to be coordinated at mRNA and protein levels.[37] However, a wide interindividual variation was superimposed on coordinated PRL/GH expression.[37] These results are consistent with our findings of a positive correlation of the expression of *Gh* and *Prl* in the Me, observed both in the results of the RNA-Seq and in the qPCR experiments. As Daudé et al,[37] we also observed a large interindividual variation in the *Prl* and *Gh* expression levels, found mainly in the groups of dams and pup-sensitized virgin females. This variation suggests that additional factors may be involved in the regulation of PRL/GH expression (eg, the circadian rhythm, the estrous cycle[51] or the different level of pup stimulation), possibilities that require further research.

The *Gh*, *Prl*, and *Pit-1* genes are related with others that are also upregulated in dams as *Pitx1*, *Tbx19*, *Ghrhr*, *Fhsb*, and *Cga* (Figure 2C; Supporting Information IIA). *Pitx1* and *Tbx19* cooperate in order to transcribe *Pomc*, the two factors binding to contiguous sites within the same regulatory element in the corticotrope/melanotrope lineages in the rostral ventral hypothalamus and in the pituitary gland.[63,64] In addition, *Pitx1* with other transcriptional regulators are required for terminal differentiation of lactotroph cells and direct regulation of the *Prl*gene in the pituitary gland.[65,66] All these genes were not described previously in the Me of female mice. *Ghrhr*, *Fhsb*, and *Cga* are discussed below.

Binding of PRL to its receptor activates the STAT5 by phosphorylation (pSTAT5), which regulates transcription.[67] Me levels of pSTAT5 indicate an increase to PRL and GH response[68,69] and this PRL response was higher in pregnant and lactating females compared to virgin females.[69] This can be related to the increase of *Prl* and *Gh* mRNA observed in our study and its posterior translation to PRL and GH in Me.[62,70-74] Although the expression of the prolactin receptor and STAT5 does not increase, the gene set analysis, taking as a group those genes implicated in the JAK/STAT cascade, shows that this functional group is upregulated. However, it should be noted that blocking peripheral prolactin production using bromocriptine results in the absence of all pSTAT5 immunoreactivity in the Me.[75] This suggests that the action of brain prolactin/growth hormone might be independent of the pSTAT5 signaling cascade, a possibility that requires further investigation. In the case of PRL, it would be possible if it binds preferentially to the short isoform of the PRL receptor, which lacks the intracellular domain activating the STAT5 cascade.[56] The lack of correlation observed in our results between the expression levels of *Prl*/*Gh* with those of *Stat5a*/*Stat5b* in the Me suggests that, at least at the level of gene expression, they are independently regulated.

Other important physiological regulation that takes place during lactation is the loss of sensitivity to PRL negative feedback in the tuberoinfundibular dopaminergic (TIDA) neurons, which is necessary to maintain hyperprolactinemia in the mother.[56] This inhibition occurs because suckling stimulus increases hypothalamic mRNA expression of CIS (also known as CISH), a member of the suppressor of cytokine signaling family of proteins, known to negatively regulate PRL signaling through JAK/STAT.[76,77] Indeed, previous studies have demonstrated that all TIDA neurons in the dorsomedial arcuate nucleus (Arc) express CIS protein, and that CIS levels in TIDA neurons are increased in lactating rats in the presence of suckling.[78] In our results we observed an overexpression in dams of *Prl* and *Cis*, probably suggesting that the PRL negative feedback can be inhibited in the Me with the same molecular mechanism of TIDA neurons.

Another adaptation in dams CNS are changes related with the neuroendocrine and behavioral stress responses. These adaptations include activation of brain OXT and PRL systems, which act to attenuate the hypothalamic-pituitary-adrenal (HPA) axis activity and emotional responsiveness in the peripartum period, coupled with a dampening of opioid and noradrenergic systems, which are the main excitatory inputs of the HPA axis.[56,79] Related with this, our result also show that Oxt expression is upregulated in dams respect to pup-sensitized virgin females (Table 2).

Furthermore, previous studies have suggested that OXT neurons express *Prlr* mRNA and are prolactin-sensitive as well, because almost all oxytocin neurons express pSTAT5 during lactation.[80] Indeed, phosphorylated STAT5 is expressed by almost all oxytocin neurons of late pregnant and lactating rats but is almost absent from OXT neurons in virgin rats.[81] PRL can also regulate the expression level and release of OXT in lactating females[80,82] and it is hypothesized that OXT stimulates PRL secretion by the pituitary gland triggering a positive feedback loop.[83] Although the regulatory mechanisms probably differ between systemic and central PRL-OXT systems, our results suggest that central PRL and OXT are also related.

Regarding other genes related with PRL signaling, it has been reported that PRL sensitizes TRPV1 channels in sensory neurons in female rats,[84] and these channels are related with glutamatergic neurotransmission. So neurons express TRPV1 channels and *Trpv1* mRNA is upregulated in this nucleus during lactation.[81] This is consistent with the result that we obtain in the RNA-seq study, where we observe an increase in *Trpv1* mRNA in dams respect to pup-sensitized virgin females (Table 2).

