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Effects of maternal care on the development of midbrain dopamine pathways and reward-directed behavior in female offspring

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Abstract

Variation within mesolimbic dopamine (DA) pathways has significant implications for behavioral responses to rewards, and previous studies have indicated long-term programming effects of early life stress on these pathways. In the current study, we examined the impact of natural variations in maternal care in Long Evans rats on the development of DA pathways in female offspring and the consequences for reward-directed behaviors. We found that tyrosine hydroxylase (TH) immunoreactivity in the ventral tegmental area was elevated by postnatal day 6 in response to maternal licking/grooming (LG), and that these effects were sustained into adulthood. Increased TH immunoreactivity was not found to be associated with altered epigenetic regulation or transcriptional activation of *Th*, but probably involved LG-associated changes in the differentiation of postnatal DA neurons through increased expression of Cdkn1c, and enhanced survival of DA projections through LG-associated increases in Lmx1b and brain-derived neurotrophic factor. At weaning, high-LG offspring had elevated DA receptor mRNA levels within the nucleus accumbens and increased conditioned place preference for a high-fat diet. In contrast, high-LG, as compared with low-LG, juvenile offspring had a reduced preference for social interactions with siblings, and haloperidol administration abolished group differences in conditioned place preference through a shift towards increased social preferences in high-LG offspring. The effects of maternal care on developing DA pathways and reward-directed behavior of female offspring that we have observed may play a critical role in the behavioral transmission of maternal LG from mother to daughter, and account for individual differences in the mesolimbic DA system.

Introduction

Variation in maternal care experienced during development profoundly impacts on the rodent brain, altering stress reactivity, cognition, and social/reproductive behavior (Meaney, 2001). In rats, maternal licking/grooming (LG) received in infancy predicts the LG behavior of females towards their own offspring, leading to the intergenerational transmission of maternal behavior (Francis *et al.*, 1999; Champagne *et al.*, 2003a). Although mechanistic studies on this transmission have focused on LG-induced hypothalamic estrogen–oxytocin receptor levels (Champagne *et al.*, 2001, 2003b, 2006; Pena *et al.*, 2013), maternal behavior is a motivated behavior, regulated by dopamine (DA) signaling from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Gaffori & Le Moal, 1979; Hansen *et al.*, 1991, 1993; Numan & Stolzenberg, 2009). Variation in LG has been linked to the mesolimbic DA system, with elevated DA release during LG and increased DA receptor levels in the NAc among high-LG dams (Champagne *et al.*, 2004). We therefore hypothesised that maternal LG would predict variation within the mesolimbic DA system of female offspring and contribute to the transmission of maternal care from mother to daughter.

In rodents, the mesolimbic DA system is not fully mature until the third postnatal week (Voorn *et al.*, 1988), and development of mesolimbic DA pathways may therefore be sensitive to the influence of early life experiences. Previous studies have demonstrated the impact on DA function of stressful experiences occurring during the prenatal and postnatal periods (Alonso *et al.*, 1994; Ortiz *et al.*, 1996; Barros *et al.*, 2004; Jahng *et al.*, 2010; Huppertz-Kessler *et al.*, 2012). Manipulation of the quality of mother–infant interactions likewise impacts on DA system development and reward-directed behaviors. Postnatal maternal deprivation in rats leads to increased basal DA levels in the NAc and stimulus-dependent variation in DA release (Afonso *et al.*, 2011). Exposure of neonatal rats to an unstable maternal environment induces a reduction in preference for palatable food stimuli (Ventura *et al.*, 2012). These findings suggest that long-term neurobiological and behavioral outcomes of early life adversity are associated with the midbrain DA system.

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The aim of the current study was to determine the impact of maternal LG on the development of mesolimbic DA pathways in female offspring. Our initial analyses indicated increased numbers of DA neurons within the VTA associated with the experience of high LG. We hypothesised that these long-lasting alterations would be organised and maintained by transcription factors critical to midbrain DA neuron differentiation, survival, and maintenance, and/or by epigenetic gene regulation. Thus, we assessed the time-course of LG-associated alterations in mRNA levels of genes implicated in these processes (*Nurr1*/*Nr4a2*, *Cdkn1c*/*p57^{kip2}*, *Lmx1b*, *Pitx3*, and *Bdnf*), and LG-associated effects on DNA methylation within a regulatory region of the tyrosine hydroxylase (TH) gene. We also examined the levels of DA receptor mRNA within the NAc, and the implications of these LG-associated changes for reward-directed behaviors. Our findings suggest that postnatal LG has programming effects within the midbrain DA system that may have implications for the expression of maternal behavior and other reward-associated responses.

