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Maternal deprivation alters expression of neural maturation gene *tbr1* in the amygdala paralaminar nucleus in infant female macaques

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Abstract

Early parental loss is associated with social–emotional dysregulation and amygdala physiologic changes. Previously, we examined whole amygdala gene expression in infant monkeys exposed to early maternal deprivation. Here, we focus on an amygdala region with immature neurons at birth: the paralaminar nucleus (PL). We hypothesized that 1) the normal infant PL is enriched in a subset of neural maturation (NM) genes compared to a nearby amygdala subregion; and 2) maternal deprivation would downregulate expression of NM transcripts (mRNA). mRNAs for *bcl2*, *doublecortin*, *neuroD1*, and *tbr1*—genes expressed in post-mitotic neurons—were enriched in the normal PL. Maternal deprivation at either 1 week or 1 month of age resulted in PL-specific downregulation of *tbr1*—a transcription factor necessary for directing neuroblasts to a glutamatergic phenotype. *tbr1* expression also correlated with typical social behaviors. We conclude that maternal deprivation influences glutamatergic neuronal development in the PL, possibly influencing circuits mediating social learning.

Keywords

ganglionic eminence; social behavior; *tbr1*; *bcl2*; *dcx*; *neuroD1*

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

1 | INTRODUCTION

Inadequate caregiving has a lasting effect on social and emotional behaviors in primate species (Harlow & Zimmermann, 1959; Spitz, 1946). Young children placed in institutionalized orphanages are a stark example of this phenomenon (Bruce, Tarullo, & Gunnar, 2009; Sheridan, Fox, Zeanah, McLaughlin, & Nelson, 2012; Tottenham et al., 2010). Follow-up studies on several populations of these children show long-lasting behavioral changes, including difficulties with emotional regulation and increased anxiety (Bruce et al., 2009; Chisholm, 1998; Zeanah et al., 2009). In monkeys, maternal deprivation also results in behavioral changes that can be depressive or anxious in nature, and are dependent on the age at which maternal absence begins, similar to children with a history of institutionalized care (Cameron, 2001; Harlow & Zimmermann, 1959; Suomi, Collins, & Harlow, 1973).

Decreased sensory inputs to developing brain structures during specific developmental time-points may result in permanent alterations in brain function (Katz & Shatz, 1996). Thus, inadequate social and emotional stimulation such as occurs in institutional rearing in humans, or maternal deprivation in monkeys, may impart long-lasting changes to infant brain structures that are actively developing, with resulting effects on function. The amygdala is a key brain substrate that is critical for normal social-emotional development (Brothers, Ring, & Kling, 1990; Dicks, Myers, & Kling, 1969; Machado & Bachevalier, 2006; Machado et al., 2008; Prather et al., 2001; Shaw et al., 2004; Tranel & Hyman, 1990). It is dysregulated in many psychopathologic conditions (Burghy et al., 2012; Schumann, Bauman, & Amaral, 2011), as well as in the aftermath of inadequate caregiving in humans (Tottenham, 2012) and monkeys (Sabatini et al., 2007). We previously found that maternal deprivation in this cohort of monkeys and others, beginning at 1 week of age has markedly different effects on social-emotional development compared to maternal deprivation beginning at 1 month of age, with the former show long-lasting decreases in social behavior, and the latter showing increases in social behaviors (Bauer, Coleman, Kupfer, & Cameron, 2000; Cameron, 2001; Cameron et al., 1998; Cameron, Coleman, Dahl, Ryan, & Kupfer, 1999; Knudsen, Heckman, Cameron, & Shonkoff, 2006; Sabatini et al., 2007). Thus, input of age-appropriate sensory and social-related information to the amygdala in early childhood may be critical to later normative development and function.

Although the amygdala is frequently referred to as a unitary structure, it is a heterogeneous collection of nuclei. In both monkeys and humans, the amygdala subregion known as the paralaminar nucleus (PL) appears very late in gestation (Kordower, Piecinski, & Rakic, 1992), and is the only amygdalar region to contain immature cellular features at birth (Ulfig, Setzer, & Bohl, 2003) and postnatally (Bernier, Vinet, Cossette, & Parent, 2000; Chareyron, Lavenex, Amaral, & Lavenex, 2012; Fudge, 2004). Specifically, the PL contains a population of immature neuroblasts. These cells can be identified morphologically by their small size and singular cell process, as well as by histochemical (Chareyron et al., 2012) and immunocytological staining for neuroblast markers (Bernier et al., 2000; Fudge, 2004).

Importantly, the PL is not reliably detected in rodents. The PL is called a “nucleus,” but cells of the PL actually form a sheet-like layer that is most apparent in the ventral amygdala near

the site of the former ganglionic eminence. From there, the PL stretches anteriorly to envelop the basal nucleus and to some extent, the lateral nucleus. The PL increases in size across primate phylogeny (accounting for body weight) and is largest in humans (Stephan & Andy, 1977; for review, deCampo & Fudge, 2012). Although the reason for this phylogenetic progression in monkeys, apes, and humans is not clear, it coincides with a relatively extended time-line for brain development, and a protracted juvenile state in these higher species (Joffe, 1997; Walker, Burger, Wagner, & Von Rueden, 2006).

Tract tracing studies indicate that the PL receives inputs from the ventral part of the lateral nucleus of the amygdala (Pitkanen & Amaral, 1998), and also from the hippocampus and entorhinal cortex (Fudge, deCampo, & Becoats, 2012; Saunders & Rosene, 1988; Saunders, Rosene, & Van Hoesen, 1988). On the efferent side, it has inputs to the lateral subregion of the central amygdala nucleus (Fudge & Tucker, 2009) and bed nucleus of the stria terminalis (deCampo & Fudge, 2013). These input/output paths place the PL at a crossroads where complex sensory and contextual information is integrated and transferred to key nodes of the “fear” system (Freese & Amaral, 2009). Although other nuclei of the amygdala also participate in “fear circuitry,” the PL is unique in containing neuroblasts—post-mitotic neurons that have not yet been incorporated into target circuits, based on markers such as doublecortin (DCX) and class III beta tubulin (TUJ1) (Bernier, Bedard, Vinet, Levesque, & Parent, 2002; Chareyron et al., 2012; deCampo & Fudge, 2012; Fudge, 2004; Ulfing, Setzer, & Bohl, 1998; Yachnis, Roper, Love, Fancey, & Muir, 2000). The percentage of immature neurons in the monkey PL begin to decline in the first year of life, whereas the proportion of mature neurons gradually increases (Chareyron et al., 2012), suggesting a period of postnatal neural development that may be particularly influenced by early life events.

Based on preliminary data demonstrating a subpopulation of immature neurons in the PL, we designed a custom gene set to probe genes involved in neural maturation (NM) to allow us to investigate the PL in both normally reared and maternally deprived infant monkeys at 3 months of age. We chose to examine early deprivation (with subsequent group-pen rearing), and the resulting effects on social behavior by 3 months of age, since this is the time period during which a secure base of attachment is forming for this species (see review by Suomi, 1997). Disruption of this primary parental social bond is therefore hypothesized to have important effects on the amygdala.

Beginning with the normal control cohort (Study 1), we first compared the expression of the NM gene set in the PL compared to the adjoining amygdalohippocampal area (AHA, which serves as a control region in having many similar connections, but few immature appearing neurons postnatally). We hypothesized that the NM gene set—or a subset of NM genes in the set—would have increased expression in the PL compared to the AHA. In Study 2, we tested the hypothesis that maternal deprivation would specifically affect genes associated with post-mitotic neural maturation in the PL.

2 | MATERIALS AND METHODS

2.1 | Animals

The animals used in this study were of the same cohort that was previously behaviorally characterized in a study of the whole amygdala gene expression. Complete details of the maternal deprivation paradigm and behavioral assessment of these animals can be found in the previous paper (Sabatini et al., 2007). We briefly review the paradigm here (Figure 1). We used 12 female infant macaques (*Macaco mulatta*) born in the breeding colony at the University of Pittsburgh Primate Laboratory. Due to the sample size, which was constrained by the need to conserve animals and costs, we used only female animals so to avoid confounds on gene expression differences. However, it is important to note that this maternal deprivation paradigm has subsequently been conducted on cohorts of both females and males. These studies have shown no sex differences in social behavior parameters measured, even when tracked into adulthood (Bauer et al., 2000; Cameron et al., 1998, 1999). The experiments were reviewed and approved by the University of Pittsburgh Animal Care and Use Committee.

2.2 | Experimental groups

Animals in all groups spent the first week of life in a single cage with their mother. After the first week of life, animals were arbitrarily designated as 1-week separated, 1-month separated, or control (maternally reared), and handled differently as described below. Although this paradigm is a “deprivation” rather than separation paradigm (see Section 2.3), we use the group names, “1-week separated” and “1-month separated” to reflect the age at which maternal deprivation began. Maternally reared monkeys stayed with their mother throughout the study duration.