Previous studies have demonstrated that GAL[85] and *Gal* mRNA[43,86] are present in the Me of male rats. Immunohistochemical detection of GAL has also been described in the Me of female (and male) mice.[87] In addition, it has been demonstrated that *Gal* labeling is increased in the BST and Me of adult compared with prepubertal male rats.[86] Galanin neurons seem to be a central regulatory node of social interactions with pups in parental behavior, specifically *Gal*-expressing neurons of medial preoptic region (MPO/A) are critical for the control of mouse parental behavior and the suppression of pup-directed aggression[88] and also in pup care behaviors.[89] Our results suggest that GAL in the Me may also be involved in the regulation of maternal behavior, as it has been shown in the MPO/A.[89]

A few genes related with reproductive hormones were found to be overexpressed in dams, such as *Fshb*, *Cga*, which encodes the FSH alpha subunit, *Gnrhr* and *Gnrh1* (Table 2). The expression of *Ghrhr*, *Fhsb*, and *Cga* showed a close relation with *Prl*, *Gh*, and *Pou1f1* (Figure 4). *Fshb* expression has been described in the brain of teleost fishes, where the FSH subunits seem to be modulated by 11-ketosterone and testosterone in male pejerrey (*Odontesthes bonariensis*),[90] however, we did not find any previous report of the expression of *Fshb* in the mammalian CNS.[43] Regarding *Cga*, its mRNA and protein have been described in the anterior lobe of the pituitary gland in sheep, mice, rat, and guinea pig[91-93] but not in the Me of female mice. In the case of *Gnrhr*, previous studies described the presence of mRNA in several brain nuclei[94,95] and in particular in the Me,[96] in agreement with our results. Nevertheless, our results also suggested that the expression of these receptors is upregulated in lactating female mice and could be involved in the neural changes associated with motherhood. Regarding *Gnrh1*, previous studies showed that injections of retrograde tracers in the amygdala labeled gonadotropin-releasing hormone (GnRH) neurons in the rostral medial septum, the caudal roots of the nervous terminalis, diagonal band, nucleus triangularis septi, nucleus interstitialis striae terminalis, and in the ventrolateral hypothalamus.[97] GnRH-l neurons are found in the forebrain in the olfactory bulb, along the terminal nerve, in the medial septum, the amygdala, the hypothalamus, and the median eminence.[98,99] These cells positive for GnRH-I project directly to the amygdala and can have an effect on the GnRH receptors present in this region.[100] It should be noted, however, that the expression of these genes related with reproductive hormones shows also the same large intragroup variation reported for *Prl* and *Gh*, and thus this results should be interpreted with caution and confirmed in future investigations.

With regard to genes related to neurotransmission, the present results show that dams overexpressed genes implicated in cholinergic, catecholaminergic and, to a lesser extent, glutamatergic synapses. Moreover, a number of genes related to peptidergic transmission (in addition to those directly related to maternal behavior discussed above) also showed a significant higher expression. Of note, the genes related to neurotransmission (including the neuropeptides *Oxt* and *Gal*) did not show the high variability in their expression observed for *Prl* and *Gh*.

Concerning the catecholaminergic neurotransmission, our RNA-seq results revealed an upregulation of *Th*, the rate-limiting enzyme in dopamine synthesis, and *Adra2b* in dams respect to pup-sensitized virgin females. *Adra2b* codes for the presynaptic noradrenergic α2B receptor, and has been found to play a role in arousal-enhanced consolidation processes, influencing amygdala and hippocampus activation immediately following an emotional event.[101-103] In particular, the noradrenergic Me innervation in rats, probably originated by the locus coeruleus, has been shown to be involved in the stress response after immobilization.[104] Thus, the *Th* and *Adra2b* overexpression might take place in the noradrenergic axon terminals of dams to facilitate the aggressive responses toward male intruders.

An alternative explanation would be the presence in the Me of TH-expressing cells, as it has been reported in

Syrian hamsters (*Mesocricetus auratus*) and prairie voles (*Microtus ochrogaster*), but not in rats, mice, or Siberian hamsters.[105,106] Northcutt et al[106] observed that posterodorsal subnucleus of the medial amygdala (MePD) contains a few TH-immunoreactive cells in male and female hamsters and male meadow voles, but not rats (they did not study their presence in mice). They observed that virgin female prairie voles had far fewer TH-immunoreactive cells in posterior BST and MePD than in males. In addition, they observed that treating ovariectomized females with testosterone substantially increased TH-immunoreactive cells in posterior BST and MePD. Consequently, they suggested that the influence of circulating gonadal hormones in TH expression in these structures may be related to changes in social behaviors across the reproductive cycle.[106] As they did not check the variations of TH cells populations in posterior BST and MePD in mice, maybe the presence of these neurons increases during lactation.

Another possibility, which may be related to the hypothetical presence of TH-expressing cells just discussed, is that PRL stimulates expression of TH in the Me, as it happens in neuroendocrine dopamine neurons,[107] where it also modulates the phosphorylation of TH, resulting in increased dopamine synthesis.[108]

Regarding cholinergic neurotransmission, the superficial layer of the Me is positive for acetyl cholinesterase.[20] Our RNA-Seq studies have shown an increase of expression of several genes related with cholinergic neurotransmission: *Chat*, *Ngfr*, *Chrna2*, *Chrna6*, *Chrnb3*, and *Chrm2* (Table 2). This kind of neurotransmission seems to be involved in the maternal-offspring bonding in sheep, mediated in part by reorganization of the olfactory bulb.[109,110] Previous studies in ewes showed that the number of mitral cells in the olfactory bulb responding to lamb odors increases after parturition, together with increased cholinergic and noradrenergic neurotransmitter release.[109] An increase in cholinergic and noradrenergic neurotransmission during lactation might also modulate the hormonal and chemosensory information processing in the Me, in relation to pup and/or to male odors. Further research is needed to address the role of cholinergic neurotransmission in maternal behaviors.

Finally, among the overexpressed genes related with peptidergic neurotransmission, *Pomc* encodes a complex precursor that comprises several peptide hormones, including MSH, ACTH, and β-endorphin.[111] Neurons expressing *Pomc* were described in several nuclei including Me and BMA[112] in the amygdala, as well as the hypothalamic Arc and the nucleus tractus solitarius in the brainstem. To the best of our knowledge, no previous reports have related the expression of POMC in the Me to maternal behavior. In situ hybridization in sheep demonstrated that *Pomc* mRNA expression in the Arc decreased at parturition and increased during lactation compared to late pregnant and ovariectomized animals, and estradiol and progesterone treatments increased *Pomc* mRNA expression compared to ovariectomized controls.[113] Although it is possible that a similar regulation of *Pomc* expression exists in Me, it is worth noting that the results in the hypothalamus reported in sheep were not replicated in rodents.[114]

4.2 | Genes downregulated in dams

The genes that were most downregulated in dams with respect to pup-sensitized virgin females were the *Inhbc* and *Inhbe*, encoding the beta-inhibins C and E (Supporting Information IIB). Some proteins in the inhibin family (A and B) are known to participate in the control of the estrous cycle,[115,116] but the function of these specific genes in the brain is unknown, so further research is needed about the role that they could play in maternal behavior.

