An epigenomic shift in amygdala marks the transition from pup-aversive to maternal-like behaviors in alloparenting virgin female mice

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ABSTRACT

In many species, adults will care for young offspring that are not their own, a phenomenon called alloparenting. However, most nonparental adults experience an initial aversion to newborns, which must be overcome before a robust display of parental-like behaviors can begin. To capture neurogenomic events underlying this dramatic behavioral transition, we analyzed brain gene expression and chromatin profiles of virgin female mice co-housed with mothers during pregnancy and after birth. After an initial display of agonistic behaviors and a surge of defense-related gene expression, we observed a dramatic shift in the chromatin landscape specifically in amygdala, accompanied by a dampening of the defense-related genes. This shift coincided with the emergence of behaviors and gene expression classically associated with maternal care. The results reveal the outlines of a neurogenomic program associated with this dramatic aversive-to-affiliative behavioral switch, and suggest molecular networks that may be relevant to human postpartum mental health.

INTRODUCTION

Interactions between newborn animals and their parents are profoundly important, being critical to the well-being of the offspring and intensely consequential to the parents as well. In most mammals, parental care is typically relegated to the female that bears the offspring, with hormonal shifts that occur during pregnancy and the early postpartum period priming her for this experience. These dramatic hormonal shifts also alter a mother's morphology, physiology, and brain structure in ways that persist far beyond the initial parenting experience (Kinsley, Bales, Bardi, & Stolzenberg, 2015; Kinsley et al., 2008). In addition to these physical changes (Brunton, 2019), mothering also alters a female's behavior, both immediately and in the longer-term. In particular, the sight, sounds, and odors of newborns – which may otherwise be perceived as aversive by adults – become intensely rewarding and motivating to the mother (C. Dulac, O'Connell, L. A., Wu, Z., 2014; Kinsley & Bridges, 1990; Numan, 2007). As with other changes associated with parenting, this shift from aversion to intense affiliation and reward is coordinated by steroid hormones and a rapid surge in neuropeptide secretion around the time of birth (Brunton et al., 2019) (Brunton, 2019). Most significantly, a surge of oxytocin, stored during pregnancy within the paraventricular and supraoptic nuclei of the hypothalamus (Russell, Leng, & Douglas, 2003), is released to target neurons within a brain circuit central to fear, aversion, reward, and the evaluation of emotional salience (Althammer & Grinevich, 2017).

New mothers are not the only individuals that can experience this switch from pup-aversive to pup-affiliative behaviors. For example, although virgin female rats display a clearly aversive response to pup stimuli, this response can be overcome by the process of "sensitization", which involves repeated and extended interactions with pups (Numan, 2007). Furthermore, although adult virgin female mice will spontaneously display certain maternal

behaviors when given first access to young pups (Gandelman, 1973; Noirot, 1972), these behaviors are significantly increased in both range and intensity after sensitization (Stolzenberg & Rissman, 2011). Like mothering itself, this experience of caring for young that are not one's own, or alloparenting, impacts future behavior. For example, juvenile female rats that have had the experience of "babysitting" younger siblings are highly motivated to display maternal behavior in future encounters with pups (Harding & Lonstein, 2016), and sensitized adult virgin female mice demonstrate superior parenting skills when they have their offspring of their own (Calemandrei, 1994; Fleming & Rosenblatt, 1974; Stolzenberg & Rissman, 2011). Indeed, many of the mechanisms that reshape a mother's brain and behavior also appear to operate in alloparenting females, where intriguingly, they are activated without the hormonal priming stimulated by pregnancy, parturition, and nursing.

Here, we investigated the functional genomics profile of the brains of virgin female mice as they transitioned to display of alloparenting behaviors toward pups. To identify genes modulated during this transition, we examined alterations in gene expression in multiple brain regions over a period of several days of continuous exposure to pups. Because histone modifications have been implicated as central to the behavior of both new mothers and sensitized virgins (Stolzenberg, Stevens, & Rissman, 2012), we also investigated chromatin accessibility profiles using H3K27Ac (histone H3 acetylated at lysine 27), a marker of open chromatin, in the same brain regions. The data reveal defense-related neurogenomic pathways that are silenced, and others that are activated, across the brains of alloparenting virgins as they transition to maternal-like behaviors and confirm an active role for chromatin remodeling in this aversive-to-affiliative behavioral switch, especially in the amygdala.

RESULTS

Antagonistic behavior, followed by active nurturance in virgins co-housed with mothers and pups.

In the traditional form of rodent pup sensitization experiments, the pups are placed into the cage of a virgin female rodent for short periods, then removed to be fed, repeatedly over the course of several consecutive days as the females' behaviors gradually transition to a more maternal-like state (Stolzenberg & Rissman, 2011). Although this paradigm is powerful and well-established, the timing of the most critical genomic events is difficult to discern in this context, and the repeated pattern of familiarization and removal add a potentially complicating dimension of stress (Aguggia, Suarez, & Rivarola, 2019; Gutman & Nemeroff, 2002; Haller, Harold, Sandi, & Neumann, 2014; Nishi, Horii-Hayashi, & Sasagawa, 2014). We therefore considered contexts that would allow virgins to be exposed to pups continuously over an extended term. Over years of mouse breeding, we had observed that females co-housed with nursing mouse dams and their litters will display maternal-like behaviors toward the pups, suggesting a way to achieve more continuous, longer-term interaction. To see whether we could observe a clear transition toward maternal-like behaviors in this context, we co-housed four pairs of virgins and pregnant dams and filmed activity in the cages from early pregnancy though the fourth postnatal day (**File S1**; Files S1 and S2 available at https://trackhub.pnri.org/stubbs/ucsc/public/allo.html).

For purposes of this study, we were primarily interested in the interactions between virgins and mothers, on the one hand, and virgins and pups one the other. As a primary indicator of these interactions, we scored the virgins for pup-grooming and mother-grooming behaviors during 5-minute intervals at the top of each hour; summing the scores in each cage over 6-hour periods coordinated with the light/dark cycle, providing a useful summary of the overall

behavioral patterns (**Fig. 1A; Table S1; File S1**). The two females were most often found together, interacting or resting in the shared nest, and grooming each other regularly while awake throughout the observation period. In contrast, although the virgins began to investigate the pups immediately after birth, they did not begin licking and grooming the pups consistently until around postnatal day 2 (P2), after which we increasingly observed the virgins engaged in pup licking/grooming behavior (**Fig. 1A; File S1, Table S1)**. To test the hypothesis that pup-focused grooming increased for the virgins while mother-focused grooming did not, we selected data binned for 6 hours around 12:00 (the beginning of the dark period during lights-out) (**Fig. 1B**). Pup-focused grooming bouts significantly differed across days P1-P4 ($F_{3,9} = 9.91$, $p = 0.003$), increasing over time, while mother-focused grooming bouts did not significantly differ across days P1-P4 ($F_{3,9} = 0.67$, $p = 0.59$).