Materials and methods

Animals

Long Evans rats (Charles River Laboratories) were maintained on a 12-h light–dark schedule (lights on at 08:00 h) with food and water provided *ad libitum*. Adult virgin females (aged 60–90 days) were mated for 1 week, and singly housed 1–2 days prior to parturition. At weaning [postnatal day (P) 21], female offspring were pair-housed. All procedures were performed in accordance with NIH guidelines, and with the approval of the Institutional Animal Care and Use Committee at Columbia University.

Maternal behavior

Home cage maternal behavior was scored as previously described (Champagne *et al.*, 2003a). Maternal behavior was observed for five 60-min observation periods daily during P1–6. Behavioral observations were made every 3 min during each observation period. The frequency of LG behavior was calculated as the number of observations of LG divided by the total number of observations. Low-LG and high-LG dams were defined as engaging in LG frequencies that were either one standard deviation below (low LG) or above (high LG) the mean LG of the cohort. Unique cohorts of litters (median dams bred per cohort, 40) contributed to each experiment, with the exception that mRNA and DNA methylation levels were examined in the same pups.

Immunohistochemistry

At P6, offspring (low LG, five pups from five litters; high LG, six ups from five litters) were decapitated, and whole brains were placed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 48 h of fixing (2 h at room temperature and then at 4 °C). Adult females were swabbed for estrous state prior to being terminally anesthetised and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were then post-fixed overnight in 4% paraformaldehyde at 4 °C, cryoprotected in 30% sucrose at 4 °C until isotonic, and stored at −80 °C. Coronal sections (40 μm) were sliced on a cryostat and floated on PBS. Sections including the ventral midbrain were washed in PBS, and blocked with normal serum before being incubated in primary antibody (rabbit anti-TH, 1 : 10 000; Santa Cruz Biotechnology) at 4 °C overnight. Sections were then washed in PBS, incubated in secondary antibody [for P6 females, Cy2-conjugated donkey anti-rabbit, 1 : 2000 (Santa Cruz Biotechnology); for adult females, biotinylated goat anti-rabbit (Vectastain); followed by avidin–biotin–peroxidase complex and horseradish peroxidase visualisation (Vector SG)], washed, and mounted on gelatin-coated slides. Slides were dehydrated in a series of ethanol washes before clearing with xylene and coverslipping. Slides were stored in a light-proof box at 4 °C before and after imaging.

Imaging and cell counting

Slides were imaged on an Olympus light microscope fitted with fluorescent filters. Six to eight sections along the anterior–posterior axis of the ventral midbrain were imaged at ×10 and analysed for each animal. Cell counts (immunoreactive cells) were performed with MCID CORE software (InterFocus Imaging, UK), based on a threshold set to include all cells with moderate to high immunoreactivity. An observer blind to condition outlined each region of interest. Nucleus-specific analysis was performed for adult sections, and included the following regions, as determined from Paxinos & Watson (2005) (Fig. S1A): rostral VTA [VTAR; parafasciculus retroflexus area (Ikemoto, 2007)], parabrachial pigmented nucleus of the VTA (PBP), paranigral nucleus of the VTA, parainterfascicular nucleus of the VTA, rostral linear nucleus of the raphe, caudal linear nucleus of the raphe, interfascicular nucleus, substantia nigra (SN) pars compacta dorsal tier, SN pars compacta medial tier, SN pars compacta ventral tier, SN pars compacta lateral part (SNL), and SN pars compacta reticular part. Statistical analyses were performed on an individual animal's average count across sections.

Tissue collection and RNA/DNA extraction

Female offspring of low-LG and high-LG dams were killed by decapitation at the day of birth (P0), at P6, at P21 at weaning, or as young adults at P66 (six or seven animals per group per age). Dissected brain tissue samples for gene expression and epigenetic analysis were obtained from female siblings sampled at multiple developmental time-points (i.e. one female per litter per time-point). Whole brains were removed, and immediately snap-frozen and stored at −80 °C. The ventral midbrain containing the VTA and SN (bregma −4.68 to −6.48), and the ventral striatum containing the NAc core and shell (bregma 2.28 to 0.72), were carefully dissected from one half of each brain in a cryostat cooled to −20 °C, and used for RNA and DNA extraction. Samples were homogenised in 700 μL of RLTplus lysis buffer (Qiagen) with 1% β-mercaptoethanol by use of a tissue homogeniser (Omni) for 15 s. RNA and DNA were extracted with a dual RNA–DNA extraction kit (Qiagen). cDNA was created from RNA with a reverse transcription kit (Applied Biosystems).

Gene expression

Relative gene expression was measured with real-time semi-quantitative polymerase chain reaction (PCR) on a 96-well 7500Fast qPCR machine (Applied Biosystems) with SybrFast. All primers were designed to span exons, and had 87–110% efficiency in a standard curve, with single melt peaks. Primer pairs are given in Table S1. Calculations of relative gene expression were conducted with the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008) with cyclophilin-A (*CypA*) and β-actin (*ActB*) as control genes, and normalisation to P0 low-LG offspring.