Other genes downregulated in dams are those characterized by their early expression, such as *Fos*, *Fosb*, *Egr1*, *Egr2*, *Egr3*, *Egr4*, and jun B proto-oncogene (*Junb*) (Figure 5). Previous studies in rats have reported an increase of *Fos* mRNA neurons in Me in response to stress[117] and conspecifics.[118] In addition, *Egr-1* it is expressed in many brain sites after the display of sexual or maternal behaviors, including in cells that do not express *Fos*.[119,120] Thus, the higher expression of these early expressed genes in pup-sensitized virgin females suggests that the expression of the genes encoding these transcription factors is inhibited by pregnancy and/or lactation.

4.3 | Genes of olfactory and vomeronasal receptors

A novel finding of this study is that the Me expresses several olfactory and vomeronasal receptors. Although the mRNA of the olfactory receptors is known to be transported to the axon terminal of the sensory neurons, and thus it can be detected in the glomeruli of the main olfactory bulbs,[121] to the best of our knowledge their expression in the amygdala has never been reported. However, a recent work has reported the expression of several olfactory and one vomeronasal receptor genes in the medial prefrontal cortex of male mice,[122] all of them different from those we found in the Me. In the case of olfactory receptors, a number of previous studies have also reported the expression of some of them in the cerebral cortex, and even in several non-neural tissues,[123] suggesting a more general function, maybe related to chemoreception of inner metabolites. It is tempting to suggest that the expression of vomeronasal receptors in Me may have a specific, unknown role in the processing of chemosensory information, but this possibility requires further investigation.

In conclusion, the results of the present RNA-Seq study are initial findings about gene expression changes in the Me of lactating female mice, which warrant further investigation to confirm whether they are induced by pregnancy and lactation. As discussed above, long-lasting gene expression changes induced by the mating experience may also play a role in inducing the reported changes. Among the differentially expressed genes, there are several cases of genes whose expression in the amygdala had not been previously reported, such as the *Fshb* and *Cga* (overexpressed in dams) or the inhibins *Inhbc* and *Inhbe* (downregulated in dams), as well as some other genes whose expression in Me had not been related to maternal behavior (such as *Prl* or *Gh*). For *Prl* and *Gh*, the qPCR results show that dams expressed higher levels than pup-naïve virgins, with pup-sensitized virgins showing intermediate levels. In summary, these results open many questions and indicate new avenues of research on the molecular changes underlying maternal behavior.

ACKNOWLEDGMENTS

The authors are grateful to Dr Adoración Hernández-Martínez for technical assistance, Dr Cecilia Pardo for her help with microdissections, Dr Sacramento R. Ferrón and Dr Jose M. Morante for their help with RNA extraction and qPCR analysis, Rafael Aparisi for his help with the histological processing, and the personnel of the Genomic section of the SCSIE of the University of València for the RNA-seq procedure.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

E. Lanuza, F. Martínez-García, and C. Agustín-Pavón designed the study. M. Abellán-Álvaro and M. Barneo-Muñoz performed the experiments. G. Ayala, M. Abellán-Álvaro, and E. Lanuza analyzed and interpreted the data. M. Abellán-Álvaro, G. Ayala, C. Agustín-Pavón, and E. Lanuza prepared the manuscript draft. E. Lanuza, C. Agustín-Pavón, and F. Martínez-García supervised the study, critically revised the manuscript, and obtained funding.

ORCID

Enrique Lanuza https://orcid.org/0000-0001-9498-7966

REFERENCES

1. Numan M, Insel TR. *The Neurobiology of Parental Behaviour*. New York: Springer; 2003:418 p. https://doi.org/10.1007/b97533
2. Lonstein JS, Gammie SC. Sensory, hormonal, and neural control of maternal aggression in laboratory rodents. *Neurosci Biobehav Rev*. 2002;*26*(8):869-888. https://doi.org/10.1016/S0149-7634(02)00087-8
3. Martín-Sánchez A, McLean L, Beynon RJ, et al. From sexual attraction to maternal aggression: when pheromones change their behavioural significance. *Horm Behav*. 2015;*68*:65-76. https://doi.org/10.1016/j.yhbeh.2014.08.007
4. Martín-Sánchez A, Valera-Marín G, Hernández-Martínez A, Lanuza E, Martínez-García F, Agustín-Pavón C. Wired for motherhood: induction of maternal care but not maternal aggression in virgin female CD1 mice. *Front Behav Neurosci*. 2015;*9*:197. https://doi.org/10.3389/fnbeh.2015.00197
5. Wyatt TD. *Pheromones and Animal Behaviour: Communication by Smell and Taste*. 2nd ed. Cambridge: Cambridge University Press; 2003:405 p. https://doi.org/10.1016/0018-506X(73)90032-9
6. Roberts SA, Simpson DM, Armstrong SD, et al. Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol*. 2010;*8*:75. https://doi.org/10.1186/1741-7007-8-75
7. Wood RI, Coolen LM. Integration of chemosensory and hormonal cues is essential for sexual behaviour in the male Syrian hamster: role of the medial amygdaloid nucleus. *Neuroscience*. 1997;*78*:1027-1035. https://doi.org/10.1016/s0306-4522(96)00629-x
8. Petrulis A. Chemosignals and hormones in the neural control of mammalian sexual behavior. *Front Neuroendocrinol*. 2013;*34*:255-267. https://doi.org/10.1016/j.yfrne.2013.07.007
9. Newman S. The medial extended amygdala in male reproductive behavior. *Ann N Y Acad Sci*. 1999;*877*:242-257. https://doi.org/10.1111/j.1749-6632.1999.tb09271.x
10. Numan M, Insel TR. Hormonal and nonhormonal basis of maternal behavior. In: M Numan, TR Insel, eds. *The Neurobiology of Parental Behavior*. New York, NY: Springer New York; 2003:8-41. https://doi.org/10.1007/0-387-21799-1_2
11. Scalia F, Winans SS. The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. *J Comp Neurol*. 1975;*161*:31-55. https://doi.org/10.1002/cne.901610105
12. Kang N, Baum MJ, Cherry JA. A direct main olfactory bulb projection to the "vomeronasal" amygdala in female mice selectively responds to volatile pheromones from males. *Eur J Neurosci*. 2009;*29*:624-634. https://doi.org/10.1111/j.1460-9568.2009.06638.x
13. Cádiz-Moretti B, Martínez-García F, Lanuza E. Neural substrate to associate odorants and pheromones: convergence of projections from the main and accessory olfactory bulbs in mice. In: East ML, Dehnhard M, eds. *Chemical Signals in Vertebrates 12*. New York, NY: Springer New York; 2013:3-16. https://doi.org/10.1007/978-1-4614-5927-9_1
14. Simerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. *J Comp Neurol*. 1990;*294*:76-95. https://doi.org/10.1002/cne.902940107
15. Salais-López H, Agustín-Pavón C, Lanuza E, Martínez-García F. The maternal hormone in the male brain: sexually dimorphic distribution of prolactin signalling in the mouse brain. *PLoS ONE*. 2018;*13*(12):e0208960. https://doi.org/10.1371/journal.pone.0208960
16. Koolhaas JM, Van Den Brink THC, Roozendaal B, Boorsma F. Medial amygdala and aggressive behavior: interaction between testosterone and vasopressin. *Aggress Behav*. 1990;*16*:223-229.