2.3 | Maternal deprivation paradigm

At 1 week of age, animals in the 1-week separated group were removed from their mothers and placed for a 5–7 days period into a single cage where they learned to bottle-feed. The cage was positioned immediately adjacent to a group-rearing pen that they would ultimately join. In the single cage, animals were provided with a soft cotton stuffed toy that could be held to provide contact comfort. After spending 5–7 days in the single cage, 1-week separated monkeys joined the group-rearing pen for weeks 3–12. Monkeys in the 1-month separated groups were introduced to the group-rearing pen with their mothers at 1 week of age. When infants turned 1 month of age, mothers were removed from the group-rearing pen, and infants were placed in the single cage adjacent to the group-rearing pen, as described for the 1-week separated monkeys. After the 5–7 days period in the single cage where they learned to bottle feed, 1-month separated monkeys were reintroduced into the group-rearing pen without their mother for weeks 6–12. After 1-week of age, control (maternally reared) monkeys were placed into the group-rearing pen, which consisted of 4–5 monkeys, with their mother where they remained for weeks 2–12. The group-rearing pen also had a nursery enclosure where only infant monkeys could enter. Bottles containing standard human artificial formula were placed in the nursery and replaced three times each day. All infant monkeys were weighed weekly and had similar weight gains over the study. For details, see Supplementary Material S1.

2.4 | Behavioral assessments

As noted, behavioral assessments on this cohort of animals were performed as part of an earlier study and took place at two time-points: 1) the week after maternal deprivation while infants were in a single cage; and 2) in the third month of life in the group-rearing pen (see methods and results section in Sabatini et al., 2007). The behaviors scored while infants were alone in the single cage included: general behaviors (e.g., locomotion, stationary, bottle drinking), where they were in the cage (e.g., adjacent to the pen) and self-comforting behaviors (e.g., toe/thumb-sucking, rocking). At the third month of life, all behaviors scored in the single cage were again scored, this time in the pen. In addition, at this time-point, time spent in social interactions was also recorded (e.g., social play, touch, groom, social huddle, aggression). In the present study, data collected only at the second time-point (3 months) was analyzed in conjunction with gene expression data. Brain tissue from the control monkeys was used for studies of the normal infant PL (Study 1) and tissue from all animals was examined in the deprivation study (Study 2).

2.5 | Gene mRNA expression-behavior correlations

Following analysis of differential gene expression in Study 2, we found that the gene *tbr1* showed significant differential expression between maternally deprived monkeys and control monkeys. Because mutations in *tbr1* are associated with autism spectrum phenotypes (O’Roak et al., 2012), we chose to look at the correlation of *tbr1* expression with the composite scores for typical social behavior (e.g., social play, touch, groom, social huddle, aggression) and self-comforting behaviors (e.g., toe/thumb sucking, rocking). Our rationale for looking at these behaviors was based on the altered social behavior, and stereotypies resembling self-comforting behaviors, that have been described in individuals with autism (Volkmar, Chawarska, & Klin, 2005). We examined the relation between *tbr1* mRNA expression using silver grain counts from in situ hybridization studies (Supplementary Material S1). Pearson correlations were performed between the number of silver grains counted from the *tbr1* in situ hybridization with either composite 1) social behaviors; or 2) self-comforting behaviors. A *t*-distribution table with $n-2$ degrees of freedom was used to determine the associated one-tailed *p* values. *r* values were considered significant when the associated *p* value was <0.05 .

2.6 | Tissue processing

At 12 weeks of age, brain tissue was collected from each monkey at the University of Pittsburgh. Monkeys were pre-anesthetized with ketamine HCl (10 mg kg⁻¹, i.m.) and then deeply anesthetized with sodium pentobarbital (30 mg kg⁻¹, i.v.). The chest was opened and a catheter placed in the heart with the tip in the ascending aorta and ~1 L of ice cold 0.9% NaCl, containing 5000 IU heparin and 2% sodium nitrite, was perfused transcatheterially. The left atrium was opened so perfusate could run out and when it ran clear the monkey was quickly decapitated. The brain was immediately removed from the skull and was hemisected by a midline sagittal cut. The right hemisphere, which was used in this study, was cut into 5-mm-thick coronal blocks, flash frozen in isopentane over dry ice, and stored at -80 °C until cryostat sectioning. For this study, tissue blocks containing the caudal amygdala at the level of the PL and amygdalohippocampal area (AHA) were selected and sectioned at 20 μm

using a cryostat. One of the 1-month separated monkeys did not have enough tissue in the block for this study, resulting in three monkeys in the 1-month separated group for microarray studies. Ten adjacent sections of the caudal amygdala were thaw-mounted onto polyethylenephtalate (PEN) foil slides (Leica Microsystems; Bannockburn, IL) for laser-capture microdissection, and frozen at -80°C . The subsequent 10 adjacent sections of each block were used for in situ hybridization.

2.7 | Laser-capture microdissection (LCM) and Microarray

We used LCM to isolate the PL and adjacent AHA on the same sections of each animal, and analyzed differential gene expression in each region using microarray (Figure 2). A custom-designed gene set enrichment approach was used to examine differential expression in the PL compared to the AHA in normal animals (Study 1) and in maternally deprived animals versus normal animals (Study 2) for the PL and AHA. We chose to use this approach to avoid the common problem in gene expression studies of identifying a list of significant, but unrelated genes. In gene set enrichment analysis, a set of genes with a common function or pathway are examined to see if there are significant differences in expression between two groups. Gene sets can be manually created, or obtained from Biocarta, KEGG, or GO databases. For this study, we manually created a list of genes that had a documented role in NM, and only examined changes in gene expression within this set. To create this custom-designed gene set, we included genes involved in NM based on 1) our previous observations of protein expression in immature neurons in the adult PL (Fudge, 2004; Fudge et al., 2012; Figure 3); and 2) primary literature searches of NM genes involved in the developing cortex and adult neurogenic zones. Genes were included in the custom-designed gene set if at least one published report showed a documented role in neural fate commitment (i.e., transition from a multi-potent precursor to committed neuronal precursor or involved in differentiation of a neural precursor into a specific neuronal cell type), migration, and/or neurite extension in post-mitotic neurons, regardless of whether specific genes had additional reported functions (Gupta, Tsai, & Wynshaw-Boris, 2002; Lie & Götz, 2009) (Supplementary Material S2). We reasoned that since most genes have more than one function, restricting the gene set to only one function might reduce discovery of important sets of genes that play a role in neural maturation in the PL.

PL and AHA boundaries were visualized using a rapid thionin staining protocol in which each section was placed in acetic acid, 0.5% thionin, and dehydrated through a series of alcohols. Total time for staining was 3–4 min. Immediately following staining, the PL and the AHA in each section (10 slides per animal), was rapidly dissected on a Leica A LMD microscope (Leica Microsystems, Bannockburn, IL). Using this method, the PL is distinguished by its small-sized cells and higher cell density (Price, Russchen, & Amaral, 1987). In separate studies, we noted that the PL visualized in this way, coincides with doublecortin (DCX)-immune reactive cells (Fudge et al., 2012) (Figure 3). The AHA is an elliptical shaped structure characterized by large cells and does not contain DCX-immuno reactive cells. A total area of 0.25 mm^2 (PL) and 0.5 mm^2 (AHA) was collected from the same section. Tissue from each region was collected into $30\text{ }\mu\text{l}$ RLT Lysis Buffer (Qiagen, Valencia, CA) with 1% β -mercaptoethanol (Sigma–Aldrich, St. Louis, MO) in under 10 min per slide, and rapidly frozen at -80°C .

Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). RNA concentration and quality were measured using an RNA 6000 Pico Chip on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The average RNA integrity value (RIN) for the PL and the AHA was 3.0 and 5.7, respectively. Material captured from two slides for the PL and one slide for the AHA were used to acquire the 5 ng of RNA needed for amplification and hybridization. We chose samples whose 28:18 peak ratio was closest to 2. Amplification and synthesis of biotinylated cDNA was performed with the WT-Ovation PicoRNA Amplification kit (Nugen, San Carlos, CA). A total of 4.5 μ g of cDNA was hybridized to Affymetrix Human Gene 1.0 ST arrays (Affymetrix Inc., Sunnyvale, CA). Following hybridization, samples were normalized using gene chip robust multi-array average (GC-RMA). Probe intensities were \log_2 transformed.