Bisulfite conversion and pyrosequencing

DNA methylation analysis was conducted with DNA extracted from the ventral midbrain (six to eight animals per group). DNA (1 μg) purified from the VTA was bisulfite-converted and cleaned with the EpiTect Bisulfite Kit (Qiagen). The *Th* promoter region was amplified by use of Pyromark PCR (Sigma) with a biotinylated reverse primer. This regulatory region contained 10 CpG sites (−269 to −94), and has been implicated in epigenetic regulation of *Th* in cell culture (He *et al.*, 2011). A portion of the bisulfite-converted DNA from each sample was run on a gel to ensure specificity. Samples were then pyrosequenced on a 24-well pyrosequencer (Qiagen) with two sets of sequencing primers (Table S1). All pyrosequencing primers were designed with PYROMARK software (Qiagen). Percentage methylation was determined from the C/T ratio at CpG sites in the sequence. Only samples that passed internal positive control standards were included in the analysis.

Conditioned place preference (CPP) behavior

In order to evaluate preference for naturally rewarding stimuli, animals were tested for CPP for a target stimulus and a control stimulus. Testing took place over 8–9 consecutive days, and included habituation, conditioning, and preference testing (Table S2). Testing of adult lactating dams began on postpartum day 1 (day of birth considered to be day 0; maternal observations continued for P1–6 as described). LG frequency had yet to be established during CPP with lactating dams, and so low-LG (n = 6), mid-LG (n = 7) and high-LG (n = 5) dams were included. Pups were kept in the home cage in the colony room under a heat lamp while dams were in the apparatus, except when present for conditioning, and were not used for subsequent testing. Testing of offspring [10–12 per group; independent cohorts used for high-fat diet (HFD) and sibling preference testing] began on P24, so that all offspring would complete testing prior to the onset of puberty. Up to two pups per litter were used, so that five to seven litters contributed pups to each group.

Apparatus

The CPP apparatus used was a three-chamber plastic box with three equally sized (20 × 20 cm) chambers separated by small openings through which subjects could move freely between chambers. The outside two chambers were decorated with distinct contextual cues that the animals could distinguish, and the center chamber had plain black walls. Fresh bedding was placed on the floor of each chamber, and the chambers were thoroughly cleaned between animals.

Habituation and exploration

In order to allow habituation to the CPP chamber and to identify any pre-existing chamber bias, animals were allowed to freely explore the entire apparatus for 30 min on day 1 of testing. Movements were recorded with ANY-maze (Stoelting), and chamber bias was assessed (defined as spending > 50% of the time in one of the two outside chambers). A chamber bias was rarely observed. In cases where there was a bias, the control stimulus was placed in the preferred chamber. In the absence of bias, the stimuli were randomly placed in either chamber.

Place conditioning of lactating dams for pups vs. familiar toy

To assess the CPP methodology using reward vs. control stimuli, which we hypothesised would significantly distinguish females, we first implemented this protocol with low-LG vs. high-LG lactating dams. The preference of postpartum rat dams for neonatal pups (< P8) is well established (Mattson *et al.*, 2001; Wansaw *et al.*, 2008), and, on the basis of characterised differences in the mesolimbic DA system and its activity during LG behavior (increased DA release in high-LG dams), we predicted that high-LG lactating females would show a stronger preference for a pup-associated chamber (Champagne *et al.*, 2004; Shahrokh *et al.*, 2010). Each dam's own litter was used as the reward stimulus, so that all pups were < P8 during conditioning. The control stimulus was a hollow plastic tube (familiar toy) that had been placed in the home cage at the time of birth of the litter. Dams were conditioned to associate one chamber with pups and the second chamber with the familiar toy (Table S2). During the 60-min conditioning trials, access to other chambers was blocked. The order of conditioning (pups first vs. familiar toy first) was counterbalanced.

Place conditioning of offspring for HFD diet vs. chow, or for sibling vs. familiar toy