https://doi.org/10.1002/1098-2337(1990)16:3/4<223::AID-AB2480160308>3.0.CO;2-%23

17. Sano K, Tsuda MC, Musatov S, Sakamoto T, Ogawa S. Differential effects of site-specific knockdown of estrogen receptor α in the medial amygdala, medial preoptic area, and ventromedial nucleus of the hypothalamus on sexual and aggressive behavior of male mice. *Eur J Neurosci*. 2013;*37*:1308-1319. https://doi.org/10.1111/ejn.12131
18. Unger EK, Burke KJJ, Yang CF, Bender KJ, Fuller PM, Shah NM. Medial amygdalar aromatase neurons regulate aggression in both sexes. *Cell Rep*. 2015;*10*:453-462. https://doi.org/10.1016/j.celrep.2014.12.040
19. Wang Y, He Z, Zhao C, Li L. Medial amygdala lesions modify aggressive behavior and immediate early gene expression in oxytocin and vasopressin neurons during intermale exposure. *Behav Brain Res*. 2013;*245*:42-49. https://doi.org/10.1016/j.bbr.2013.02.002
20. Paxinos G, Franklin KBJ. *The Mouse Brain in Stereotaxic Coordinates*. 2nd ed. Amsterdam: Academic Press; 2004. https://doi.org/10.1016/S0306-4530(03)00088-X
21. Schroeder A, Mueller O, Stocker S, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol*. 2006;*7*:3. https://doi.org/10.1186/1471-2199-7-3
22. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*. 2011;*17*:10-12. https://doi.org/10.14806/ej.17.1.200
23. Flicek P, Amode MR, Barrell D, et al. Ensembl 2014. *Nucleic Acids Res*. 2014;*42*(D1):D749-D755. https://doi.org/10.1093/nar/gkt1196
24. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol*. 2013;*14*(4):R36. https://doi.org/10.1186/gb-2013-14-4-r36
25. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics*. 2009;*25*(9):1105-1111. https://doi.org/10.1093/bioinformatics/btp120
26. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*. 2012;*9*(4):357-359. https://doi.org/10.1038/nmeth.1923
27. Morgan M, Pagès H, Obenchain V & Hayden N. Rsamtools: Binary alignment (BAM), FASTA, variant call (BCF), and tabix file import. R package version 2.8.0. https://bioconductor.org/packages/Rsamtools
28. Lawrence M, Huber W, Pagès H, et al. Software for computing and annotating genomic ranges. *PLoS Comput Biol*. 2013;*9*(8):e1003118. https://doi.org/10.1371/journal.pcbi.1003118
29. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;*26*(1):139-140. https://doi.org/10.1093/bioinformatics/btp616
30. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc*. 1995;*57*(1):289-300. https://doi.org/10.2307/2346101
31. Goeman JJ, Bühlmann P. Analyzing gene expression data in terms of gene sets: methodological issues. *Bioinformatics*. 2007;*23*(8):980-987. https://doi.org/10.1093/bioinformatics/btm051
32. Warde-Farley D, Donaldson SL, Comes O, et al. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res*. 2010;*38*(suppl_2):214-220. https://doi.org/10.1093/nar/gkq537
33. Murphy M, Brown G & Wallin C Gene help: integrated access to genes of genomes in the reference sequence collection. In: *Gene Help [Internet]*. Bethesda, MD: National Center for Biotechnology Information (US); 2005. Available from: https://www.ncbi.nlm.nih.gov/books/NBK3841/
34. Medina L, Abellán A, Vicario A, Castro-Robles B, Desfilis E. The Amygdala. In: Kaas JH, ed. *Evolution of Nervous Systems*. Vol *1*, 2nd ed. Amsterdam: Academic Press; 2017:427-478. https://doi.org/10.1016/B978-0-12-804042-3.00019-1
35. Pavlidis P, Noble WS. Analysis of strain and regional variation in gene expression in mouse brain. *Genome Biol*. 2001;*2*(10):RESEARCH0042. https://doi.org/10.1186/gb-2001-2-10-research0042
36. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res*. 2007;*35*:W71-W74. https://doi.org/10.1093/nar/gkm306
37. Daude N, Lee I, Kim TK, et al. A common phenotype polymorphism in mammalian brains defined by concomitant production of Prolactin and growth hormone. *PLoS ONE*. 2016;*11*(2):1-27. https://doi.org/10.1371/journal.pone.0149410
38. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;*55*(4):611-622. https://doi.org/10.1373/clinchem.2008.112797
39. Goni R, García P, Foissac S. The qPCR data statistical analysis. *Integromics White Pap*. 2009;*1*:1-9. http://www.gene-quantification.eu/integromics-qpcr-statistics-white-paper.pdf
40. Jozefczuk J, Adjaye J. Quantitative real-time PCR-based analysis of gene expression. *Methods Enzymol*. 2011;*500*:99-109. https://doi.org/10.1016/B978-0-12-385118-5.00006-2
41. Robinson MD, Smyth GK. Moderated statistical tests for assessing differences in tag abundance. *Bioinformatics*. 2007;*23*:2881-2887. https://doi.org/10.1093/bioinformatics/btm453
42. Robinson MD, Smyth GK. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics*. 2008;*9*:321-332. https://doi.org/10.1093/biostatistics/kxm030
43. Lein ES, Hawrylycz MJ, Ao N, et al. Genome-wide atlas of gene expression in the adult mouse brain. *Nature*. 2007;*445*:168-176. https://doi.org/10.1038/nature05453
44. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. *Nat Genet*. 2000;*25*:25-29. https://doi.org/10.1038/75556
45. Arbeitman MN. Maternal experience leads to lasting gene expression changes in some regions of the mouse brain. *G3 Genes, Genomes, Genet*. 2019;*9*(8):2623-2628. https://doi.org/10.1534/g3.119.400249
46. Li Y, Mathis A, Grewe BF, et al. Neuronal representation of social information in the medial amygdala of awake behaving mice. *Cell*. 2017;*171*:1176-1190. https://doi.org/10.1016/j.cell.2017.10.015
47. Clapp C, Torner L, Gutierrez-Ospina G, et al. The prolactin gene is expressed in the hypothalamic-neurohypophyseal system and the protein is processed into a 14-kDa fragment with activity like 16-kDa prolactin. *Proc Natl Acad Sci*. 1994;*91*:10384-10388. https://doi.org/10.1073/pnas.91.22.10384