2.8 | Microarray analyses

2.8.1 | Study 1: Differential expression of NM genes in the PL versus AHA in normal infant animals—In Study 1, we hypothesized that the NM gene set as a whole, or a subset of genes within it, would be enriched (upregulated) in the PL compared to the AHA in control animals. To determine whether the gene set as a whole was differentially expressed in the two regions, the average \log_2 ratio (ALR) was computed between the PL and AHA for every gene on the microarray ($|\text{ALR}| = 1$ corresponds to a twofold increase or decrease, $|\text{ALR}| = 0.585$ represents a 1.5-fold change, $|\text{ALR}| = 0.383$ corresponds to a 1.3-fold change). These fold-changes are considered biologically relevant, and are detectable by quantitative polymerase chain reaction (qPCR) (Shelton et al., 2011). The “average ALR” for the NM gene set was computed by averaging the ALRs of each gene in this customized gene set. This average was then compared to the average of the ALRs for all genes on the chip using an unpaired one-tailed Student’s *t*-test with a *p* value threshold set at *p* < 0.05. We did not correct for multiple comparisons.

We then wanted to identify the individual genes within the NM gene set that were differentially expressed between the PL and the AHA. We used a two-criteria approach to determine differential expression (Mirnics, Levitt, & Lewis, 2006). First, an individual gene was considered to be differentially expressed if the ALR between the PL and the AHA was greater than 30% ($|\text{ALR}| > 0.383$). This criterion was chosen based on previous studies indicating that gene expression fold changes of 1.3 ($\text{ALR} > 30\%$) are biologically significant and can be verified with either real-time PCR or in situ hybridization at this level (Shelton et al., 2011). Genes that survived this first criterion were subjected to an unpaired 1-tailed Student’s *t*-test comparing expression levels between the two regions, with *p* value < 0.05. Genes that met criteria were validated using qPCR and/or in situ hybridization (Supplementary Material S1) to determine whether they represented true biological changes.

2.8.2 | Study 2: Maternal deprivation effects on gene expression in the PL and AHA—In Study 2, the effects of maternal deprivation on gene expression of the entire customized NM gene set in the PL was examined in two different comparisons: 1) 1-week separated monkeys relative to maternally reared monkeys (1-week separated vs. control); and 2) 1-month separated monkeys relative to maternally reared monkeys (1-month separated vs. control; Figure 2). As in Study 1, for every gene on the microarray the average

\log_2 ratio (ALR) was calculated for each of the two comparisons (1-week separated vs. control, 1-month separated vs. control). The “average ALR” for the NM gene set as a whole was calculated by averaging the ALRs of all genes in the gene set. The average was then compared to the average of the ALRs for all genes on the chip using an unpaired one-tailed Student’s *t*-test with a *p* value threshold set at $p < 0.05$.

We then identified individual genes within the customized gene set that showed differential expression in the PL for each of the two comparisons (1-week separated vs. control, 1-month separated vs. control). We considered an individual gene within our customized gene set to be significant if it fulfilled our two criteria: 1) the gene’s ALR between the separated (separated at 1-week or at 1-month) and the maternally reared groups was greater than 30% ($|\text{ALR}| > 0.383$); and 2) the *p*-value for a one-way ANOVA (1-tailed) was $p < 0.05$.

Next, we wanted to determine whether genes that showed differential expression in the two comparisons were similar with respect to changes in direction and magnitude. We therefore plotted the ALR values of the most changed genes ($|\text{ALR}| > 0.383$) in the 1-week separated (vs. control) with corresponding values in the 1-month separated (vs. control). Pearson correlations were used to determine the *r* and *p* values. A *t*-distribution table with *n*-2 degrees of freedom was used to determine the one-tailed *p* values. *r* values were considered significant when the associated *p* was < 0.05 . Identical analyses were conducted on tissue collected from the AHA to identify the most changed genes in this region for each comparison. Differentially expressed genes are included in the results. However, as our focus was on the PL, AHA correlation plots are not shown.

To determine whether maternally deprived gene expression changes in the PL and AHA were region specific for the two comparisons, we correlated ALR values from one region with corresponding values in the other region, restricting our correlations to $|\text{ALR}| > 0.383$. This allowed us to see whether or not the most changed genes in the PL following maternal deprivation were correlated with most changed genes in the AHA. If a set of most changed genes in the PL were also changed in the same direction and of similar magnitude in the AHA, we interpreted this to mean that the effects of maternal deprivation on gene expression were non-specific to the PL. We used a Pearson correlation to determine *r*-values. A one-tailed *p*-value was used. *r* values were considered significant when the associated $p < 0.05$.

To validate some of the findings, qPCR and in situ hybridization were performed. qPCR was run for *dcx*, *tbr1*, and *neuroDI*, based on microarray results for both Study 1 and 2. In situ hybridization of *tbr1* mRNA was done on frozen sections adjacent to those used for LCM (Additional Methods in Supplementry Material S1).

3 | RESULTS

3.1 | Study 1: Differential expression of NM genes in the PL versus AHA in normal infant animals

In the normal infant amygdala, the NM gene set was decreased in the PL compared to the AHA (average ALR of all genes in the gene set compared to the average of the ALRs for all genes on the chip). This decrease (12.8%) (ALR = -0.174 , $p = 0.0001$) in the NM gene set

as a whole was in a direction contrary to our hypothesis. We reasoned that since many genes in the NM gene set had additional functions, a second level of analysis could be used to determine whether individual genes within the NM gene set had a higher expression in the PL relative to the AHA. From this analysis, four genes were found to have enriched expression in the PL based on the two criteria for differential expression ($|\text{ALR}| > 0.383$ and $p < 0.05$), and were confirmed by qPCR. They were: *bc12* (ALR = 0.433, $p = 0.01$), *dcx* (ALR = 0.88, $p = 0.01$), *neuroD1* (ALR = 0.84, $p = 0.02$), and *tbr1* (ALR = 0.84, $p = 0.01$) (Figure 4A, red). qPCR confirmed the microarray findings, showing a significant enrichment in the expression of *dcx* of approximately fivefold ($-\text{ddC}_t$ 2.35, $p = 0.02$), *tbr1* of approximately sixfold ($-\text{ddC}_t$ 2.62, $p = 0.01$), and *neuroD1* approximately 11-fold ($-\text{ddC}_t$ 3.51, $p = 0.001$) in the PL relative to expression in the AHA (Figure 4B). Unfortunately, there was inadequate sample to confirm *bc12* expression differences by qPCR. Thus, a specific subset of NM genes expressed in post-mitotic neurons in the fetus, and in adult neuro-genetic zones (see Section 4), was enriched in the PL compared to the AHA, in normal 3-month old animals. Interestingly, a number of genes from the NM gene set were relatively enriched in the AHA versus the PL (Figure 4A, blue). These genes participate in axonal outgrowth and cytoskeletal stabilization, developmental processes that are somewhat different than those implicated by gene expression in the PL (see Section 4).

3.2 | Study 2: Maternal deprivation effects on gene expression in the PL and AHA

3.2.1 | Maternal deprivation effects on gene expression in the PL—The effects of early maternal deprivation on PL gene expression were then examined. The NM gene set as a whole was examined first and was decreased in both maternal deprivation groups, although changes were small. For the 1-week separated versus control comparison, there was a 7.2% decrease (ALR = -0.10 , $p = 0.0001$) and for the 1-month separated versus control comparison, there was a 4% decrease (ALR = -0.06 , $p = 0.0005$) in the gene set in the PL compared to the ALR of all genes.

As in Study 1, we then identified a subset of NM genes with an $|\text{ALR}| > 0.383$ for each deprivation group within the PL (Figure 5). In the 1-week separated versus control comparison, we identified 13 genes meeting this criterion for fold-change. In the 1-month separated versus control comparison, we identified nine genes that met this criterion. Five of the genes in the 1-week versus control and 1-month versus control comparison were the same, displaying $|\text{ALR}| > 0.383$ in both cases (Figure 5, gray). The genes downregulated in both comparisons were: *apc*, *ncam1*, *nrx1*, *rap1a*, *tbr1*. Together these genes comprised a common core of genes involved in NM that were altered by early maternal deprivation in the PL.

We then determined whether changes in gene expression in the two maternally deprived groups were similar (i.e., in the same direction and magnitude). Plotting the ALR values of the 13 most changed genes in the 1-week separated (vs. control) against their corresponding ALR values in the 1-month separated (vs. control), we found a correlation of $r = 0.76$, $p = 0.001$ (Figure 5A). Performing the reverse correlation (i.e., plotting values for the nine most changed genes in the 1-month separated (vs. control) comparison, against the corresponding ALR values in the 1-week separated (vs. control) comparison), gave a similar positive

correlation $r = 0.66$, $p = 0.03$ (Figure 5B). These findings indicate that as a group, the most changed genes in the PL (relative to control values) had similar changes in direction and magnitude.