In order to evaluate the effect of maternal LG on offspring preference for natural rewards during the juvenile period, CPP for two different pairs of stimuli was assessed: (i) HDF (reward) vs. standard chow (control); or (ii) a sibling/cagemate (reward) vs. a familiar toy (control). Conditioning took place over 6 days, during which offspring were conditioned to associate one side of the chamber with the reward stimulus and the other side of the chamber with the control stimulus. HFD is a palatable natural reward, and preference for an HFD is mediated by activation of NAc DA receptors (Baker *et al.*, 2001; Zhang *et al.*, 2003; Teegarden & Bale, 2007; Teegarden *et al.*, 2009). To ensure that animals would explore and eat the novel HFD, all animals were exposed to the HFD (D12492; Research Diets) in their home cage 2 days prior to habituation. Two pellets were introduced into the home cage and checked 4 h later to examine consumption. All animals consumed some if not all of the HFD pellets. Four pellets of HFD or chow were placed on the bottom of the assigned chamber during each conditioning session. CPP testing for sibling/cagemate vs. familiar toy was conducted in a separate cohort of offspring (i.e. offspring not previously tested for CPP). Like a preference for HFD, social play is regulated by the mesolimbic DA system, and has previously been found to be naturally rewarding among juveniles (Einon *et al.*, 1981; Thor & Holloway, 1984; Calcagnetti & Schechter, 1992; Trezza *et al.*, 2010; El Rawas *et al.*, 2012; Peartree *et al.*, 2012). Social play increases at around P18, and reaches a peak at around P30–40 during the juvenile period (Panksepp, 1981; Spear & Brake, 1983). In the sibling/cagemate conditioning, the social partner was a sibling pair-housed with the test subject at the time of weaning (P21). The familiar toy (control) was a hollow plastic tube that had been present in the home cage since the time of weaning. During conditioning, the animal was restricted to one side of the chamber (containing the stimulus) for 60 min. The stimulus that was presented first during training was counterbalanced (Table S2).

Preference testing

On the eighth day of chamber exposure, animals were tested for chamber preference. During the preference test (60 min in duration), animals were permitted to explore the entire apparatus in the absence of any stimuli, and movements were recorded with ANY-maze. A preference score was calculated as the time spent in the reward-associated chamber divided by the time spent in both outer

chambers together: preference score = $(T_{reward})/(T_{reward} + T_{control})$. This interpretation of preference (which is a construct that can be defined by the use of multiple methodological approaches) takes into account activity in both conditioned chambers. In order to evaluate whether D2-like receptors mediated preference in the sibling vs. familiar toy condition, chamber preference was evaluated on the ninth day, following an injection of either dimethylsulfoxide (vehicle) or the D2 antagonist haloperidol (0.3 mg/kg, intraperitoneal; Sigma) (Beatty *et al.*, 1982; Stern & Taylor, 1991), 20 min prior to testing.

Statistics

All statistical analyses were performed with SPSS (version 18.0; PASWS, IBM). A two-tailed Student's *t*-test was used for single comparisons among high-LG and low-LG animals. Main effects were determined with ANOVA, with independent and dependent variables as noted. Repeated-measures ANOVA was used to determine effects, with multiple time-points being sampled (behavior and gene expression) where described. For experiments in which up to two pups were used from the same litter, significant group differences were verified with ANOVA, with litter as a covariate. All significance thresholds were set at $P < 0.05$.

Results

Postnatal maternal behavior

In all cohorts, no differences were found in average number of pups per litter or male/female pup ratio among low-LG and high-LG dams ($P > 0.6$). LG frequency was significantly reduced in low-LG compared to high-LG dams during the postpartum period (representative cohort with litters used for gene expression and methylation analysis: $t_{1,13} = 8.23$, $P < 0.001$). Repeated-measures analysis with postpartum day as a within-subject factor and maternal LG as a between-subject factor indicated a significant effect of day ($F_{5,65} = 6.54$, $P < 0.001$) and a significant effect of maternal LG ($F_{1,13} = 50.74$, $P < 0.001$), but not a significant interaction between the two ($P > 0.21$). Consistent with previous studies (Jensen Peña & Champagne, 2013), LG decreased across postpartum days in both low-LG and high-LG dams, and group differences in LG were apparent across P1–6.

Maternal behavior influences TH immunoreactivity in the VTA

TH is an enzyme that catalyzes the conversion of tyrosine to the DA precursor L-DOPA, and serves as a marker of DA neuron cell bodies. In the VTA of P6 offspring, there was a significant effect of maternal LG on the average number of cells expressing TH ($t_{1,9} = 3.13$, $P < 0.05$; Fig. 1A) and on cell density ($t_{1,9} = 2.90$, $P < 0.05$), such that high-LG offspring had elevated levels of TH-immunoreactive cells as compared with low-LG offspring. This effect was not statistically significant within the SN (Fig. 1B). No significant differences were found in the size of the VTA or SN ($P > 0.36$) at P6. In adult offspring, estrous cycle state was used as a covariate in the analysis. There was a significant effect of maternal LG on TH immunoreactivity in the VTA ($F_{1,11} = 11.32$, $P < 0.01$; Fig. 1A), such that high-LG adult offspring had an elevated number of TH-immunoreactive cells as compared with low-LG adult offspring. Similarly to the findings in P6 offspring, there was no significant effect of maternal LG on TH immunoreactivity in the SN (Fig. 1B). To determine the anatomical localisation of the effects of maternal LG in the adult brain, we assessed TH immunoreactivity in specific nuclei within the VTA and SN (Fig. S1). The number of TH-immunoreactive cells was significantly greater in high-LG compared to low-LG adult offspring in the PBP ($F_{1,11} = 9.73$, $P < 0.01$), VTAR ($F_{1,11} = 12.75$, $P < 0.01$), and SNL ($F_{1,11} = 7.12$, $P < 0.05$); no significant effects were found in any other region (Fig. S1B).