48. Emanuele NV, Jurgens JK, Halloran MM, Tentler JJ, Lawrence AM, Kelley MR. The rat prolactin gene is expressed in brain tissue: detection of normal and alternatively spliced prolactin messenger RNA. *Mol Endocrinol*. 1992;*6*:35-42. https://doi.org/10.1210/mend.6.1.1738369
49. Schachter BS, Durgerian S, Harlan RE, Pfaff DW, Shivers BD. Prolactin mRNA exists in rat hypothalamus. *Endocrinology*. 1984;*114*:1947-1949. https://doi.org/10.1210/endo-114-5-1947
50. Ray S, Tzeng R-Y, DiCarlo LM, et al. An examination of dynamic gene expression changes in the mouse brain during pregnancy and the postpartum period. *G3 Genes, Genomes, Genet*. 2016;*6*:221-233. https://doi.org/10.1534/g3.115.020982
51. DiCarlo LM, Vied C, Nowakowski RS. The stability of the transcriptome during the estrous cycle in four regions of the mouse brain. *J Comp Neurol*. 2017;*525*:3360-3387. https://doi.org/10.1002/cne.24282
52. Wilson DM 3rd, Emanuele NV, Jurgens JK, Kelley MR. Prolactin message in brain and pituitary of adult male rats is identical: PCR cloning and sequencing of hypothalamic prolactin cDNA from intact and hypophysectomized adult male rats. *Endocrinology*. 1992;*131*:2488-2490. https://doi.org/10.1210/endo.131.5.1339346
53. Torner L, Neumann ID. The brain prolactin system: involvement in stress response adaptations in lactation. *Stress*. 2002;*5*:249-257. https://doi.org/10.1080/1025389021000048638
54. Torner L, Toschi N, Nava G, Clapp C, Neumann ID. Increased hypothalamic expression of prolactin in lactation: involvement in behavioural and neuroendocrine stress responses. *Eur J Neurosci*. 2002;*15*:1381-1389. https://doi.org/10.1046/j.1460-9568.2002.01965.x
55. Bridges RS, Grattan DR. 30 years after: CNS actions of prolactin: sources, mechanisms and physiological significance. *J Neuroendocrinol*. 2019;*31*:1-12. https://doi.org/10.1111/jne.12669
56. Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function, and regulation of secretion. *Physiol Rev*. 2000;*80*:1523-1631. https://doi.org/10.1152/physrev.2000.80.4.1523
57. Montgomery DW. Prolactin production by immune cells. *Lupus*. 2001;*10*:665-675. https://doi.org/10.1191/096120301717164895
58. Nowakowski BE, Maurer RA. Multiple Pit-1-binding sites facilitate estrogen responsiveness of the prolactin gene. *Mol Endocrinol*. 1994;*8*:1742-1749. https://doi.org/10.1210/mend.8.12.7708061
59. Torner L, Nava G, Duenas Z, et al. Changes in the expression of neurohypophyseal prolactins during the estrous cycle and after estrogen treatment. *J Endocrinol*. 1999;*161*:423-432. https://doi.org/10.1677/joe.0.1610423
60. Pfaffle RW, DiMattia GE, Parks JS, et al. Mutation of the POU-specific domain of Pit-1 and hypopituitarism without pituitary hypoplasia. *Science*. 1992;*257*:1118-1121. https://doi.org/10.1126/science.257.5073.1118
61. Voss JW, Rosenfeld MG. Anterior pituitary development: short tales from dwarf mice. *Cell*. 1992;*70*:527-530. https://doi.org/10.1016/0092-8674(92)90422-9
62. Hojvat S, Baker G, Kirsteins L, Lawrence AM. Growth hormone (GH) immunoreactivity in the rodent and primate CNS: distribution, characterization and presence posthypophysectomy. *Brain Res*. 1982;*239*:543-557. https://doi.org/10.1016/0006-8993(82)90529-7
63. Bancalari RE, Gregory LC, Mccabe MJ, Dattani MT. Pituitary gland development: an update. *Endocr Dev*. 2012;*23*:1-15. https://doi.org/10.1159/000341733
64. Liu J, Lin C, Gleiberman A, et al. Tbx19, a tissue-selective regulator of POMC gene expression. *Proc Natl Acad Sci U S A*. 2001;*98*:8674-8679. https://doi.org/10.1073/pnas.141234898
65. Quentien MH, Manfroid I, Moncet D, et al. Pitx factors are involved in basal and hormone-regulated activity of the human prolactin promoter. *J Biol Chem*. 2002;*277*:44408-44416. https://doi.org/10.1074/jbc.M207824200
66. Tremblay JJ, Lanctot C, Drouin J. The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. *Mol Endocrinol*. 1998;*12*:428-441. https://doi.org/10.1210/mend.12.3.0073
67. Yip SH, Eguchi R, Grattan DR, Bunn SJ. Prolactin signalling in the mouse hypothalamus is primarily mediated by signal transducer and activator of transcription factor 5b but not 5a. *J Neuroendocrinol*. 2012;*24*:1484-1491. https://doi.org/10.1111/j.1365-2826.2012.02357.x
68. Furigo IC, Metzger M, Teixeira PDS, Soares CRJ, Donato J. Distribution of growth hormone-responsive cells in the mouse brain. *Brain Struct Funct*. 2017;*222*:341-363. https://doi.org/10.1007/s00429-016-1221-1
69. Saláis-López H, Lanuza E, Agustín-Pavón C, Martínez-García F. Tuning the brain for motherhood: prolactin-like central signalling in virgin, pregnant, and lactating female mice. *Brain Struct Funct*. 2017;*222*:895-921. https://doi.org/10.1007/s00429-016-1254-5
70. Harlan RE, Shivers BD, Fox SR, Kaplove KA, Schachter BS, Pfaff DW. Distribution and partial characterization of immunoreactive prolactin in the rat brain. *Neuroendocrinology*. 1989;*49*:7-22.
71. Paut-Pagano L, Roky R, Valatx JL, Kitahama K, Jouvet M. Anatomical distribution of prolactin-like immunoreactivity in the rat brain. *Neuroendocrinology*. 1993;*58*:682-695. https://doi.org/10.1159/000126609
72. Siaud P, Manzoni O, Balmefrezol M, Barbanel G, Assenmacher I, Alonso G. The organization of prolactin-like-immunoreactive neurons in the rat central nervous system - light- and electron-microscopic immunocytochemical studies. *Cell Tissue Res*. 1989;*255*:107-115. https://doi.org/10.1007/BF00229071
73. Toubeau G, Desclin J, Parmentier M, Pasteels JL. Compared localizations of prolactin-like and adrenocorticotropin immunoreactivities within the brain of the rat. *Neuroendocrinology*. 1979;*29*:374-384. https://doi.org/10.1159/000122948
74. Toubeau G, Desclin J, Parmentier M, Pasteels JL. Cellular localization of a prolactin-like antigen in the rat brain. *J Endocrinol*. 1979;*83*:261-266.
75. Brown RSE, Herbison AE, Grattan DR. Differential changes in responses of hypothalamic and brainstem neuronal populations to prolactin during lactation in the mouse. *Biol Reprod*. 2011;*84*:826-836. https://doi.org/10.1095/biolreprod.110.089185
76. Krebs DL, Hilton DJ. SOCS: physiological suppressors of cytokine signaling. *J Cell Sci*. 2000;*113*:2813-2819.
77. Pezet A, Favre H, Kelly PA, Edery M. Inhibition and restoration of prolactin signal transduction by suppressors of cytokine signaling. *J Biol Chem*. 1999;*274*:24497-24502. https://doi.org/10.1074/jbc.274.35.24497