We then identified which genes within the 13 gene (1-week separated) and nine gene (1-month separated) subsets met additional criteria for significance ($p < 0.05$). In the PL, examination of differential gene expression values revealed that *tbr1*, a gene expressed in post-mitotic neurons destined to be projection neurons (Hodge, Kahoud, & Hevner, 2012), was significantly altered. *tbr1* was also found to be relatively enriched in the normal PL in Study 1 (Figure 4). A one-way ANOVA showed that there was a significant effect of rearing condition on *tbr1* expression in the PL at $p < 0.05$ [$F(2,8) = 8.036$, $p = 0.0122$]. Post hoc comparison indicated that *tbr1* expression was significantly lower in monkeys deprived at 1 week and at 1 month of age compared to controls, with an ALR = -0.86 for each comparison. There was no significant difference in *tbr1* expression between 1-week- and 1-month-separated comparisons. Expression levels of *neuroD1* and *dcx* genes which are also expressed in post-mitotic neurons (Hodge et al., 2012), had meaningful decreased expression the 1-week separated versus control animals, but were not significant [*neuroD1* ALR = -0.48 , $F(2,8) = 2.16$, $p = 0.18$; *dcx* ALR = -0.86 , $F(2,8) = 1.21$, $p = 0.35$]. Lack of statistical significance may mean that there is no true effect of group on *neuroD1* and *dcx* expression. Alternately, the magnitude of the fold changes, and the fact that these genes are temporally and biologically linked (along with *tbr1*) in neuroblasts (Hodge et al., 2012), raises the strong possibility that there was insufficient power to detect statistically significant effects due to low sample size.

In addition to significant decreased expression of *tbr1* in the 1-month separated versus control comparison, *dcc* expression was significantly lower in monkeys deprived at 1 month of age [$F(2,8) = 14.56$, $p = 0.002$, ALR = -0.49]. *dcc* encodes a membrane bound receptor that, when bound to netrin-1, is important in maintaining axonal growth along stereotyped paths during brain development (Fazeli et al., 1997; Keino-Masu et al., 1996). As yet, the developmental expression of *dcc*, or its ligands, in the immature primate brain have not been described.

3.2.2 | Maternal deprivation also alters gene expression in the AHA—As a whole, the custom-designed NM gene set was downregulated in the AHA in the 1-week separated group (decreased by 12%; -0.16 ALR, $p = 0.05$), but upregulated in the 1-month separated group (increased by 3.5%; 0.05 ALR, $p = 0.05$) relative to maternally reared monkeys. Again, because many NM genes serve broad functions, to better evaluate specific gene expression changes, we identified the most changed genes in the AHA for each comparison as had been done for the PL. *cables1*, which plays a role in neurite outgrowth in the central nervous system (Zukerberg et al., 2000), was significantly altered in the AHA for both deprived groups compared to control animals [$F(2,8) = 12.54$, $p = 0.01$]. Although there were several other genes that were altered in either 1-week or 1-month separated comparisons, *cables1* was the only gene to show a significant change in both comparisons, with its expression significantly lower in maternally deprived groups compared to controls (ALR = -0.49 for both comparisons). *Cables1*, which was also enriched in the AHA relative to the PL in normal animals (Study 1), is localized to growth cones where it modulates

axonal outgrowth. Maternal deprivation at both 1 week and 1 month of age was associated with reduced *cables1* expression in the AHA suggesting region-specific effects on axonal outgrowth. Maternal deprivation in the 1-week separated, but not 1-month separated comparison revealed several other significantly changed gene transcripts: *bdnf* (ALR = -0.51, $p = 0.03$) and *cdk5* (ALR = -0.66, $p = 0.01$), which were enriched in the normal AHA, were downregulated in the 1-week separated monkeys. Additionally, some genes not found to be enriched in Study 1, including *ncam1* (ALR = -0.40, $p = 0.02$), *rap1b* (ALR = -0.90, $p = 0.02$), *apc* (ALR = -0.82, $p = 0.01$) and *st8sia2* (ALR = +0.44, $p = 0.01$), were also altered in the 1-week separated comparison only.

3.2.3 | Gene signatures in maternally deprived animals are distinct for the PL and AHA

—Finally, to determine whether the most changed genes in the PL ($|\text{ALR}| > 0.383$) for each of the two comparisons were similar or different than those in the AHA (i.e., the specificity of gene expression changes in each region), we analyzed whether the ALR values from the PL (1-week separated vs. control, and 1-month separated vs. control) were similar in magnitude and direction compared to the values found in the AHA (Figure 6). For the 1-week separated versus control comparison, the correlation between the most changed genes in the PL with their corresponding values in the AHA was $r = -0.011$, $p = 0.98$; for the 1-month versus control comparison, the correlation was $r = -0.3$, $p = 0.21$. Therefore, in both comparisons, overall gene expression changes in the PL did not as a group, correspond to similar changes in the AHA. One striking difference was that *tbr1* was downregulated in the PL of monkeys deprived at both 1 week and 1 month, but not the AHA (Figure 6, boxed). *neuroD1* and *dcx*, genes which were also enriched in the PL of normal animals (Study 1), were uniquely downregulated in the PL compared to the AHA of 1-week separated monkeys, but did not reach significance (Figure 6A).

Analysis of qPCR data for *tbr1*, *dcx*, and *neuroD1* across rearing conditions for the PL and AHA was also performed. There was a significant effect of rearing condition on *tbr1* expression [$F(2,7) = 4.973$, $p = 0.045$] (Figure 7A). Post hoc comparison using Fisher's LSD test indicated that *tbr1* expression was significantly lower in monkeys that were deprived at 1 week and 1 month compared with control animals, but not between 1-week and 1-month separated animals. Supporting microarray findings, *tbr1* was not downregulated in the AHA in either the 1-week separated versus control or 1-month separated versus control comparisons. As with microarray data, *dcx*, and *neuroD1* did not show significant changes in gene expression across group comparisons for the PL or the AHA.

3.2.4 | *tbr1* mRNA expression in PL correlates with social behavior at 3 months of age

—The maternal deprivation paradigm had no effects on the animals' developmental trajectories of physical parameters such as weight and locomotor behavior. These were similar among all animals at the time brain tissue was collected (ANOVA, $F = 0.284$, $p = 0.76$; Sabatini et al., 2007). As previously reported for this cohort of monkeys, the percent time spent in typical social behaviors by 3 months of age was abnormal for all maternally deprived monkeys. Also as previously reported, 1-week separated animals demonstrated more contact-comforting behavior with a soft-cotton stuffed toy and decreased social contact initially (i.e., at time of deprivation), while later increasing self-comforting

behaviors (thumb-/toe-sucking, rocking) and having decreased social-contact behaviors. One-month separated animals in contrast, initially showed increases in social-comforting behaviors and continued to engage in more social behavior (i.e., ventral contact, proximity, grooming) than 1-week separated or control groups (Cameron, 2001).

Spontaneous mutations in *tbr1* are associated with autism, a condition that often presents with altered social behaviors and stereotypies that are thought to be self-comforting (O’Roak et al., 2012). We therefore examined correlations between *tbr1* mRNA expression obtained from in situ hybridization methods with total typical social behaviors (e.g., play, grooming, touch, aggression, social huddle) and total self-comforting behaviors (e.g., toe/thumb sucking, rocking) measured at 3 months of age in the group-rearing pen. In situ hybridization confirmed that the density of silver grains was relatively higher over the PL than adjacent regions, including the AHA, in all animals (Figure 7B and C). Although there were no significant group differences in *tbr1* mRNA expression by in situ hybridization [$F(2,8) = 1.66, p = 0.25$], these results showed a trend in the same direction as the significant changes detected by qPCR and microarray. The time spent in typical social behaviors by 3 months of age across all animals was significantly positively correlated with *tbr1* PL expression ($r = 0.56, p = 0.045$; Figure 7D). Self-comforting behaviors and *tbr1* expression were not significantly correlated ($r = -0.47, p = 0.08$).

4 | DISCUSSION

4.1 | Summary of main findings

In female infant monkeys, the pattern of NM gene enrichment (Study 1) indicates that in the first months of postnatal life, genes involved in the relatively early differentiation of neuroblasts predominate in the PL. Maternal deprivation (Study 2) during this time is associated with downregulation of *tbr1*, which regulates the production and differentiation of neuroblasts into glutamatergic neurons (Hodge et al., 2012) (Table 1). The percent time spent in typical social behaviors by 3 months was decreased in maternally deprived animals and correlated positively with *tbr1* expression at this age.

4.2 | Study 1: Differentially expressed genes identified in infant PL suggest postnatal maturation of glutamatergic neurons

In normal infant monkeys, we identified a subset of NM genes that distinguish the PL from its near-neighbor, the relatively developed AHA (Table 1). The particular gene signature of the PL supports the concept that it is a zone where maturational processes in post-mitotic neurons occur. As the caudal ganglionic eminence recedes in the last few months of human fetal life, the PL is progressively defined (Ulfig et al., 1998). Recent work in developing non-human primates has noted several immature neural characteristics of the PL, with changes over post-natal development. Chareyron et al. recently used histochemical and stereologic methods to show that the PL of monkeys between birth and 1 year of age contains only 15% of the total mature neurons present in young adulthood (5–9 years). In adulthood, there is an increase in the number of mature neurons paralleled by a decrease in the number of immature neurons. Importantly, the total number neurons in the PL does not

differ between ages, suggesting that by adulthood, a significant number of immature neurons have matured (Chareyron et al., 2012).