We also noted some additional behaviors that did not occur frequently enough for statistical analysis, but are worth noting. For example, we observed that the virgins displayed some clear signs of early antagonism toward the pups, grabbing pups in their mouths and actively tossing them out of the shared nest; a variant of this behavior involved the virgin pushing pups away and under the bedding of the nest, where the mother would eventually find and retrieve them. One or both variants of this behavior was shown by all of the virgin females we observed sometime during the first two postnatal days **(Table S1, examples in File S2)**. However, consistent with the increased frequency of pup grooming after P3, the virgins were observed in classic hunched or prone nursing postures over the pups (**File S1, Table S1**); similar postures have been documented for female rats sensitized in the traditional scheme by other groups (Lonstein, 1999). Together, these observations suggested that we could indeed capture

the transition between pup aversion to active pup affiliation between postnatal days 1 and 3 in this continuous-exposure paradigm.

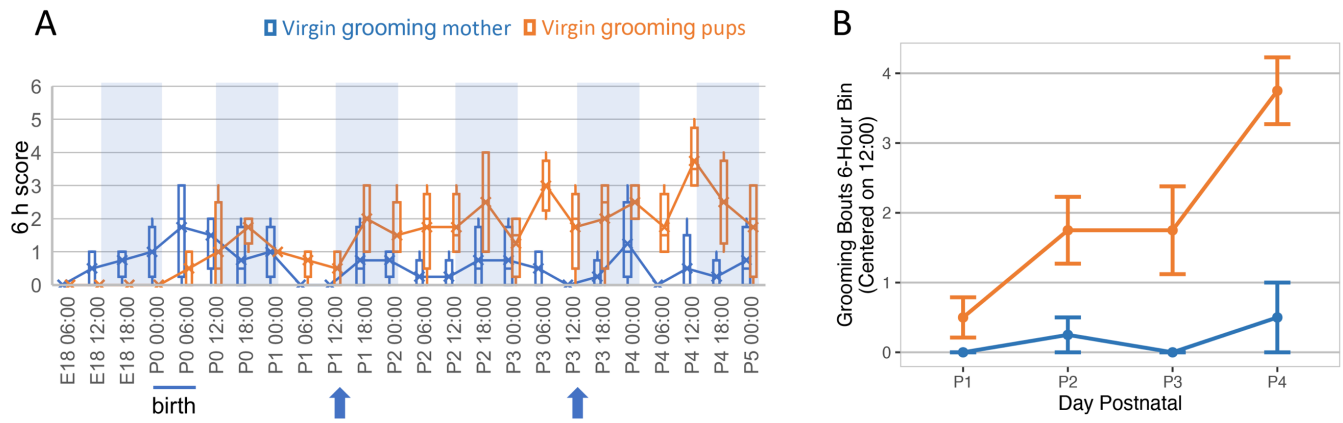

Figure 1. Behaviors exhibited by virgin females co-housed with new mothers and pups over six days beginning the day before birth. (A) Four cages of co-housed mothers and virgin females were recorded over a period of several days before and after birth and grooming behaviors (virgins to pups, plotted in orange; or virgins to mother, blue) were scored (0 or 1) in 5-minute intervals at the beginning of each hour from 12:00 am (0:00) the day before the birth (E18) through the end of the fourth postnatal day (P4), then scores were summed over 6 h periods. To generate the graph, 6 h summed scores were plotted for the four cages as box-and-whisker plots. Times shown mark the end of each 6h period scored. Blue shading in each plot shows the "lights out" periods (12 h beginning at 12:00 pm) for each day. All pups were born within a 6-hour period at the beginning of the light phase on the day designated as P0 for that particular cage, as marked with a bar below each graph. Behaviors plotted and colors used are shown above each graph. Blue arrows below each graph show the times of day that samples were taken from similarly co-housed pairs for gene expression and chromatin analysis. **(B)** The frequency with which co-housed virgins groomed pups (plotted in orange) increased significantly over days (p=0.003), as illustrated by a plot focused on 12:00 pm (start of lights out), while the frequency with which virgins groomed mothers (blue) did not change. Values plotted are mean ± standard error.

Hormone- and neurotransmitter-related genes are dynamically expressed throughout the virgin brain during the first three days of pup interaction.

To understand the genomic underpinnings of this behavioral transition, we collected RNA from the brains of five virgin females co-housed with a pregnant dam at each of three time points: before birth (2 hr into the dark period of embryonic day 18, or E18) and at the same time during postnatal day 1 (P1) and P3. We collected and sequenced RNA from four brain regions involved in pup response, aversion, affiliation and reward: hypothalamus, amygdala, striatum, and frontal cortex.

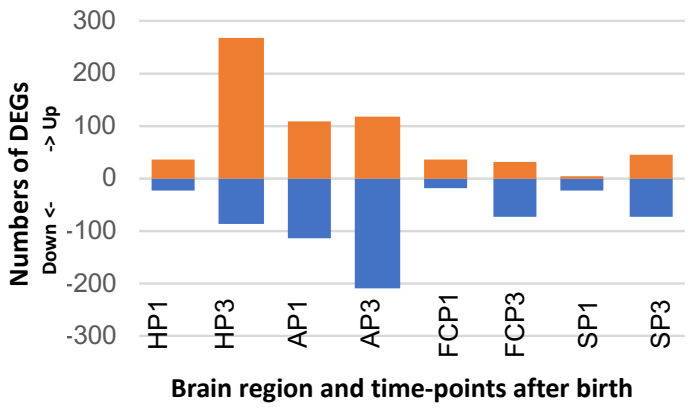

Figure 2. Numbers of genes up- (orange) or down (blue)-regulated in brains of pup-exposed compared to non-exposed virgin females over time. Numbers represent all genes identified as differentially expressed at fdr ≤ 0.05 in each set of pairwise comparisons. H=hypothalamus; A=amygdala; FC=frontal cortex; S=striatum; P1 = postnatal day 1, P3=postnatal day 3.

At P1, we saw an intense transcriptomic response in amygdala with very little expression change in other brain regions; by P3, relatively large numbers of differentially expressed genes (DEGs) were detected in both amygdala and hypothalamus (**Fig. 2; Table S2**). The transcriptomic response did not correlate simply with expression of immediate early genes (IEGs) such as *Fos*, which is classically used to mark neuronal activity (Sagar, Sharp, & Curran, 1988). However, the IEG *Npas4,* which has been implicated specifically in social recognition (Heslin & Coutellier, 2018) and reward-related behaviors (Funahashi et al., 2019), was upregulated at P3 in all brain regions tested (**Fig. 3**). Focusing first on the hypothalamus, genes encoding neuropeptide hormones that are central to initiation of maternal response - oxytocin (*Oxt*) and prolactin (*Prl*) (Bridges, 2015; Rosenblatt, Mayer, & Giordano, 1988) - were first up-regulated at P3, when the virgins were beginning to consistently display maternal-like behaviors (**Fig. 1, Fig. 3**). At P1, dopaminergic (DA) signaling components including *Drd1* were down-regulated along with related Gene Ontology (GO) and functional categories such as morphine addiction and behavioral despair. However, *Drd1* returned to pre-exposure levels in hypothalamus at P3, at the same time that genes related to the activity of dopaminergic neurons were significantly up-regulated.