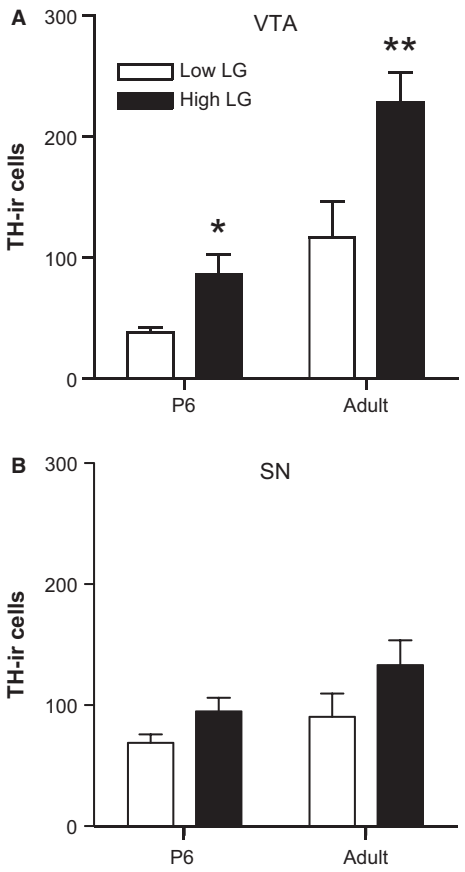

FIG. 1. TH-immunoreactive (TH-ir) cells in the VTA and SN of P6 and adult offspring. Number of cells (mean ± standard error of the mean) expressing TH counted in the (A) VTA and (B) SN of low-LG and high-LG offspring. *$P < 0.05$, **$P < 0.01$.

Maternal LG alters the expression of genes critical for midbrain DA neuron differentiation and maintenance

The differential TH cell counts within the VTA associated with variation in postnatal maternal care suggest that developing DA pathways are shaped by maternal LG. To examine the potential mechanisms of this effect, we determined the expression during postnatal development (P0, P6, P21, and P66) of transcription factors that regulate the maturation of the DA system, including Nurr1 (Nr4a2), Cdkn1c, Lmx1b, Pitx3, and brain-derived neurotrophic factor (BNDF). Nurr1 and Cdkn1c interact in DA neuron differentiation (Joseph *et al.*, 2003), Lmx1b and Pitx3 are involved in DA maintenance and survival (Smidt *et al.*, 2000), and BDNF is a secreted neurotrophic factor that is additionally involved in reward and motivation (Nestler & Carlezon, 2006). We found a main effect of age on *Nurr1* expression ($F_{3,53} = 39.24$, $P < 0.001$; Fig. 2A), with relative mRNA levels decreasing with age, but no significant effect of maternal LG ($F_{1,53} = 2.21$, $P = 0.14$). *Cdkn1c* expression likewise decreased with increasing age, with a trend for a main effect of

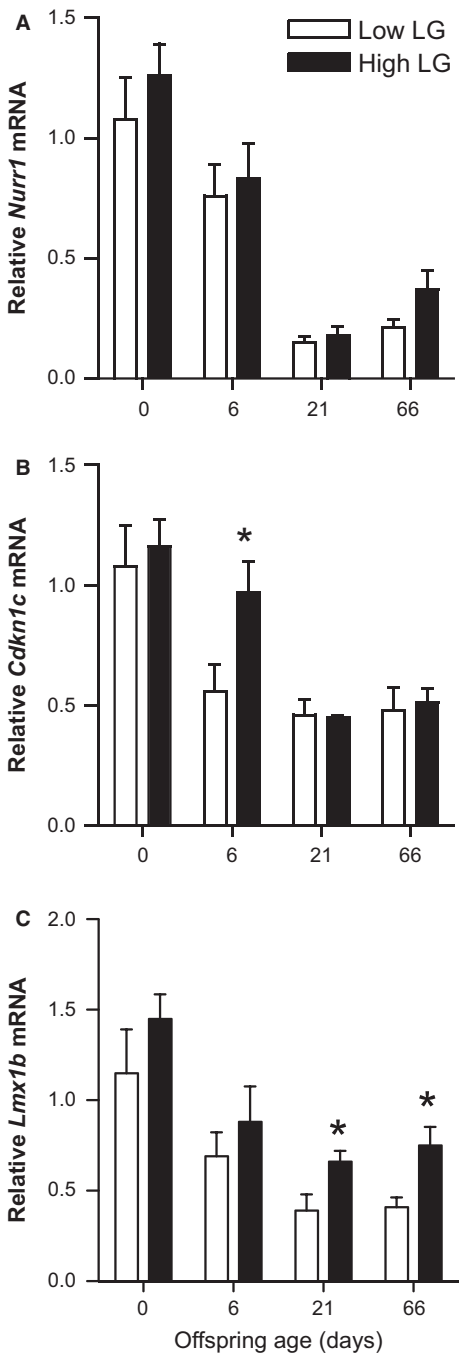

FIG. 2. Offspring gene expression in the ventral midbrain. Relative mRNA expression (mean ± standard error of the mean) of (A) *Nurr1*, (B) *Cdkn1c*, and (C) *Lmx1b*, as determined with semi-quantitative real-time PCR, in low-LG and high-LG offspring across postnatal development. *$P < 0.0$.