Building on existing knowledge of the infant PL, we found that several transcripts involved in neuroblast fate determination, neurite extension, migration, terminal differentiation, and layer specification are relatively enriched in the PL of normal 3 month old animals (see below, this Section). Two of these gene products, *bcl2* and *dcx*, have been previously described at the protein level in the PL of young adult monkeys (Bernier et al., 2002; Fudge, 2004; Fudge et al., 2012) and in humans (Yachnis et al., 2000). *bcl2*, while best-known for its anti-apoptotic activity (Zhong et al., 1993), is independently involved in differentiation of committed neurons and in neurite formation (Zhang, Westberg, Holtta, & Andersson, 1996). *bcl2* is broadly expressed throughout human fetal brain (Merry, Veis, Hickey, & Korsmeyer, 1994), declines through gestation, and after birth is highly expressed only in restricted regions such as the hippocampal subgranular zone and PL in both humans and monkeys (Bernier et al., 2002; Fudge, 2004; Yachnis et al., 2000). *dcx* codes a microtubule-associated protein that is critically involved in neural migration (Gleeson, Lin, Flanagan, & Walsh, 1999), and its genetic mutation in humans results in severe disruption of cortical layering (des Portes et al., 1998; Gleeson et al., 1998). *dcx* is expressed in both radially and tangentially migrating neurons, possibly at different stages of migration or in different neuronal subclasses (i.e., interneurons and pyramidal cells) (Meyer, Perez-Garcia, & Gleeson, 2002).

neuroD1 and *tbr1* transcripts, which have not previously been described in the postnatal primate brain, are involved in fate specification of post-mitotic neurons. *neuroD1* encodes the neuronal differentiation 1 protein, which is required for terminal neuronal differentiation in the classic postnatal neurogenesis zones, namely the olfactory bulb (Boutin et al., 2010; Roybon, Deierborg, Brundin, & Li, 2009) and hippocampus (Miyata, Maeda, & Lee, 1999) based on work in the rodent. In these regions, as well as in the hippocampus and cortex of the fetal brain, the temporal expression of *neuroD1* precedes and overlaps subsequent *tbr1* expression (Hodge et al., 2012). *tbr1* codes a transcription factor required for the development and laminar positioning of glutamatergic projection neurons in the cortex (Bulfone et al., 1995; Hevner et al., 2001; Hodge et al., 2012), cerebellum, and subgranular zone (Hodge et al., 2012) and marks precursors destined to become glutamatergic neurons. In fetal monkeys (Donoghue & Rakic, 1999), humans (Bulfone et al., 1995), and mice (Bulfone et al., 1995; Kolk, Whitman, Yun, Shete, & Donoghue, 2005), *tbr1* expression is localized to the fetal subplate and deep cortical plate, both transient regions in which gene expression and transient efferent and afferent connections orchestrate formation of the mature cortex (Sidman & Rakic, 1973; Ulfig, Neudorfer, & Bohl, 2000). *tbr1* expression in the PL may suggest a similar transient “way station” in the development of the amygdala in primates.

bcl2, *dcx*, *neuroD1*, and *tbr1* transcripts are all closely temporally regulated during the final phases of neuronal differentiation and positioning in developing cortex and in adult neurogenic zones (Hodge et al., 2012), which is a specific phase of neural development prior to the integration into final circuits. The relative enrichment of these four transcripts in the postnatal PL in monkey suggests that continuing neural maturation processes in this

subregion occur after birth, as the young animal begins its early interactions with the environment. Given the fact that the PL is minimal to non-existent in the rodent, and large in the human, our results highlight the importance of using non-human primate models to study normal and abnormal amygdala development that may pertain to humans, and human disease (deCampo & Fudge, 2012).

In contrast to genes involved in cell fate specification and differentiation (PL), genes enriched in AHA are more identified with molecular pathways involving neurite outgrowth and the cytoskeletal shifts that underlie neurite outgrowth (Figure 4A, blue). Several combinations of these genes participate in intracellular cascades including actin stabilization/polymerization (e.g., Hussain et al., 2001; Sumi, Matsumoto, Takai, & Nakamura, 1999) and neurite outgrowth (Cheung, Chin, Chen, Ng, & Ip, 2007; Fujino, Wu, Lin, Phillips, & Nedivi, 2008; Leemhuis et al., 2010; Meiri, Saffell, Walsh, & Doherty, 1998; Strittmatter, Fankhauser, Huang, Mashimo, & Fishman, 1995; Zuker-berg et al., 2000). Although these genes are critical in CNS development, they also participate in processes such as synaptic plasticity and learning (e.g., reviews by Dhavan & Tsai, 2001; Park & Poo, 2013).

4.3 | Study 2: *tbr1* is highly enriched in the normal infant PL and downregulated following maternal deprivation

The PL has a fundamentally different maturation profile compared to the rest of the amygdala, with PL neurons at a much earlier phase of development compared to the rest of the amygdala, particularly in the first few years of life (Bernier et al., 2002; Chareyron et al., 2012; Fudge, 2004). Our previous study of this cohort, using an exploratory approach to examine the entire amygdala found that differential expression of several genes distinguished the two separation groups, and that there were correlations with specific behavioral profiles typical of each group (Sabatini et al., 2007). In the present study, we dissected out the PL and found that the timing of deprivation (i.e., beginning at 1 week and 1 month) made no difference on *tbr1* gene expression between 1-week and 1-month separated animals. We interpret this to mean that the very immature status of PL neurons in the first three months of life makes them sensitive to maternal deprivation at either time-point in the first month of life, specifically with respect to *tbr1* expression. Decreased *tbr1* expression implies an alteration in the maturation of glutamatergic neurons in the PL, which could affect these circuits, and/or their efferent and afferent contacts.

Although our focus was not on the AHA, in this nucleus, we found that *cables1* (enriched in the AHA in normal animals in Study 1) was significantly downregulated in both maternal deprivation groups. *Cables1* is a cdk5-interacting protein that is highly expressed in the brain and largely localized to growth cones (Zukerberg et al., 2000). Decreases in *cables1* may suggest a negative influence on neurite outgrowth and circuit development in the AHA following early maternal deprivation. *bdnf* and *cdk5*—also relatively highly enriched in the AHA of normal animals—were additionally downregulated in monkeys deprived at 1 week of age. Although the downregulation of *bdnf*, *cables 1*, and *cdk5* in 1-week separated monkeys may be entirely independent, it is intriguing that both *bdnf* and *cables1* interact with *cdk5* (Cheung et al., 2007; Zukerberg et al., 2000) to influence neurite growth.

4.3.1 | Maternal deprivation, *tbr1*, and development of typical social behavior

—We found that *tbr1* expression in the PL was positively correlated with the frequency of typical social behaviors (e.g., ventral contact, proximity, grooming) at 3 months of age, suggesting a potential link between glutamatergic neuron development in the PL and the expression of social behavior. *tbr1* has been identified as one of several genes whose disruption is associated with autism phenotypes (Huang & Hsueh, 2015; O’Roak et al., 2012), possibly through effects on developing glutamatergic cells. Autism spectrum disorders are heterogeneous disorders with varying phenotypic expression and severity, but a core feature is disrupted development of typical social behaviors (Volkmar et al., 2005). Similar deficits can be present in children raised in suboptimal caregiving environments in infancy (Rutter, Kreppner, & O’Connor, 2001).

Although it has long been known that normal amygdala development is critical for instantiation of appropriate social responses in monkeys (Bauman, Lavenex, Mason, Capitanio, & Amaral, 2004; Goursaud & Bachevalier, 2007; Prather et al., 2001; Schumann et al., 2011), the molecular-anatomic correlates of typical social learning and response in the infant have been unclear. Here, we find evidence that disrupted expression of *tbr1* in the PL is associated with maternal deprivation and decreased time spent in typical social behavior by 3 months of life. Whether *tbr1* is directly involved in the emergence of social behaviors, part of a larger gene network involved in social behavior, or a secondary effect of maternal deprivation and/or social impairment remains to be seen. We also do not yet know whether the gene-behavior correlation between *tbr1* and time spent in age-appropriate social behavior persists over time. However, this association may be an important clue to determining how early maternal deprivation is translated to altered brain circuits with effects on social behavior. Whether disrupted gene expression in the PL is due to “stress-related” consequences of maternal deprivation such as cortisol activation (Feng et al., 2011) and inflammation (Cole et al., 2012)—or to deficits in social and sensory inputs required for normal PL development—also remain to be tested.