Therefore, a switch from repressed to increased dopamine-related gene expression was coordinated with the increase of *Oxt* and *Prl* expression in the hypothalamus. This pattern is similar to that observed in mothers at the time of birth, and is consistent with the role of DA signaling in OXT and PRL release (Stolzenberg & Champagne, 2016; Stolzenberg & Numan, 2011). It is also consistent with recent observations from single-cell sequencing that show DA neurons in the hypothalamic preoptic area to be activated in maternally behaving animals (Moffitt et al., 2018). The data suggested that a shift to a neuropeptide and neurotransmitter environment favoring stable maternal behavior was developing in the hypothalamus at P3, concomitant with increased expression of maternal behaviors in the virgin mice. Furthermore, in light of the hypothesized role of histone modifications on maternal behavior (Mayer et al., 2019; Stolzenberg et al., 2012; Stolzenberg, Stevens, & Rissman, 2014), it is also worth noting the low-level but coordinated up-regulation of genes encoding chromatin remodeling and binding proteins (*Hdac10, Hdac7, Sirt6,* and *Sirt7*, *Kmt5c*, *Smarcd3, Atrx, L3mbtl1*) which we observed in the virgin hypothalamus at P3. This coordinated shift suggested the existence of a subtle but significant epigenetic response in the hypothalamus around or before that time (**Table S2A**).

In striking contrast to hypothalamus, components of DA signaling were coordinately *up-regulated* in the amygdala at P1, along with genes encoding endogenous opioids, proenkephalin (*Penk*), and prodynorphin (*Pdyn)*. The combined up-regulation of these neurotransmitters, neuropeptides and functionally-related genes led to P1 enrichment of multiple functional categories related to conditioned behavior, drug addiction, depression and anxiety (**Fig. 3; Table S3**). Together the transcriptomic data indicated a substantial level of genomic activity in the

amygdala of the virgin females during the first day of interaction with pups, strongly suggesting that the females were experiencing stress and anxiety.

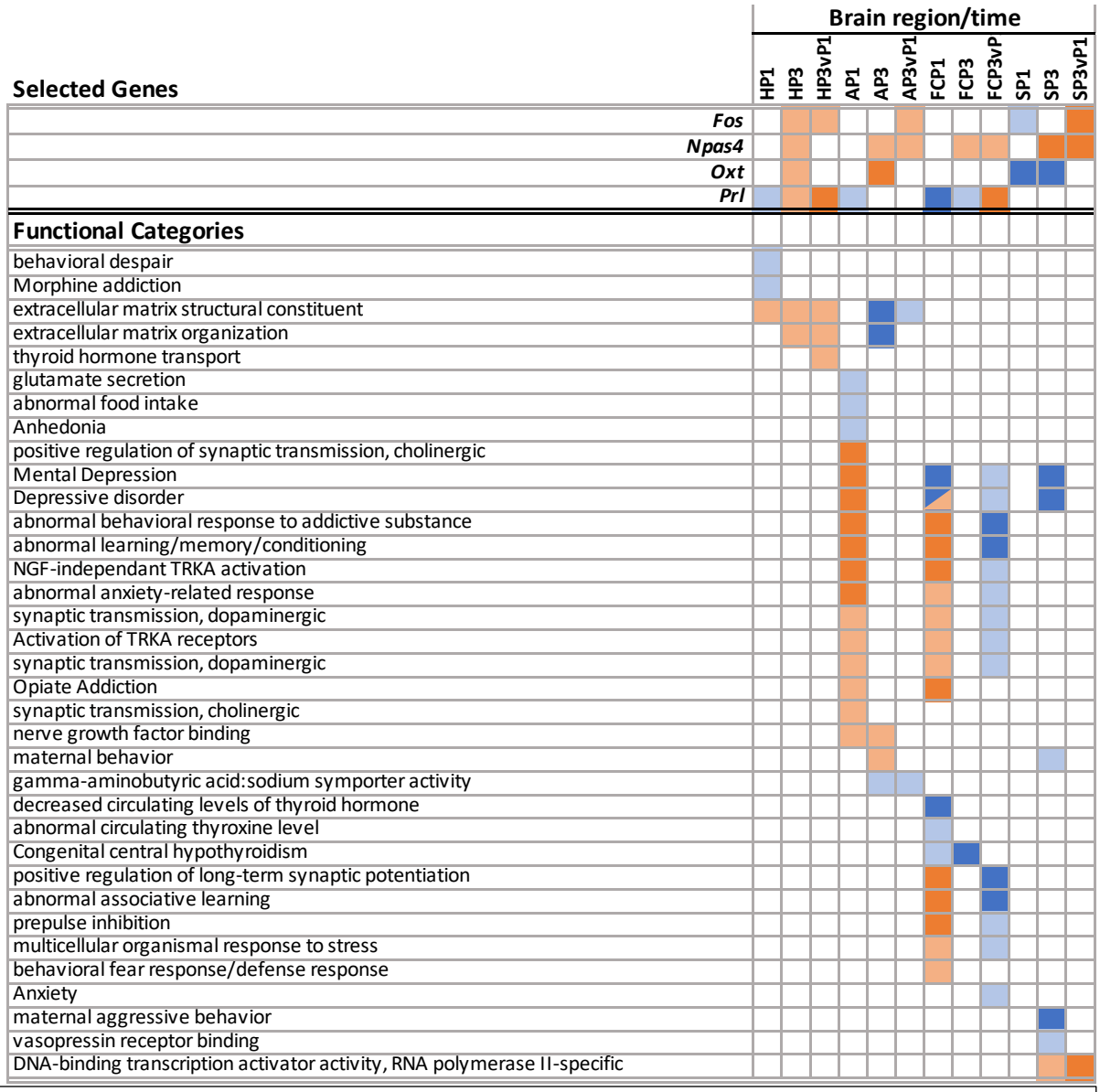

Figure 3. Enrichment of differentially expressed genes in functional categories. Differentially expressed genes (identified at fdr≤0.05 and with absolute value of fold change ≥1.5) were used to identify enriched functional categories using the ToppCluster tool (Kaimal et al., 2010), as described in Methods. Top panel shows differential expression levels for selected genes, as described in the text. Categories shown are a representative subset of the full report included in Table S3, with up- or down-regulation and category enrichment levels displayed as a heat map. Colored cells denote degree of up- (orange) or down-regulation (blue) for genes or Functional Categories in each brain region/time. In the top panel, key markers differentially expressed at fdr ≤0.05 are shown; darker colors highlight DEGs with ≥2-fold change. For functional categories, darker colors signify ToppCluster enrichment of -log10 fdr >10, lighter colors -log10 fdr between 3 and 10.

At P3, many of the anxiety-related amygdala DEGs had returned to pre-exposure levels or were down-regulated compared to E18 controls, suggesting that the initial P1 surge of transcription for these genes might be actively silenced. At the same time that the surge of anxiety-related genes was suppressed, the GO biological process category "maternal behavior" was identified as being enriched in P3 up-regulated genes (**Fig. 3**).

Frontal cortex followed the amygdala in terms of DEGs, direction of change, and enriched functional categories very closely, with a few notable exceptions. In particular, genes related to thyroid hormone activity were uniquely downregulated in the virgin frontal cortex at P3, a finding that is especially interesting given the known role of thyroid hormone in maternal care (Stohn et al., 2018). Furthermore, in addition to the dopamine-related genes similarly up-regulated in amygdala and cortex (**Table S2**), a second cadre of genes associated with depressive states, but related to abnormal thyroid hormone signaling, were down-regulated; the result was that depression-related functional categories were both up and down-regulated in the frontal cortex DEG set. (**Fig. 3; Table S3**).