maternal LG ($F_{1,53} = 2.95$, $P = 0.09$; Fig. 2B). Further analysis revealed a significant difference in *Cdkn1c* expression at P6 ($t_{1,11} = 2.42$, $P < 0.05$), such that low-LG offspring expressed lower levels of *Cdkn1c* than high-LG offspring. We found an effect of age ($F_{3,53} = 14.10$, $P < 0.001$) and maternal LG ($F_{1,53} = 8.46$, $P < 0.01$; Fig. 2C) on *Lmx1b* expression. *Lmx1b* mRNA was elevated among high-LG as compared with low-LG offspring at P21 ($t_{1,12} = 2.45$, $P < 0.05$) and P66 ($t_{1,12} = 3.00$, $P < 0.05$). There was a main effect of age ($F_{3,50} = 3.98$, $P < 0.05$; Fig. S2A) on relative *Pitx3* mRNA levels, and an effect of maternal LG ($F_{1,53} = 7.75$, $P < 0.01$; Fig. S2B) but not of age ($F_{3,53} = 2.09$, $P = 0.12$) on relative VTA *Bdnf* expression, primarily because of elevations in BDNF mRNA in adult high-LG offspring ($P < 0.05$).

Maternal LG affects DA receptor gene expression in the NAc during development

DA neurons of the VTA involved in reward and motivated behaviors project to the ventral striatum (Björklund & Dunnett, 2007). To determine whether maternal LG also influenced development of the mesolimbic DA system at the level of DA receptors, we analysed relative mRNA levels of the dopamine receptors D1, D2, and D3, as well as the BDNF receptor (TrkB), in the ventral striatum. There was a main effect of age on levels of the three DA receptors examined ($F_{3,52} > 15.47$, $P < 0.001$; Fig. 3A–C). Analysis of *Drd1* expression indicated an interaction between age and maternal LG ($F_{3,52} = 2.73$, $P = 0.06$), primarily because of significantly increased *Drd1* mRNA levels in high-LG offspring at P21 ($t_{1,12} = 2.51$, $P < 0.05$; Fig. 3A). A similar pattern of findings was evident for *Drd2* (age × maternal LG interaction: $F_{3,52} = 3.18$, $P < 0.05$; Fig. 3B) and *Drd3* (age × maternal LG interaction: $F_{3,52} = 3.56$, $P < 0.05$; Fig. 3B), with elevations in *Drd2* and *Drd3* expression at P21 in high-LG offspring ($P < 0.05$). Analyses of Trkb expression in the NAc across development indicated a significant effect of age ($F_{3,52} = 9.58$, $P < 0.001$) but not of maternal LG ($F_{3,52} = 2.35$, $P = 0.08$; Fig. S3).

Th promoter DNA methylation across development

To further explore the mechanism through which maternal LG shapes the development of DA pathways (as indicated by the increased number of TH-immunoreactive cells in the VTA in high-LG offspring), we determined whether postnatal LG was associated with variation in epigenetic programming of *Th* expression through DNA methylation of the *Th* promoter. Analyses of *Th* mRNA indicated a main effect of age ($F_{3,53} = 26.46$, $P < 0.001$; Fig. 4A) but no significant effect of maternal LG ($P > 0.14$) on expression of the enzyme. Analysis of a region in the *Th* promoter containing 10 CpG sites (−269 to −94) revealed a consistent pattern of DNA methylation across postnatal development at P0, P6, and P21 (Fig. 4C). CpG9 was 100% methylated in nearly all samples at all ages. There was a significant main effect of age on average DNA methylation collapsed across the 10 CpG sites ($F_{2,36} = 18.08$, $P < 0.001$; Fig. 4B), such that DNA methylation increased with age. This analysis did not reveal a significant main effect of maternal LG on CpG methylation of the *Th* promoter region examined ($P > 0.8$).