Finally, it is important to recognize that downregulation of *tbr1* expression following early maternal loss may point to either regression or acceleration of young neurons along their developmental trajectory. Future studies will be needed to determine whether there are fewer or more mature neurons in the PL of monkeys undergoing early social stress. Several groups have suggested a hastening of development of neural growth and/or connectivity following a stressful early life experience (Gee et al., 2013; Schumann et al., 2011).

There are several limitations to this study. The most important is related to the sample size, necessitated by the value of the primate animals used and their extended gestational period. Larger animal numbers would clearly have enhanced statistical power to detect changes in other gene transcripts. In order to minimize variability in this small sample, the study was limited to female monkeys, and a larger future study should examine both sexes. Although we have not seen significant behavioral differences between the sexes in subsequent cohorts (Bauer et al., 2000; Cameron et al., 1998, 1999), it will be important to study gene expression and brain changes in both males and females, including into adulthood. In larger studies, the potential impact of nutritional differences between maternally deprived and maternally reared infants also needs to be examined, given growing recognition of

nutritional differences between breast milk and standard human formula. Standard infant formula, such as used in this study, has decreased amounts of fatty acids, which are required for normal behavioral and neural development (Champoux et al., 2002). This fact has important implications for maternal deprivation paradigms in general and warrants further exploration.

On the microarray side, the relatively low RIN numbers for our samples indicate that our ability to detect other potentially important gene expression changes, particularly in the PL, is reduced (Schroeder et al., 2006). However, despite these challenges, several genes emerged in both Study 1 and Study 2 that were validated by qPCR. Although important genes were likely undetectable due to low sample size and lower than optimal RIN, we were able to show several robust subregion-specific gene expression changes between the PL and AHA in normal control animals, and altered gene expression of *tbr1* in maternally deprived groups in the PL. Importantly, there was also differential expression of genes that are known to be temporally and/or biologically linked. Additionally, two of four genes in the NM gene set that were relatively highly expressed in normal PL in Study 1, *bcl2*, *dcx*, have also been demonstrated at the protein level, further validating those findings. Furthermore, the remaining two of the four genes were new discoveries: *neuroD1* and *tbr1*. In a larger sample and with higher RIN, we would expect additional NM gene transcripts specific to the PL to be revealed. Despite limitations, both Study 1 and 2 highlight that the particular developmental “signature” of the amygdala varies by subregion, a point that needs to be considered in future studies of developmentally vulnerable circuitry.

Finally, we used a hypothesis-driven approach, focusing on genes involved in NM. The findings of this study do not diminish the possibility that other neurobiological processes in the PL are co-occurring in the 3-month animal, or are influenced by early maternal deprivation. In fact, we have previously published that maternal deprivation leads to time-dependent changes in gene expression in other non-NM genes in other subregions of the amygdala (Sabatini et al., 2007). Our future studies will utilize more broad pathway analysis to interrogate other biologic processes associated with these PL-specific findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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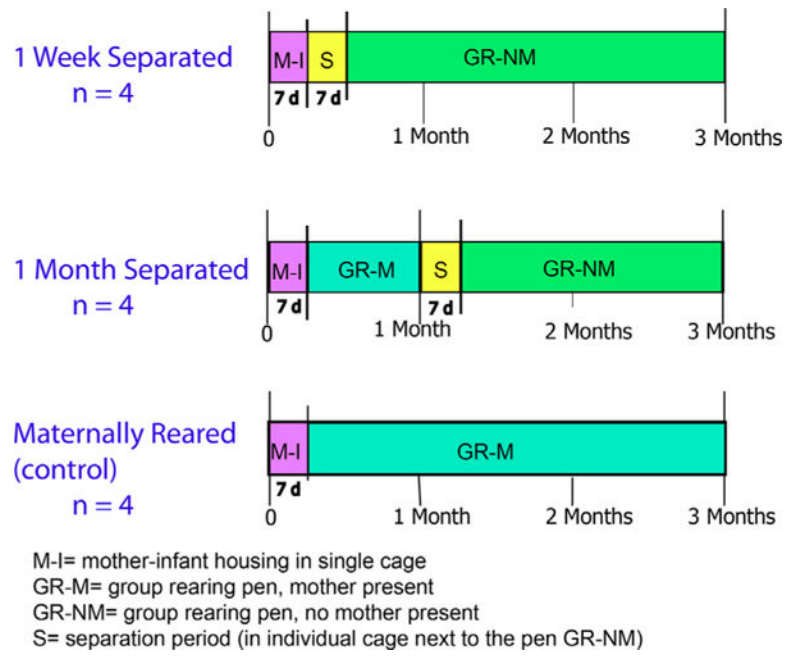
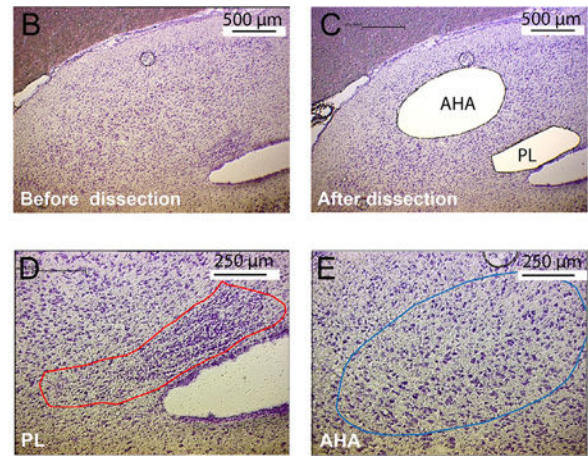
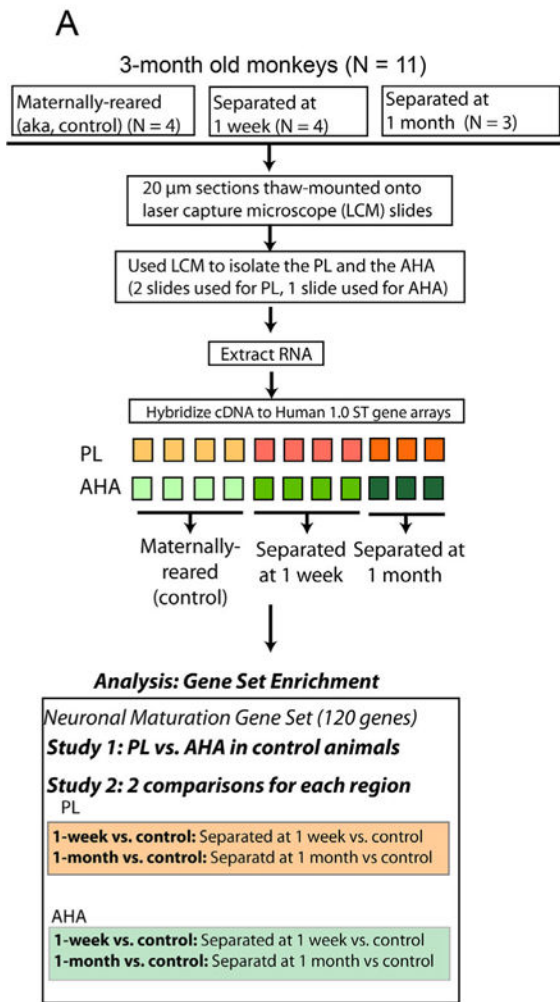


FIGURE 1. Timeline of maternal deprivation paradigm (Sabatini et al., 2007). Tissue was collected at 3 months of age



Study 2: Correlation of gene expression changes

Determine gene expression differences across rearing conditions (Figure 5)

PL

Figure 5A Correlation between ALR values of the most changed genes in 1-week (vs. control) with corresponding ALR in the 1 month (vs. control).

Figure 5B Correlation between ALR values of most changed genes in 1 month (vs. control) with corresponding ALR in 1 week (vs. control).

Determine whether gene expression changes across regions (Figure 6)

Figure 6A Correlation between ALR values of the most changed genes in the PL, 1-week (vs. control) with corresponding ALR in the AHA, 1-week (vs. control).

Figure 6B Correlation between ALR values of most changed genes in PL, 1 month (vs. control) with corresponding ALR in the AHA 1 week (vs. control).

Study 2: Correlation of *tbr1* expression with behavior (Figure 7)

1. Correlation of *tbr1* silver grain count with typical social behaviors (measured at 3 months)
2. Correlation of *tbr1* silver grain count with self-comforting behaviors (measured at 3 months)

FIGURE 2.