Finally, in P3 striatum, down-regulated categories were centered on neuropeptide-related genes including those encoding vasopression receptor (*Avpr1a*) and prolactin receptor (*Prlr*) (**Fig. 3**). On the other hand, the gene encoding neuropeptide cholecystokinin (*Cck*), which positively regulates striatal dopamine signaling in *Drd2*-expressing neurons (Kombian, Ananthalakshmi, Parvathy, & Matowe, 2004, 2005) was up-regulated in striatum at P3 compared to P1. This event is notable, since *Cck* plays a critical role in the postnatal maintenance of maternal behaviors (Mann, Felicio, & Bridges, 1995) and mediates responses to anxiety and reward (Rotzinger & Vaccarino, 2003; Vaccarino, 1994). Together these data indicated that a transcriptomic signature consistent with a "maternal response" - as it is classically defined by

neuropeptide and neurotransmitter gene expression -was observed in the virgin mice beginning around P3. In particular, the P1 burst of anxiety-related genes was down-regulated to pre-exposure levels in amygdala and frontal cortex by this time. In contrast with this response in amygdala, DA signaling was *down-regulated* at P1, then *up-regulated* at P3 in the hypothalamus of the pup-exposed virgins, concordant with the onset of maternal-like behaviors in those mice**.**

Comparison to published datasets.

Parallels to gene expression in brains of new mothers. As referenced above, expression of several key markers that have been identified in new mothers was also observed in the alloparenting virgins at the P3 time point. An obvious next question was whether and how gene expression aligned more globally between maternal and alloparenting virgin brains. Most published maternal datasets were generated with distinct hypotheses and biological questions in mind, investigating brain regions and time points very different from ours, complicating direct comparisons. Nevertheless, two published data series warrant some discussion. In the first series, Gammie and colleagues used microarrays to compare gene expression between virgins (not exposed to pups) and nursing females at P7, after maternal behaviors have been robustly established (Driessen et al., 2014; Eisinger, Driessen, Zhao, & Gammie, 2014; Eisinger, Zhao, Driessen, Saul, & Gammie, 2013; Zhao, Eisinger, Driessen, & Gammie, 2014). The same group later completed a meta-analysis of their data to identify genes that were commonly dysregulated across the maternal brain. Despite the differences in methods, time and brain regions examined, DEGs identified in the meta-analysis were enriched in GO categories, pathways, and disease associations very similar to those we identified as most pronounced in the pup-exposed virgin

brains: neuron development, addiction, mental health disorders, and pathways involving oxytocin, vasopressin, prolactin, and opioids (Gammie, Driessen, Zhao, Saul, & Eisinger, 2016).

A second published data series examined maternal gene expression over a wide range of time points pre-and post-partum including P1 and P3, and importantly, used experimental and statistical methodology very similar to ours (Ray et al., 2015). However, cortex (neocortex in the maternal study, which includes frontal cortex and additional cortical regions) and hypothalamus were the only brain regions examined commonly in both studies. Using a hypergeometric test to compare gene expression in pup-exposed virgins and mothers (**Table S2B**), we found that DEGs up-regulated in the hypothalamus of P3 virgins correlated positively and most significantly with genes up-regulated in the maternal hypothalamus at P10 (**Table 1**).

Table 1. Significant correlations between differential gene expression in specific brain regions of pup-exposed virgins and new mothers, or virgins and socially challenged male mice. Hypothalamus (H), Amygdala (A), Frontal cortex (FC) and Neocortex (NC). Highest correlations for all comparisons involving at least 3 overlapping genes are shown, for a full list of comparisons see Table S2.

Virgin dataset/ Maternal Dataset[1]	***p* value**	**example genes**
HP3-up/HP10-up	1.21E-16	*Prl, En1, Slc6a3, Slc10a4, Cryab, Mif, Mfge8*
HP3-up/HP10-down	9.12E-10	*Egr1, Fos, Junb, Lamb2, Col6a1, Col6a2*
HP3-up/HP3-up	2.16E-07	*Prl, Nxph4*
HP3-up/HP1-up	2.99E-07	*Prl, Nxph4*
HP1-up/HP10-down	1.23E-06	*Magel2, Nr1d1, Slc13a4, Ogn*
FCP3-up/NCP1-down	1.59E-25	*Arc, Fos, Npas4, Celsr3, Igsf9b, Robo3*
FCP3-up/NCP3-down	1.06E-21	*Arc, Fos, Npas4, Celsr3, Igsf9b, Robo3*
FCP1-up/NCP1-up	9.32E-15	*Gpr88, Pde10a, Ppp1r1b, Tac1, Rasd2*
FCP3-down/NCP10-down	2.54E-11	*Sgk1, Nnat, Calb2, Igsf1*
Virgin dataset/ Social challenge dataset[2]	***p* value**	**example genes**
AP1-up/A120-up	3.67E-54	*Drd1, Drd2, Rarb, Grp88, Ppp1r1b, Tac1, Tcf7l2*
FCP1-up/FC60-up	1.33E-48	*Drd1, Drd2, Gpr88, Ppp1r1b, Rxrg, Tac1, Penk*
AP3-down/A120-up	4.16E-36	*Cdh1, Ogn, Ccn2, Igf2, Fmod, Sgk1, Grin2b*
AP1-down/A120-up	7.16E-35	*Avp, Ccn2, Grin2b, Gucy1a2*
FCP3-down/FC120-up	1.65E-21	*Igsf1, Calb2, Nnat, Trh, Gabrq*
AP3-down/A120-dn	3.09E-16	*Slc17a7, Tbr1, Nrn1, Sv2b, Lmo3, Tafa1*

The overlapping genes included several involved in DA neuron development and function (*En1*, *Slc6a3* and *Slc10a4*), and neuroprotection and neuroinflammatory processes (*Cryab, Mif,* and

Mfge8). On the other hand, *up-regulated* hypothalamic DEGs from P3 virgins also overlapped with genes that were *down-regulate*d in the maternal hypothalamus at P10 (**Table 1**); IEGs (*Egr1, Fos,* and *Junb)* dominated this list along with genes encoding extracellular matrix (ECM) proteins.

We further identified both positively and negatively-correlated overlaps in comparisons between virgin frontal cortex and maternal neocortex. DEGs *up-regulated* in virgin frontal cortex at P3 overlapped significantly with DEGs *down-regulated* in neocortex of mothers at P1 and P3 (**Table 1**); as in hypothalamus, this group of oppositely regulated genes was dominated by IEGs (*Npas4, Arc)* and genes involved in ECM, and more particularly ECM proteins involved in axon pathfinding (*Celsr3, Igsf9b, Robo3*). Interestingly, there was also significant overlap between DEGs *up-regulated* in both virgin P1 frontal cortex and P1 maternal neocortex. This cluster included genes related to the anxiety-related response that, as noted above, were also up-regulated in the virgin P1 amygdala (*Adora2a, Gpr88, Pde10a, Rasd2, Ppp1r1b, Tac1, Syndigl1*) (**Fig. 3; Table S2C**).

More generally, DEGs across the maternal brain showed enrichment in many of the same functional categories detected in brains of the alloparenting virgin mice (Ray et al., 2015). Although direct comparison of the same brain regions at similar time points will be required for further clarification, the data are consistent with the idea that, with the exception of activation of IEGs and plasticity-related ECM genes, virgin and maternal brains activate many of the same pathways in response to pups.