78. Anderson ST, Barclay JL, Fanning KJ, Kusters DHL, Waters MJ, Curlewis JD. Mechanisms underlying the diminished sensitivity to prolactin negative feedback during lactation: reduced STAT5 signaling and up-regulation of cytokine-inducible SH2 domain-containing protein (CIS) expression in tuberoinfundibular dopaminergic neurons. *Endocrinology*. 2006;*147*(3):1195-1202. https://doi.org/10.1210/en.2005-0905
79. Slattery DA, Neumann ID. No stress please! Mechanisms of stress hyporesponsiveness of the maternal brain. *J Physiol*. 2008;*586*:377-385. https://doi.org/10.1113/jphysiol.2007.145896
80. Augustine RA, Seymour AJ, Campbell RE, Grattan DR, Brown CH. Integrative neurohumoural regulation of oxytocin neurone activity in pregnancy and lactation. *J Neuroendocrinol*. 2018;*30*:1-15. https://doi.org/10.1111/jne.12569
81. Augustine RA, Bouwer GT, Seymour AJ, Grattan DR, Brown CH. Reproductive regulation of gene expression in the hypothalamic supraoptic and paraventricular nuclei. *J Neuroendocrinol*. 2016;*28*:1-12. https://doi.org/10.1111/jne.12350
82. Ghosh R, Sladek CD. Prolactin modulates oxytocin mRNA during lactation by its action on the hypothalamo-neurohypophyseal axis. *Brain Res*. 1995;*672*:24-28. https://doi.org/10.1016/0006-8993(94)01340-N
83. Kennett JE, Mckee DT. Oxytocin: an emerging regulator of prolactin secretion in the female rat. *J Neuroendocrinol*. 2012;*24*:403-412. https://doi.org/10.1111/j.1365-2826.2011.02263.x
84. Patil MJ, Ruparel SB, Henry MA, Akopian AN. Prolactin regulates TRPV1, TRPA1, and TRPM8 in sensory neurons in a sex-dependent manner: contribution of prolactin receptor to inflammatory pain. *Am J Physiol Metab*. 2013;*305*:E1154-E1164. https://doi.org/10.1152/ajpendo.00187.2013
85. Melander T, Hökfelt T, Rökaeus A. Distribution of galanin-like immunoreactivity in the rat central nervous system. *J Comp Neurol*. 1986;*248*:475-517. https://doi.org/10.1002/cne.902480404
86. Planas B, Kolb PE, Raskind MA, Miller MA. Activation of galanin pathways across puberty in the male rat: galanin gene expression in the bed nucleus of the stria terminalis and medial amygdala. *Neuroscience*. 1994;*63*:851-858. https://doi.org/10.1016/0306-4522(94)90529-0
87. Pérez SE, Wynick D, Steiner RA, Mufson EJ. Distribution of galaninergic immunoreactivity in the brain of the mouse. *J Comp Neurol*. 2001;*434*:158-185. https://doi.org/10.1002/cne.1171
88. Wu Z, Autry AE, Bergan JF, Watabe-Uchida M, Dulac CG. Galanin neurons in the medial preoptic area govern parental behaviour. *Nature*. 2014;*509*:325-330. https://doi.org/10.1038/nature13307
89. Tsuneoka Y, Maruyama T, Yoshida S, et al. Functional, anatomical, and neurochemical differentiation of medial preoptic area subregions in relation to maternal behavior in the mouse. *J Comp Neurol*. 2013;*521*:1633-1663. https://doi.org/10.1002/cne.23251
90. Miranda LA, Strüssmann CA, Guilgur LG, Strobl-Mazzulla PH, Somoza GM. Cloning of FSH-β, LH-β and glycoprotein hormone α subunits in pejerrey *Odontesthes bonariensis* (Valenciennes): expression profile and relationship with GnRH expression and plasma sex steroid levels in male fish. *J Fish Biol*. 2007;*71*:1571-1589. https://doi.org/10.1111/j.1095-8649.2007.01621.x