Overview of experimental methods. A) Schematic of methods used for this study (tissue from one animal deprived at 1 month was not used, $n = 3$). B and C) Coronal sections of the caudal amygdala, using a rapid thionin stain to identify the PL and AHA on the same section, before B), and after C), LCM. D and E) High-power image showing the PL D), and the AHA E), prior to dissection

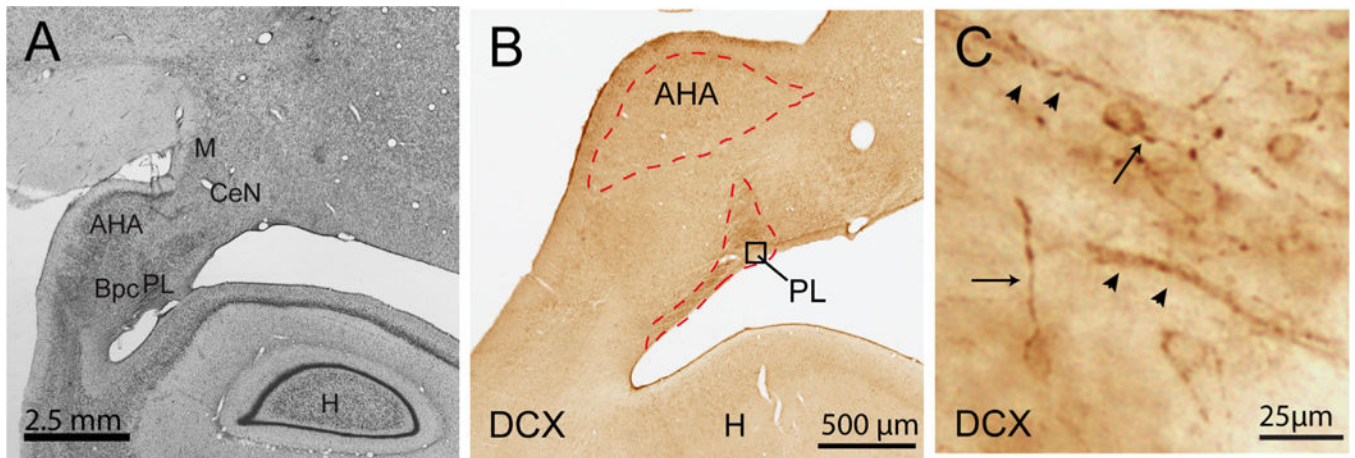
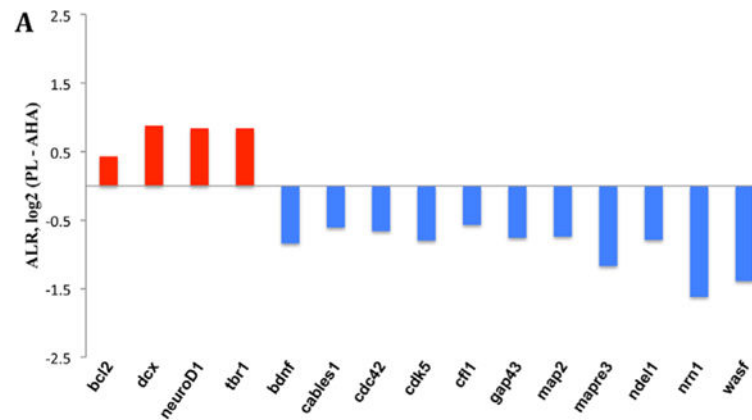


FIGURE 3.

Photomicrographs of the PL and AHA. A) Coronal section of the caudal primate amygdala stained with cresyl violet. Scale bar = 2.5 mm. B) The caudal amygdala, at the level in B) seen at higher power in a section labeled for doublecortin (DCX) immunoreactivity. Laser capture microdissection of the PL and AHA was conducted at this amygdala level. C) Higher power view of DCX positive cells in the PL, boxed region in B). Morphologically, cells have a rounded soma and thin process (arrow). Cells are frequently found in close apposition to fibers of passage (arrowheads). CeN, central nucleus; Bpc, parvocellular subdivision of the basal nucleus; H, hippocampus; M, medial nucleus



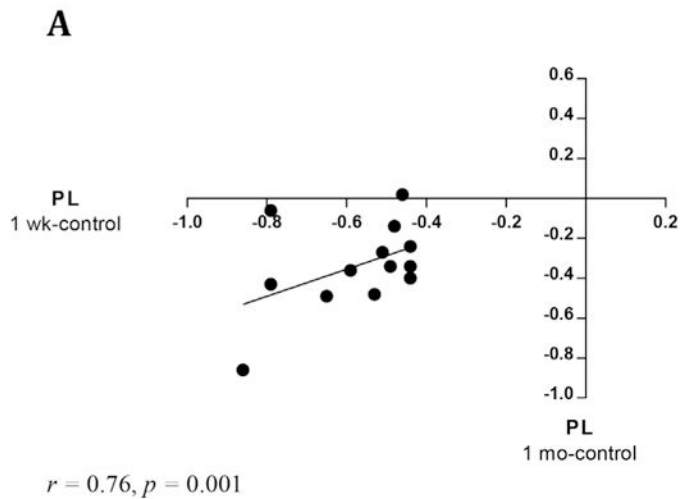
B

Summary of ddC, and Microarray Values for *tbr1*, *dcx*, *neuroD1* for Study 1

Gene	Microarray		qPCR			
	ALR	Fold change	P-value	-ddCT	Fold change	P-value
<i>dcx</i>	0.88	1.84	0.01	2.35	5.10	0.02
<i>neuroD1</i>	0.84	1.79	0.02	3.51	11.40	0.001
<i>tbr1</i>	0.84	1.79	0.01	2.62	6.14	0.01
<i>bcl2</i>	0.43	1.35	0.01	*	*	*

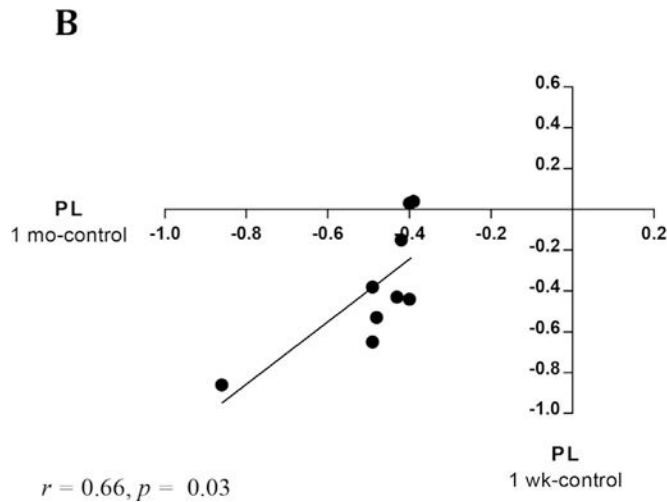
FIGURE 4.

Subsets of differentially expressed genes in the NM gene set differ between the PL and AHA in control animals. A) Most changed genes in the PL versus the AHA based on our two criteria method: *bcl2*, *dcx*, *neuroD1*, *tbr1* (red = increase). Eleven other genes were decreased in the PL relative to the AHA (blue = decreased). B) Microarray and qPCR values for most enriched genes in the PL relative to AHA in normal controls. **bcl2* was not validated by qPCR due to inadequate sample



13 most changed genes in the 1-week separated vs. control comparison with corresponding ALR in the 1-month separated vs. control comparison		
Gene	PL 1-week separated vs. control (13 genes)	PL 1-month separated vs. control
actr2	-0.79	-0.06
apc	-0.53	-0.48
cdkn1b	-0.44	-0.24
dclk1	-0.49	-0.34
dcx	-0.44	-0.34
fyn	-0.59	-0.36
gsk3b	-0.51	-0.27
map2	-0.46	0.02
ncam1	-0.44	-0.40
neurod1	-0.48	-0.14
nrm1	-0.65	-0.49
rap1a	-0.79	-0.43
tbr1*	-0.86	-0.86

*ALR > 0.383 and significance of $p < 0.05$

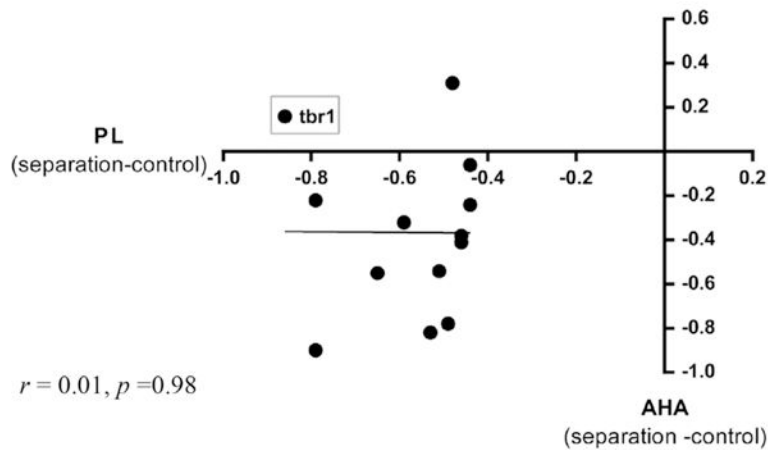


9 most changed genes in the 1-month separated vs. control comparison with corresponding ALR in the 1-week separated vs. control comparison		
Gene	PL 1-month separated vs. control (9 genes)	PL 1-week separated vs. control
abi2	-0.42	-0.15
apc	-0.48	-0.53
dcc*	-0.49	-0.38
efnb2	-0.40	0.03
ncam1	-0.40	-0.44
nrm1	-0.49	-0.65
nrp1	-0.39	0.04
rap1a	-0.43	-0.71
tbr1*	-0.86	-0.86