Gene expression in P1 virgins closely parallels that of socially challenged males. The similarities between gene expression in mothers and the P3 virgins fits well with the fact that the

virgins were beginning to exhibit maternal-like behaviors around this time. However, the molecular events in the virgin frontal cortex and amygdala around P1 remained something of a puzzle. We noted some similarities between DEGs in the virgin P1 amygdala and frontal cortex and DEGs previously identified in the same brain regions taken from of male mice undergoing a territory threat (Saul and Seward et al., 2017), and a hypergeometric test confirmed a very robust correlation (**Table 1; Table S2D**). DEGs up-regulated in P1 virgin amygdala -and particularly those down-regulated in P3vP1 comparisons - showed especially high levels of overlap with genes up-regulated in the amygdala of the socially challenged males; frontal cortex DEGs followed a similar pattern. The overlapping amygdala genes included those associated with dopamine and cholinergic signaling (*Drd1, Drd2, Slc18a2, Slc5a7*) as well as a large cohort of TF-encoding genes (*Isl1, Foxp1, Neurod2, Nkx2-1, Rarb, Tbr1, Tcf7l2, Zic1*) (**Table S2E**). The common up-regulation of these genes in the two social contexts suggests an especially important and common role in the defense/pup aversion response. The data are consistent with the interpretation that at P1, the virgin females are experiencing emotions related to fear and threat, marked by a genomic response that is remarkably similar to that operating in the brains of males involved in territory defense. Notably, this threat-related P1 transcriptomic response was largely extinguished in the virgins at P3, as the females began to display maternal behavior toward the pups.

DEGs cluster into network modules, suggesting regulatory factors with coordinated roles. To gain insights into the coordination and interactions of regulatory factors involved in these brain transcriptomic events, we used a weighted gene correlation network analysis (WGCNA) approach (Langfelder & Horvath, 2008, 2012) to generate a co-expression network, including 25

co-expression modules connected by positive or negative links (**Fig. 4A**, **Table S4A-D**). DEGs from particular brain regions and time points clustered strongly within certain network modules, indicating the coordinated regulation of functionally inter-related genes **(Table S4E**). In particular, the "threat-related" genes that were up-regulated in the virgin amygdala at P1 and then down-regulated at P3 compared to P1, were especially highly enriched in module 3, with modules 7 and 8 showing a similar but less robust enrichment pattern (**Fig. 4B**). These modules included most of the genes that were similarly expressed in P1 virgins and socially challenged males (**Table S2D**). The three positively-correlated modules also included several sets of known interacting genes and DEGs with related functions. For example, Module 3 includes *Drd1* and *Penk* together with TF genes *Rarb* and *Foxp1***,** both of which are important to development and activity of development of dopaminergic neurons (Araujo et al., 2015; Kitaoka et al., 2011). Module 7 includes *Drd5* and TF-encoding DEG *Tcf7l2,* which has been implicated in fear learning (Savic et al., 2011); Module 8 includes *Drd2, Pdyn, Tac1,* and *Rxrg,* the latter encoding RARB dimerization partner, RXRG. Therefore, the DEGs cluster into modules with inter-related functions, including TFs with known regulatory interactions.

In contrast, genes down-regulated at both P1 and P3 clustered together especially in Modules 4 and 6 (**Table S4E**); suggesting potential regulators of this pattern, Module 6 contains many TF-encoding DEGs, several of which have been implicated in social behavior and emotional learning. For example, *RORA* mutations are associated with human autism (Guissart et al., 2018; Sayad, Noroozi, Omrani, Taheri, & Ghafouri-Fard, 2017), and enhanced stress response (Frederic, Chianale, Oliver, & Mariani, 2006). Other module 6 TF genes including *Tfap2b, Neurod2,* and *Tbr1* have also been associated with emotional learning and social

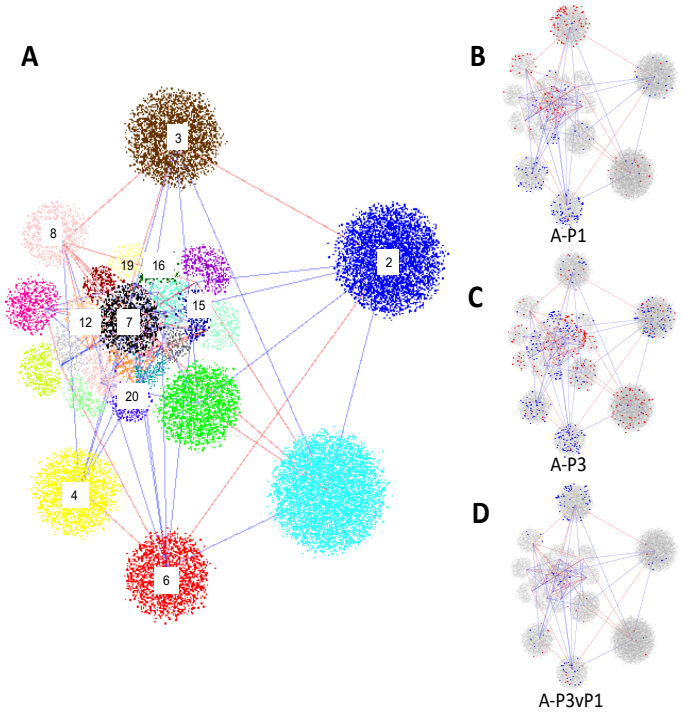

Figure 4. Weighted gene correlation network representing gene expression in four brain regions of virgin female mice. (A) The network, composed of genes expressed in Amygdala, Frontal Cortex, Hypothalamus, and Striatum of pup-exposed and non-exposed virgin controls, consists of 25 modules, each represented by clusters of different color and joined by lines representing positive (blue) or negative (red) eigengene correlations (in both cases showing only those correlations ≥0.6). Numbers have been added to label modules with particular enrichments in amygdala (A) DEGs as referred to in the text. (B-D) representations of the same network but showing the module membership of up- (blue dots) or down-regulated (red dots) DEGs identified in P1 v E18 (A-P1, B), P3 v E18 (A-P3, C), or P3vP1 (A-P3vP1, D) transcriptomic comparisons. Full details of network structure, membership and correlations are provided in Table S4.

behavior phenotypes (Damberg, 2005; Damberg et al., 2000; Huang et al., 2014; Lin et al., 2005). Additionally, P3 up-regulated genes clustered separately, with especially high concentration in Module 15; this module includes the heat shock factor regulator, *Hsf1,* a neuroprotective factor involved in adaptation to stressful experience (Zhu et al., 2008). Other modules displaying more modest levels of amygdala DEG enrichment reflect brain expression patterns that are strongly correlated with, or anticorrelated to, Modules 3, 6 or 15 and might thus also include regulatory factors involved in cross-module gene activation or repressive effects (**Table S4E**).

A dramatic shift in chromatin landscape during the long-term nurturance experience.

Dynamic changes in amygdala chromatin at the P3 time point. The behavioral adaptations that follow maternal and alloparenting experiences have long been thought to involve epigenetic factors (Stolzenberg & Champagne, 2016; Stolzenberg et al., 2012). We therefore expected that

histone modifications could play a key role in establishing and silencing the threat-related transcriptional program. In particular, we hypothesized that the key genes involved in the aversion/anxiety/fear reaction we observed at P1 might be actively silenced by these mechanisms as the virgins began to display maternal-like behaviors at P3. We tested this hypothesis by carrying out chromatin immunoprecipitation (ChIP) in chromatin from each of the four brain regions from virgin females co-housed with mothers at E18 and P3. For these ChIP experiments, we used an antibody specific to histone 3 acetylated at lysine 27 (H3K27Ac), a general marker for accessible chromatin (Hon, Hawkins, & Ren, 2009).