CPP in low-LG vs. high-LG lactating dams

Consistent with our predictions, the LG status of lactating dams predicted CPP for pups vs. a familiar toy. We found a significant effect of dam LG status on time spent in the pup-associated chamber ($F_{2,15} = 3.75$, $P < 0.05$; Fig. S4A) and on time spent in the toy-associated chamber ($F_{2,15} = 5.56$, $P < 0.05$), but not on time spent in the center chamber ($P > 0.21$), such that high-LG dams spent significantly more time in the pup-associated chamber than low-LG dams ($P < 0.05$), and significantly less time in the toy-associated chamber than mid-LG and low-LG dams ($P < 0.01$). There was also a significant effect of dam LG status on latency to enter the pup-associated chamber ($F_{2,15} = 5.12$, $P < 0.05$), such that low-LG dams were significantly slower [31.1 ± 6.9 s (standard error of the mean), $P < 0.05$] to enter the pup-associated chamber than mid-LG (9.0 ± 5.1 s) and high-LG (6.3 ± 5.8 s) dams. There was no effect

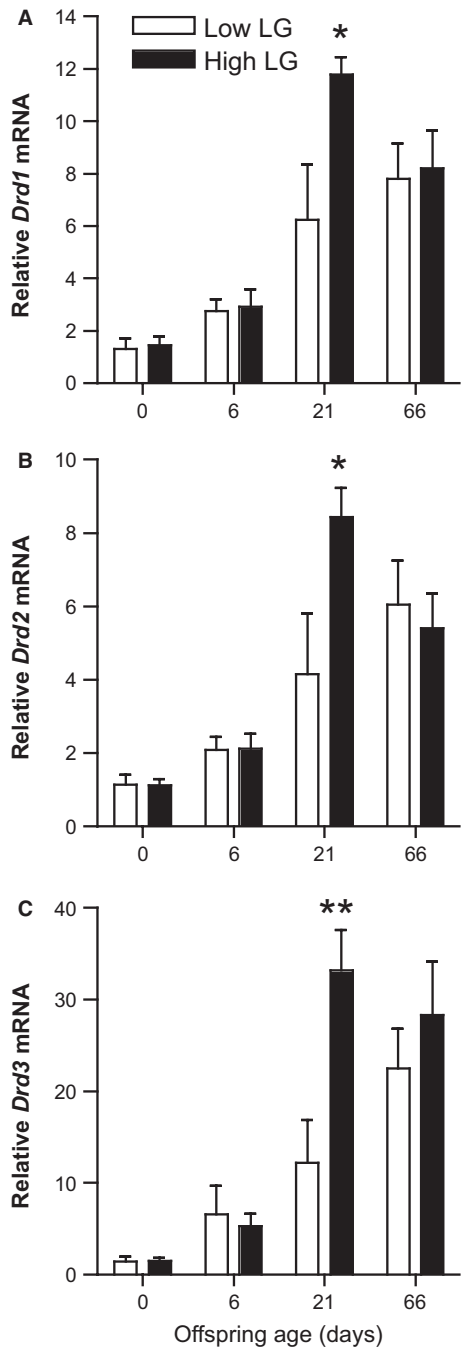

FIG. 3. Offspring gene expression in the NAc. Relative mRNA expression (mean ± standard error of the mean) of (A) *Drd1*, (B) *Drd2*, and (C) *Drd3*, as determined with semi-quantitative real-time PCR, in low-LG and high-LG offspring across postnatal development. $*P < 0.05$, $**P < 0.01$.

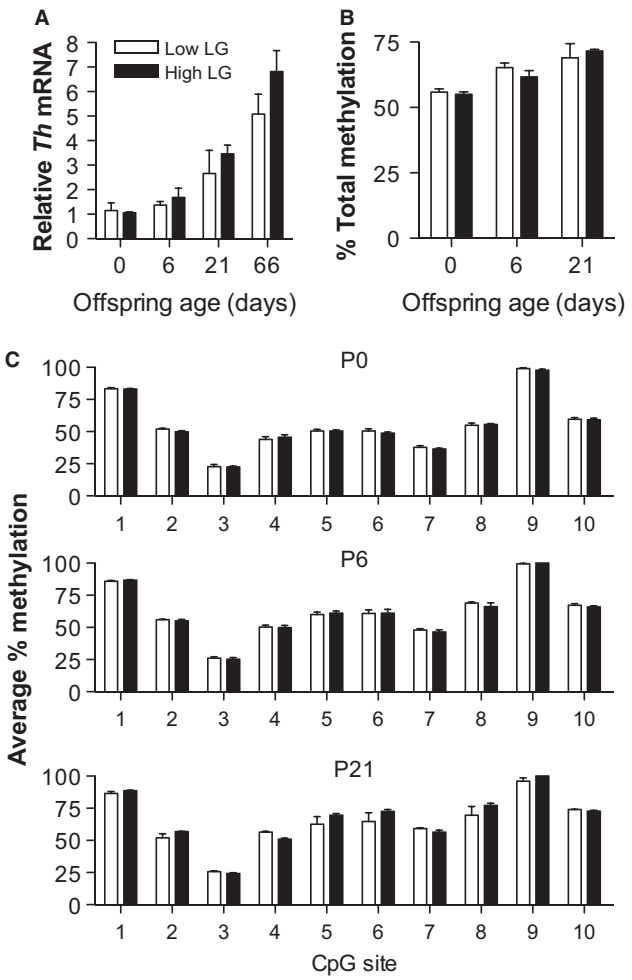

FIG. 4. *Th* expression and DNA methylation within the *Th* promoter of low-LG and high-LG offspring. (A) Relative mRNA expression and (B) average percent CpG methylation (mean ± standard error of the mean) of *Th* at P0, P6, P21, and P66 (mRNA only). (C) Percentage DNA methylation (mean ± standard error of the mean) at individual CpG sites in low-LG and high-LG offspring at each developmental stage.