*ALR > 0.383 and significance of $p < 0.05$

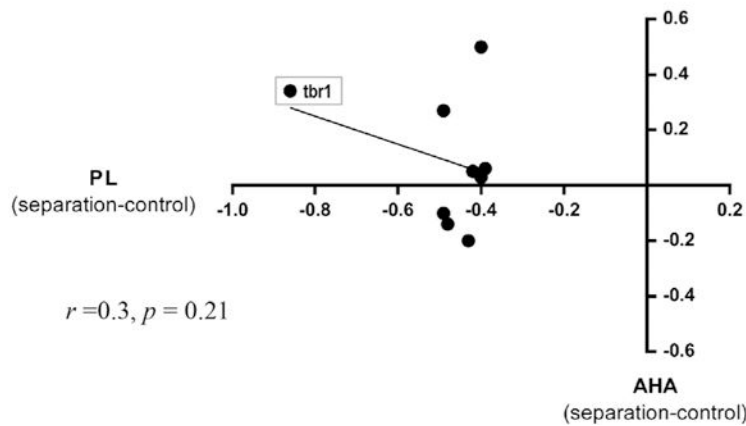
FIGURE 5.

Most changed genes in PL following maternal deprivation, 1-week and 1-month separated subgroups. A) Plot of the most changed genes in the PL in 1-week separated animals (x-axis) versus their corresponding change in the PL of 1-month separated animals (y-axis) ($r = 0.76, p = 0.001$). B) Plot of the most changed genes in the PL in 1-month separated animals (x-axis) versus their corresponding change in the PL of 1-week separated animals ($r = 0.66, p = 0.03$). Tables of the individual genes are to the right of each plot. Gray boxes highlight gene changes common to both 1-week and 1-month separated groups (ALR > 0.383, with same directionality)



1 week-separated	ALR (PL)	ALR (AHA)
actr2	-0.79	-0.22
apc	-0.53	-0.82
cdkn1b	-0.44	-0.24
dclk1	-0.49	-0.78
dcx	-0.44	-0.06
fyn	-0.59	-0.32
gsk3b	-0.51	-0.54
map2	-0.46	-0.41
ncam1	-0.44	-0.40
neurod1	-0.48	0.31
nrn1	-0.65	-0.55
rap1a	-0.79	-0.9
tbr1*	-0.86	0.16

*ALR > 0.383 and significance of $p < 0.05$.



1 month-separated	ALR (PL)	ALR (AHA)
abi2	-0.42	0.05
apc	-0.48	-0.14
dcc*	-0.49	-0.10
efnb2	-0.40	0.5
ncam1	-0.40	0.03
nrn1	-0.49	0.27
nrp1	-0.39	0.06
rap1a	-0.43	-0.2
tbr1*	-0.86	0.34

*ALR > 0.383 and significance of $p < 0.05$.

FIGURE 6.

Most changed gene profiles in the PL versus AHA following maternal deprivation. Plots of the most changed genes ($|\text{ALR}| > 0.383$) in the PL (x-axis) versus their change in the AHA (y-axis). A). One-week versus control comparison ($r = -0.011, p = 0.98$). B) One-month versus control comparison ($r = -0.3, p = 0.21$). Gray boxes on graphs highlight the position of *tbr1* for each comparison. Tables of the individual genes are to the right of each plot

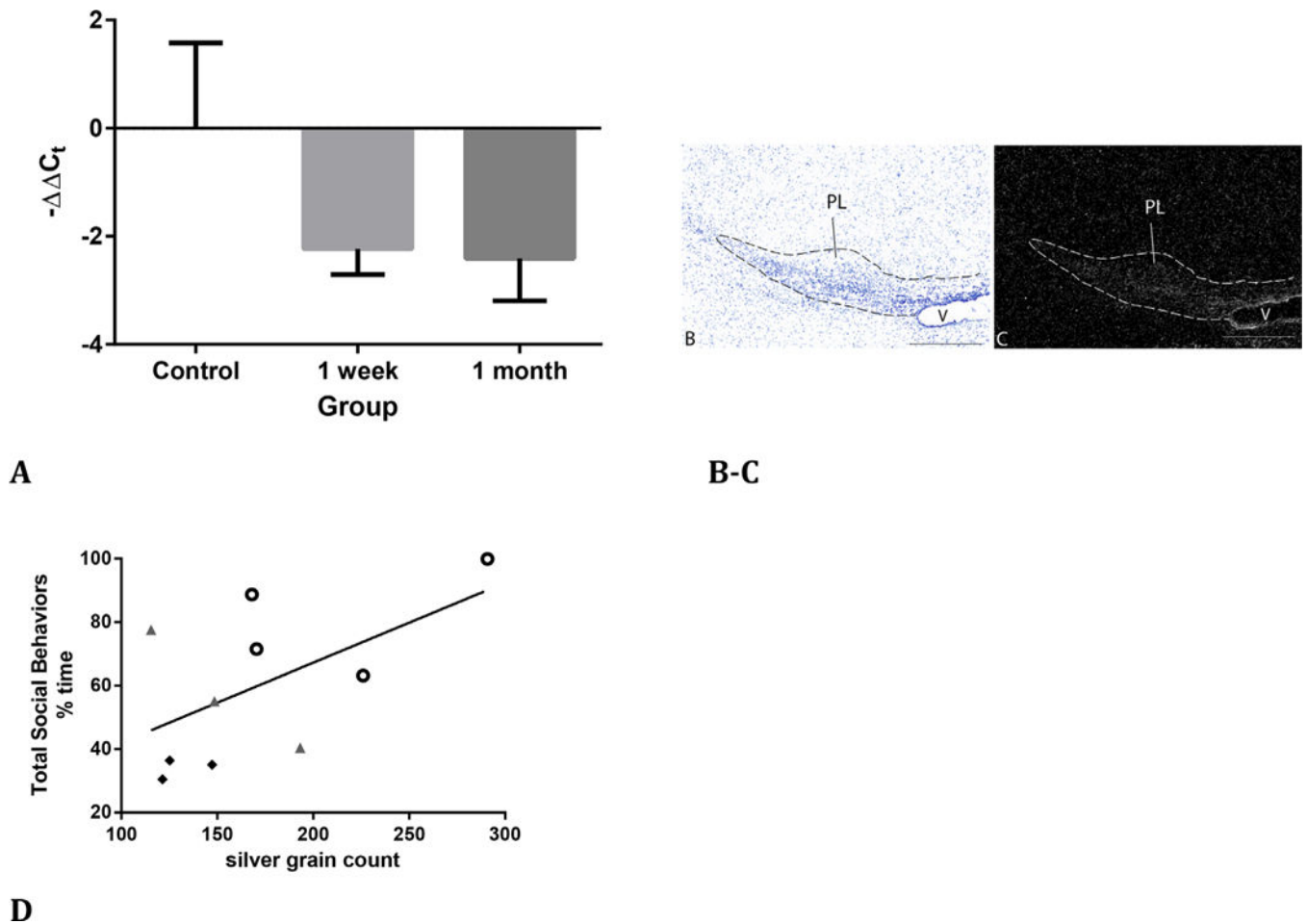


FIGURE 7. *tbr1* mRNA and correlations with social behaviors at 3 months of age. A) qPCR of *tbr1* transcript fold-changes in maternally deprived animals; 1-week separated versus control, $p = 0.04$ and, 1-month separated versus control, $p = 0.03$. B and C) Adjacent sections near the LCM site stained for cresyl violet, B) and processed for *tbr1* mRNA. C) Dotted line indicates the PL. V, ventricle. Bar = 500 μm . D) Correlation between total social behaviors at 3 months and silver grain count (corrected for background) in the PL for the *tbr1* probe across all animals. Black filled, diamonds 1-week separated monkeys; gray filled triangles, 1-month separated monkeys; open circles, maternally reared monkeys ($r = 0.56$, $p = 0.045$)

TABLE 1

Summary table of the main microarray findings that met dual-criteria for gene expression changes.

<u>Study 1</u>	<u>Study 2</u>	
Maternally reared (control) PL versus AHA	Maternal deprivation (1 week and 1 month combined) versus control PL	AHA
<i>neurod1</i> ↑		
<i>dcx</i> ↑		
<i>bcl2</i> ↑		
<i>tbr1</i> ↑	<i>tbr1</i> ↓	<i>cables 1</i> ↓

Summary of microarray data meeting dual criteria for gene change ($|\text{ALR}| > 0.383$ and $p < 0.05$).

Gene expression changes shown, except *cables 1* and *bcl2*, were additionally verified by qPCR.