Consistent with our previous results (Saul and Seward et al., 2017), the ChIP profiles revealed tens of thousands of open-chromatin peaks in every brain region for both pup-exposed and non-exposed females (**Table S5**). Since differentially accessible peaks (DAPs) offer a unique window into chromatin dynamics that may drive the brain response, we paid special attention to DAP regions – defined as genomic regions in which the relative levels of H3K27Ac were at least two-fold higher or lower in brains collected at P3 compared to E18 consistently in biological replicate samples (**Table S6**). Surprisingly, DAPs were virtually absent in the chromatin samples from hypothalamus, and were found in relatively low numbers in frontal cortex and striatum. But in striking contrast, chromatin from the P3 amygdala contained thousands of DAPs, either increased (5325 DAPs) or decreased in accessibility (7209 DAPs) at P3 compared to E18 (**Fig. 5A, Table S6A**). To maximize the chances of linking DAPs to DEGs accurately, we focused our attention on the smaller number of DAPs located within 5 kb of the TSS of an annotated gene (called TSS-DAPs). Altogether we found 2738 TSS-DAPs with decreased H3K27Ac at P3 compared to E18 abbreviated hereafter as P3-closed DAPs), and 1040

TSS-DAPs with increased levels of H3K27Ac accumulation at P3 compared to E18 (P3-open DAPs).

Interestingly, all genes associated with P3-open or P3-closed TSS-DAPs, respectively, clustered into network modules that were also enriched for up- or down-regulated DEGs. For example, amygdala P3-closed DAPs were particularly enriched for linkage genes in modules 3 and 6, whereas P3-open DAPs were most likely to be associated with genes in Modules 7 and 8 (**Table S4F**). The TSS-DAPs were associated with 138 amygdala DEGs, including 22 TSS containing P3-open DAPs, 115 TSS containing P3-closed DAPs, and 1 TSS hosting DAPs of both types (**Table S6B**). Given that amygdala DEGs were up-and down-regulated in roughly equal numbers (Fig. 2) the preponderance of down-regulated genes in TSS-DAPs

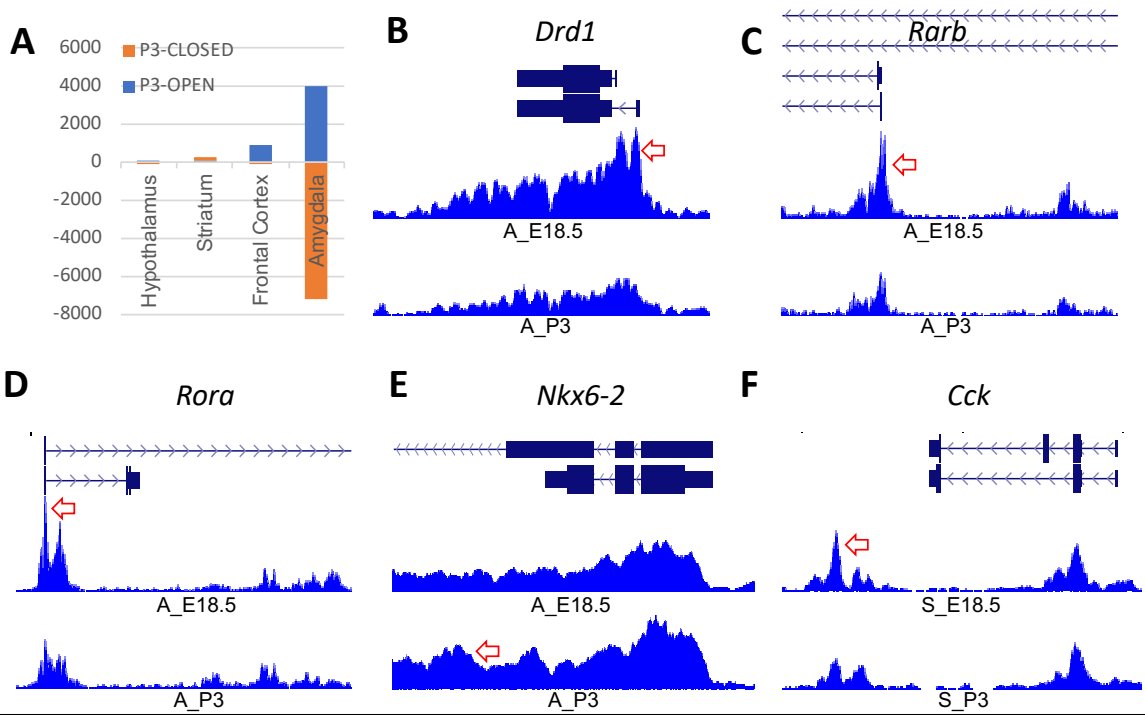

Figure 5. Dramatic changes in the amygdala chromatin landscape accompanies the transition to maternal-like behaviors in alloparenting virgin mice. (A) Relative numbers of TSS-associated differentially accessible peaks (DAPs), as measured by >2-fold change in detected levels of H3K27Ac, in the four brain regions tested in this study. Positive numbers represent peaks more accessible at P3 than E18 (P3-open DAPs); negative numbers represent less accessible (P3-closed) peaks. (B-E) Examples of DAPs in *Drd1, Rarb1, Rora, and Nkx6-2* genes, showing normalized H3K27Ac profiles in amygdala chromatin at E18 (A_E18, top track) and P3 (A_P3, bottom track) for each gene. (F) An example of a DAP in chromatin from Striatum (S_E18, S_P3) within the *Cck* gene. Red arrows in each panel point to examples of significant differentially accessible peaks. Full ChIP profiles are available online as a UCSC Browser track hub at https://trackhub.pnri.org/stubbs/ucsc/public/allo.html), and data are available in Tables S5 and S6.

suggested that alterations in the chromatin landscape were primarily focused on silencing amygdala genes. The DEGs associated with P3-closed DAPs were enriched specifically and significantly in network module 3 (hypergeometric p = 2.57E-17), suggesting an especially important role for histone de-acetylation in silencing this coregulated cluster of threat-associated genes.

Striking and potentially relevant examples included amygdala P3-closed DAPs associated with TSS of Module 3 genes, *Drd1* and *Rarb* (**Fig 4B, 3C**), P3-closed DAPs associated with the primary promoter of *Rora* (Module 6 TF gene down-regulated at both P1 and P3) (**Fig. 4D**), and P3-open DAPs associated with *Nkx6-2* (Module 1 TF gene up-regulated at the P3 timepoint) (**Fig. 4E**). Together the data suggest that activities of many key genes involved in pup response are regulated by differential chromatin accessibility, specifically in the amygdala.

Although much smaller in numbers, some DAPs associated with DEGs in other brain regions also deserve some mention. For example, *Rarb* was also down-regulated in frontal cortex P3 vs. P1 comparisons, and displayed a pattern of DAPs in cortex very similar to that seen in amygdala (**Table S6A**). Additional DAPs that may be relevant to gene expression were discovered in brain regions other than amygdala by lowering the fold-change cutoff to 1.5 instead of 2 (1.5X vs 2X), while keeping the same replicate FDR significance threshold. For example, a 2X P3-open DAP in striatal chromatin was identified approximately 25 Kb downstream of *Cck*, and several 1.5X P3-closed DAPs were found within and closer to the gene (**Table S6A; Fig. 4F)**. Since *Cck* was up-regulated in striatum at P3, this chromatin configuration suggests the possible role for chromatin dynamics in the regulation of this critical gene.