91. Böckers TM, Bockmann J, Fauteck J-D, Kreutz MR, Bock R, Wittkowski W. Pars tuberalis-specific cells in the ovine pituitary do express the common α-chain of glycoprotein hormones: an in situ hybridization and immunocytochemical study. *Eur J Endocrinol*. 1994;*131*:540-546. https://doi.org/10.1530/eje.0.1310540
92. Kendall SK, Gordon DF, Birkmeier TS, et al. Enhancer-mediated high level expression of mouse pituitary glycoprotein hormone α-subunit transgene in thyrotropes, gonadotropes, and developing pituitary gland. *Mol Endocrinol*. 1994;*8*:1420-1433. https://doi.org/10.1210/me.8.10.1420
93. Stoeckel ME, Hindelang C, Klein MJ, Poissonnier M, Félix JM. Expression of the α-subunit of glycoprotein hormones in the pars tuberalis-specific glandular cells in rat, mouse and guinea-pig. *Cell Tissue Res*. 1994;*278*:617-624. https://doi.org/10.1007/BF00331382
94. Albertson AJ, Navratil A, Mignot M, Dufourny L, Cherrington B, Skinner DC. Immunoreactive GnRH type I receptors in the mouse and sheep brain. *J Chem Neuroanat*. 2008;*35*:326-333. https://doi.org/10.1016/j.jchemneu.2008.03.004
95. Jennes L, Dalati B, Michael CP. Distribution of gonadrotropin releasing hormone agonist binding sites in the rat central nervous system. *Brain Res*. 1988;*452*:156-164. https://doi.org/10.1016/0006-8993(88)90020-0
96. Wen S, Götze IN, Mai O, Schauer C, Leinders-Zufall T, Boehm U. Genetic identification of GnRH receptor neurons: a new model for studying neural circuits underlying reproductive physiology in the mouse brain. *Endocrinology*. 2011;*152*:1515-1526. https://doi.org/10.1210/en.2010-1208
97. Jennes L. Sites of origin of gonadotropin releasing hormone containing projections to the amygdala and the interpenduncular nucleus. *Brain Res*. 1987;*404*:339-344. https://doi.org/10.1016/0006-8993(87)91391-6
98. Gore AC. Neuroanatomy of the GnRH-1 system. In: Gore AC, ed. *GnRH: The Master Molecule of Reproduction*. Boston: Springer; 2002:9-27.
99. Muske LE. Evolution of gonadotropin-releasing hormone (GnRH) neuronal systems. *Brain Behav Evol*. 1993;*42*:215-230. https://doi.org/10.1159/000114156
100. Sanchez MA, Dominguez R. Differential effects of unilateral lesions in the medial amygdala on spontaneous and induced ovulation. *Brain Res Bull*. 1995;*38*:313-317. https://doi.org/10.1016/0361-9230(95)00094-U
101. Anderson AK, Wais PE, Gabrieli JD. Emotion enhances remembrance of neutral events past. *Proc Natl Acad Sci*. 2006;*103*:1599-1604. https://doi.org/10.1073/pnas.0506308103
102. Mineur YS, Cahuzac EL, Mose TN, et al. Interaction between noradrenergic and cholinergic signaling in amygdala regulates anxiety- and depression-related behaviors in mice. *Neuropsychopharmacology*. 2018;*43*:2118-2125. https://doi.org/10.1038/s41386-018-0024-x
103. Todd RM, Palombo DJ, Levine B, Anderson AK. Genetic differences in emotionally enhanced memory. *Neuropsychologia*. 2011;*49*:734-744. https://doi.org/10.1016/j.neuropsychologia.2010.11.010
104. Ma S, Morilak DA. Norepinephrine release in medial amygdala facilitates activation of the hypothalamic-pituitary-adrenal axis in response to acute immobilisation stress. *J Neuroendocrinol*. 2005;*17*:22-28. https://doi.org/10.1111/j.1365-2826.2005.01279.x
105. Asmus SE, Kincaid AE, Newman SW. A species-specific population of tyrosine hydroxylase-immunoreactive neurons in the medial amygdaloid nucleus of the Syrian