Enrichment of binding motifs support a significant role for differentially expressed TFs. To obtain further information regarding the potential activity of TFs in the pup response, we searched for enrichment of known TF binding motifs (TFBMs) in the P3-open and P3-closed amygdala TSS DAPs. The search identified REST/NRSF binding motifs as the top enrichment within *P3 closed* TSS-DAPs (E18 enriched compared to P3); although *Rest* itself was not identified as differentially expressed, the data suggest that REST TFBMs were being closed between E18 and P3 in amygdala of the pup-exposed virgins. This finding is of interest, because REST is a central regulatory of neuron differentiation and plasticity (Lepagnol-Bestel et al., 2007), and also plays a role in stress resilience in adult brain (Singh-Taylor et al., 2018). The search also identified enrichments for motifs recognized by proteins encoded by genes that were detected as amygdala DEGs, including *Mef2c*, *Rora*, and *Ebf4,* in the set of P3-closed DAPs. *Rora* and *Mef2c* genes were down-regulated in the amygdala of pup-exposed virgins at P3 (Table S2); consistently, the TSS of both genes were associated with P3-closed DAPs (**Fig. 4D; Table S7B**). Together these data indicate that histone deacetylation events evident at P3 serve to not only reduce levels of *Mef2c* and *Rora* gene expression levels, but simultaneously, to reduce the accessibility of both TFs to their target genes. Notably, both TF genes have been associated with deficits in social behavior (Adachi, Lin, Pranav, & Monteggia, 2016; Harrington et al., 2020; Sarachana, Xu, Wu, & Hu, 2011), supporting a possible functional role. In contrast, *Ebf4* was upregulated in the P3 amygdala despite the reduced accessibility of its binding sites; the function of this gene in brain is not known.

Some notable TFBM Enrichments were also detected in P3-open DAPs. For example, EBF motifs were also enriched in the P3-open DAPs, suggesting a potentially important and novel role for *Ebf4* in this context. Further, we noted enrichment of FOX family TFs including

the specific TFBM of DEG, *Foxp1* which was up-regulated in amygdala at P1 (**Table S7**). Also enriched in the P3-open DAPs was the TFBM of TCF7; this TFBM is shared with TCF7L2, encoded by a DEG that is up-regulated at P1 in the virgin amygdala; both *Foxp1* and *Tcf7l2* were also up-regulated in the amygdala of socially challenged males (Saul and Seward et al., 2017) (Table S2D), suggesting a role for these TFs in regulating shared aspects of the aversion/threat response. Because we did not measure chromatin at P1, the initial timing of these epigenetic events is not discernible. However, the data would suggest that TSS-linked targets of REST, RORA and MEF2C became less accessible, targets of FOX family proteins and TCF7L2 became more accessible, and targets of EBF family factors were differentially affected by epigenetic modification during the postnatal period.

DISCUSSION

With the goal of understanding molecular mechanisms that underlie a transition from aversive to affiliative behaviors, we investigated the behavioral, transcriptomic, and chromatin response of virgin females as they were co-housed with mothers and newborn pups over a period of several days. Our data revealed dramatic and dynamic neurogenomic shifts that coincided with displayed behaviors. In particular, consistent with the virgins' agonistic behavior around P1, gene expression suggested a striking signal of threat, fear and anxiety in hypothalamus and amygdala, in a pattern that correlated with high significance to that previously observed in brains of territorially-challenged males (Saul and Seward et al., 2017). The data are thus consistent with the hypothesis that pup aversion and defensive behaviors share a common brain circuitry (C. Dulac, O'Connell, & Wu, 2014), and suggest a common molecular mechanism as well. Several TF genes implicated by gene expression, network co-expression, chromatin analysis,

and/or motif-enrichment analysis were similarly up- or down regulated in the pup-exposed virgins and socially challenged males, suggesting crucial roles in this shared molecular signature of social threat.

Published data support the roles of several of these TFs in threat/anxiety response. For example, *Tcf7l2* has been demonstrated as important for fear-learning and social dominance phenotypes in mice (Savic et al., 2011), and the RARB:RXRG dimer's activities in amygdala have been linked to expression of anxiety-related phenotypes (Krzyzosiak et al., 2010). Additionally, the association of *Rora* with enhanced fear response in humans (Miller, Wolf, Logue, & Baldwin, 2013) and mice (Frederic et al., 2006), together with the fact that *Rora* mutant mouse mothers do not retrieve, care for, or suckle their young (Guastavino, 1983), support the further examination of this gene's role in the amygdala of mothers. Furthermore, other TF genes implicated in the shared threat signature are associated with social-behavior phenotypes, including *Tbr1* (den Hoed et al., 2018), *En1* (Kuemerle, Gulden, Cherosky, Williams, & Herrup, 2007) and *Foxp1* (Hamdan et al., 2010) suggesting coordinated functional roles.

We hypothesize that these and other networked TFs work together to modulate the pup-aversion response. The network developed from our dataset suggests a robust framework of positive and negative gene interactions that coordinate this behavioral switch over time. The expression of the threat-related TF genes was extinguished along with the pulse of dopamine signaling after the rise of oxytocin, prolactin, and other neuropeptides by P3, paving the way for a shift in the virgin females' behavior toward the pups. Further, we hypothesize that this transcriptomic shift was driven, at least in part, by the substantial level of chromatin remodeling we observed in the amygdala of the pup-exposed virgins. Having taken chromatin profiles only

at two time points, it is possible that earlier chromatin remodeling events may have played an important role in the transition to a maternal-like state. However, many of the genes that returned to normal expression levels between P1 and P3 in amygdala were associated with differentially accessible chromatin, consistent with their active epigenetic silencing in that brain region at the later time point.

Chromatin remodeling has been implicated in the development of maternal behaviors in both mothers and alloparenting virgins, although most published studies have focused on the hypothalamic MPOA as the primary site of this epigenetic response (Mayer et al., 2019; Stolzenberg et al., 2012, 2014). These studies have shown that *suppression* of HDAC activity - or *inhibition* of chromatin silencing - in hypothalamus is key to inhibiting the females' aversion response. Surprisingly therefore, we found no evidence of chromatin remodeling in the hypothalamus at the P3 time point, and the massive chromatin remodeling we did observe in amygdala was weighted toward *de-acetylation,* or chromatin closure, and the silencing of differentially expressed genes. These findings would suggest that HDAC activity plays a positive role in quenching the aversion response. However, histone modifications at earlier time points or in different brain regions could easily have influenced the virgins' behavior. For example, although deacetylation in amygdala may be critical in quenching the aversion/defensive response once established, *suppression* of deacetylation in hypothalamus (and/or other brain regions) before P1 might have prevented the establishment of the aversive/fear response in the first place. Possibly relevant to this hypothesis is the coordinated up-regulation of histone deacetylase and chromatin remodeling genes we detected in the hypothalamus at P3; this signal could reflect the trace of earlier epigenetic events associated with the expression of fear and anxiety in the virgins before they transitioned to active pup care.

This is the first study to investigate global gene expression in the amygdala in the context of alloparental care, and supports the idea of including amygdala in future studies with mothers as well. Our studies highlight a special role for the amygdala in the switch from aversion to affection in this behavioral context, a hypothesis that is consistent with the known functions of amygdala in maternal behavior and bonding (Atzil et al., 2017; Wonch et al., 2016). As underscored by human brain imaging studies, maternal behavior involves a global brain response that unfolds over an extended period of pre- and postnatal time (Dufford, Erhart, & Kim, 2019); in the pup-exposed virgins, we detected just the start of this behavioral transition during the third postnatal day. Nevertheless, the mechanisms involved in transition from aversion to intensive pup care in the virgin mice could be relevant to a successful transition to motherhood as well. Although it is not yet possible to determine whether a similar response is activated, or actively suppressed, at some time around birth in the maternal amygdala, this question is an important one in the context of maternal bonding and infant care. We speculate that a similar active suppression of the threat/aversion program may occur in the amygdala of new mothers, and that dysregulation of this program could underlie the failure of mother-infant bonding, post-partum anxiety and depression. Addressing this hypothesis will offer a novel perspective on the causes of these very common and painful human maladies.

MATERIALS AND METHODS

Mice and behavioral analysis

All work with mice was done under the approval of the IACUC at the University of Illinois, Urbana Champaign. Mice were housed in a temperature-controlled room in a reverse 12h/ 12h light-dark cycle. Six-week-old female mice (C57BL/6J x C3HJ F1 hybrids, with an agouti coat

to allow clear distinction with the black-coated virgins) purchased from the Jackson Laboratory were impregnated, and co-housed with age-matched virgin female C57BL/6J mice during pregnancy and through the early post-partum pregnancy. To record behavior, four pairs were filmed in clear-topped cages continuously using a Samsung SCB-2000 CCTV Camera with iSpy 64 v7.2.1.0 CCTV software. Behavior was scored in each cage (0 or 1) in 5-minute snapshots at the top of each hour from the day before and until the end of the fourth day after birth, with scores combined over 6 h periods for each cage to generate the illustrative plot in Fig. 1. To test the hypothesis that pup-grooming behaviors increased over time, while mother-grooming behaviors did not, we performed one-way repeated measures ANOVA in R (v4.0.4) using the rstatix package (v0.7.0). The anova_test() function in rstatix automatically assesses repeated measures data for the assumption of sphericity. Scores for these and other behaviors are also presented in Table S1. The 5-minute video snapshots are provided as File S1 with video clips of specific and unusual behaviors noted in the text provided as File S2; additional video is available on request.

Dissections and RNA preparation

Dissections were performed as described in detail previously (Saul and Seward et al., 2017) with the addition of the striatum. Briefly, mice were euthanized by cervical dislocation followed by rapid decapitation. Their brains were removed and sectioned in a coronal slicing mouse brain matrix. A total of three cuts were made: two cuts separated by 4 mm defined by the rostral and caudal aspects of the hypothalamus and a third cut bisecting these two cuts. The hypothalamus, frontal cortex, striatum, and amygdala were dissected from the resultant brain slices (**Supplementary Figure S1**). Upon completion of these dissections, focal brain regions were

placed into cryotubes, snap-frozen in liquid N2, and stored at -80°C until downstream processing. Samples were prepared for sequencing from the four dissected brain regions of five mice per condition (E18, P1, P3). RNA isolation and QC were completed as described previously, with libraries prepared robotically at the Roy J. Carver Biotechnology Center at University of Illinois, also as described in Saul and Seward et al., 2017.

Gene expression analysis

Illumina Sequencing libraries were generated with the TruSeq Stranded mRNA HT kit (Illumina) using an Eppindorf ePMotion 5075 robot, and were sequenced to a depth 45-60 million reads per sample on Hi-Seq 2500 instruments at the Roy J. Carver Biotechnology Center at the University of Illinois. All sequencing data generated in this study have been deposited to the GEO database under Accession number *(in progress)*. Pairwise comparisons of E18, P1 and P3 samples were completed as previously described in detail (Saul and Seward et al., 2017), with results provided in Table S2. For functional analysis, genes that were found to be differentially expressed at fdr < 0.05 were first filtered for absolute fold change >1.5, and uploaded to the ToppCluster web analysis tool (Kaimal, Bardes, Tabar, Jegga, & Aronow, 2010)) using default conditions (Bonferroni correction, fdr <0.05). Selected categories are summarized in Table 2, with full ToppCluster Results reported in Table S3.

WGCNA Analysis

We used signed WGCNA (Langfelder & Horvath, 2008) to generate networks from the data from all individuals, brain regions, and time points, as described in depth in our previous study (Saul and Seward et al., 2017). Eigengenes calculated for each module were used to generate

module correlations; details of module structure, module gene content, eigengene correlations, and hypergeometric enrichments are presented in Table S4. After log-transforming our data using voom+limma, we filtered zero variance genes, selected a soft thresholding coefficient of 3, then used a signed Pearson correlation analysis with a minimum module size of 30. Images in Figure 3 were generated using version 3.7.1 of Cytoscape (Shannon et al., 2003).

ChIP Tissue Preparation, Chromatin Immunoprecipitation, and Library Preparation

ChIP was performed essentially as described in detail in our previous study (Saul and Seward et al., 2017). Briefly, brain tissue dissected from 3 animals was pooled, homogenized, and fixed in PBS with 1% formaldehyde for 10 minutes. Nuclei were prepared from the fixed cells and stored at -80° C until use. Thawed nuclei were sonicated using a BiorupterTM UCD-200 (Diagenode, Liège, Belgium) sonicator, and fragmented chromatin was processed for ChIP with 2 ug histone H3K27Ac antibody per sample (Abcam ab4729), using one million nuclei for each IP. IPs were performed in biological replicate, with one pool of 3 samples in each replicate, as previously described. Libraries were prepared from eluted DNA using KAPA LTP library kits (KK8230) using Bioo Scientific index adapters, size-selected using AmpureXP beads (Beckman Coulter, Brea, CA, USA) and quality checked by Qubit 2.0 and Bioanalyzer (Agilent 2100). Samples were sequenced to a depth of 20-30M reads per replicate on an Illumina HiSeq 2500 sequencer using a TruSeq SBS sequencing kit, version 4, in single-end format with fragment length of 100 bp. Base calling and demultiplexing into FASTQ files was done using bcl2fastq v1.8.4 software (Illumina, San Diego, CA, USA).

ChIP-Seq Bioinformatics

ChIP sequencing reads were mapped with Bowtie2 (Langmead & Salzberg, 2012) to the UCSC Mus musculus mm9 or mm10 genome, using default settings and analyzed for peaks using HOMER (Hypergeometric Optimization of Motif EnRichment) v4.7 (Heinz et al., 2010), as previously described (Saul and Seward et al., 2017). Differential chromatin peaks were identified in biological replicates using the HOMER getDifferentialPeaksReplicates.pl script, looking for any peaks that changed at least two-fold between conditions with an FDR cutoff of 0.05. Known motif discovery was performed with the HOMER findMotifsGenome.pl script using default settings with 201bp peak regions extracted from all histone peaks or only differential histone peaks. Chromatin profiles are available online as a UCSC Genome Browser track hub at https://trackhub.pnri.org/stubbs/ucsc/public/allo.txt).

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