hamster. *Brain Res*. 1992;*575*:199-207. https://doi.org/10.1016/0006-8993(92)90080-S

106. Northcutt KV, Wang Z, Lonstein JS. Sex and species differences in tyrosine hydroxylase-synthesizing cells of the rodent olfactory extended amygdala. *J Comp Neurol*. 2007;*500*:103-115. https://doi.org/10.1002/cne.21148
107. Arbogast LA, Voogt JL. Hyperprolactinemia increases and hypoprolactinemia decreases tyrosine hydroxylase messenger ribonucleic acid levels in the arcuate nuclei, but not the substantia nigra or zona incerta. *Endocrinology*. 1991;*128*:997-1005. https://doi.org/10.1210/endo-128-2-997
108. Ma FY, Grattan DR, Goffin V, Bunn SJ. Prolactin-regulated tyrosine hydroxylase activity and messenger ribonucleic acid expression in mediobasal hypothalamic cultures: the differential role of specific protein kinases. *Endocrinology*. 2005;*146*:93-102. https://doi.org/10.1210/en.2004-0800
109. Kendrick KM, Levy F, Keverne EB. Changes in the sensory processing of olfactory signals induced by birth in sheep. *Science*. 1992;*256*:833-836. https://doi.org/10.1126/science.256.5058.833
110. Lim MM, Young LJ. Neuropeptidergic regulation of affiliative behavior and social bonding in animals. *Horm Behav*. 2006;*50*:506-517. https://doi.org/10.1016/j.yhbeh.2006.06.028
111. Navarro S, Soletto L, Puchol S, Rotllant J, Soengas JL, Cerdá-Reverter JM. 60 YEARS OF POMC: POMC: an evolutionary perspective. *J Mol Endocrinol*. 2015;*56*:T113-T118. https://doi.org/10.1530/jme-15-0288
112. Niikura K, Zhou Y, Ho A, Kreek MJ. Proopiomelanocortin (POMC) expression and conditioned place aversion during protracted withdrawal from chronic intermittent escalating-dose heroin in POMC-EGFP promoter transgenic mice. *Neuroscience*. 2013;*236*:220-232. https://doi.org/10.1016/j.neuroscience.2012.12.071
113. Broad KD, Kendrick KM, Sirinathsinghji DJS, Keverne EB. Changes in pro-opiomelanocortin and pre-proenkephalin mRNA levels in the ovine brain during pregnancy, parturition and lactation and in response to oestrogen and progesterone. *J Neuroendocrinol*. 1993;*5*:711-719. https://doi.org/10.1111/j.1365-2826.1993.tb00544.x
114. Nahi F, Arbogast LA. Prolactin modulates hypothalamic pre-proenkephalin, but not proopiomelanocortin, gene expression during lactation. *Endocrine*. 2003;*20*:115-122. https://doi.org/10.1385/ENDO:20:1-2:115
115. Meunier H, Cajander SB, Roberts VJ, et al. Rapid changes in the expression of inhibin α-, βA-, and βB-subunits in ovarian cell types during the rat estrous cycle. *Mol Endocrinol*. 1988;*2*:1352-1363. https://doi.org/10.1210/mend-2-12-1352
116. Woodruff TK, Besecke LM, Groome N, Draper LB, Schwartz NB, Weiss J. Inhibin A and inhibin B are inversely correlated to follicle-stimulating hormone, yet are discordant during the follicular phase of the rat estrous cycle, and inhibin A is expressed in a sexually dimorphic manner. *Endocrinology*. 1996;*137*:5463-5467. https://doi.org/10.1210/endo.137.12.8940372
117. Ons S, Rotllant D, Marín-Blasco IJ, Armario A. Immediate-early gene response to repeated immobilization: Fos protein and arc mRNA levels appear to be less sensitive than c-fos mRNA to adaptation. *Eur J Neurosci*. 2010;*31*:2043-2052. https://doi.org/10.1111/j.1460-9568.2010.07242.x
118. Arakawa H, Arakawa K, Deak T. Oxytocin and vasopressin in the medial amygdala differentially modulate approach and avoidance behavior toward illness-related social odor. *Neuroscience*. 2010;*171*:1141-1151. https://doi.org/10.1016/j.neuroscience.2010.10.013
119. Hasen NS, Gammie SC. Maternal aggression: new insights from Egr-1. *Brain Res*. 2006;*1108*:147-156. https://doi.org/10.1016/j.brainres.2006.06.007
120. Numan M, Numan MJ, Marzella SR, Palumbo A. Expression of c-fos, fos B, and egr-1 in the medial preoptic area and bed nucleus of the stria terminalis during maternal behavior in rats. *Brain Res*. 1998;*792*:348-352.
121. Barnea G, O'Donnell S, Mancia F, et al. Odorant receptors on axon termini in the brain. *Science*. 2004;*304*:1468. https://doi.org/10.1126/science.1096146
122. Pallé A, Zorzo C, Luskey VE, Mcgreevy KR, Fernández S, Trejo JL. Social dominance differentially alters gene expression in the medial prefrontal cortex without affecting adult hippocampal neurogenesis or stress and anxiety-like behavior. *FASEB J*. 2019;*33*:6995-7008. https://doi.org/10.1096/fj.201801600R
123. Oh SJ. System-wide expression and function of olfactory receptors in mammals. *Genomics Inf*. 2018;*16*:2-9. https://doi.org/10.5808/GI.2018.16.1.2

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Abellán-Álvaro M, Ayala G, Barneo-Muñoz M, Martínez-García F, Agustín-Pavón C, Lanuza E. Motherhood-induced gene expression in the mouse medial amygdala: Changes induced by pregnancy and lactation but not by pup stimuli. *FASEB J*. 2021;35:e21806. https://doi.org/10.1096/fj.202100163RR