of LG status on total distance traveled during the 1-h test ($P > 0.75$). Consistent with this pattern of chamber exploration, we found a main effect of LG status on preference score ($F_{2,15} = 7.19$, $P < 0.01$; Fig. S4B), such that high-LG dams had greater preference for pups than both low-LG and mid-LG dams ($P < 0.05$).

Maternal LG affects offspring CPP for rewards

To determine the functional impact of maternal LG-associated changes in the mesolimbic DA system on reward/motivated behavior, low-LG and high-LG offspring were tested for CPP for: (i) HFD; and (ii) social interaction and play with a sibling/cagemate.

HFD vs. standard chow CPP

Following conditioning, high-LG offspring spent a higher percentage of time in the HFD-paired chamber than low-LG offspring ($t_{1,22} = 3.14$, $P < 0.01$; Fig. 5A), whereas low-LG offspring spent a higher percentage of time exploring the chow-paired chamber ($t_{1,22} = 3.48$, $P < 0.01$). Group differences in percentage of time exploring the center chamber were not observed ($P = 0.75$). High-LG offspring were found to have a significantly greater HFD preference score than low-LG offspring ($t_{1,22} = 3.65$, $P < 0.001$; Fig. 5B). Low-LG and high-LG offspring did not differ in their latencies to enter either the HFD-paired or chow-paired chamber ($P > 0.45$) or in the total distance traveled ($P > 0.26$).

Sibling/cagemate vs. toy CPP

High-LG offspring were found to spend a higher percentage of time in the toy-paired chamber than low-LG offspring ($t_{1,18} = 2.69$, $P < 0.05$; Fig. 6A), whereas there was a trend for low-LG offspring

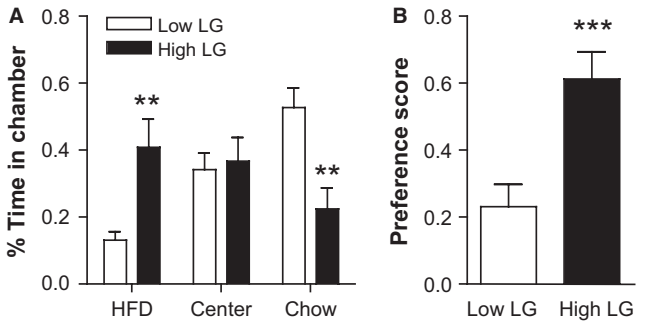

FIG. 5. Conditioned place preference for an HFD vs. standard chow in low-LG and high-LG offspring. (A) Percentage of time (mean ± standard error of the mean) spent in each chamber (HFD-associated, center, and chow-associated) by low-LG and high-LG offspring. (B) Preference scores [$T_{HFD}/(T_{HFD} + T_{chow})$] of low-LG and high-LG offspring. $^{**}P < 0.01$, $^{***}P < 0.001$.

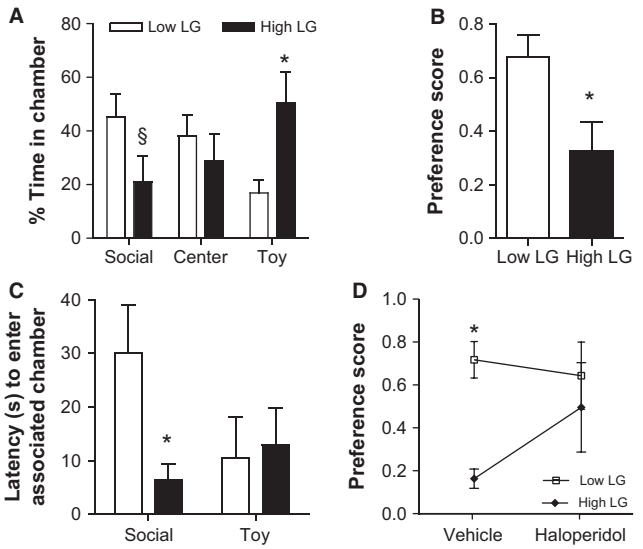

FIG. 6. Conditioned place preference for sibling (social) vs. toy in low-LG and high-LG offspring. Percentage of time (mean ± standard error of the mean) spent in each chamber (sibling-associated, center, and toy-associated) by low-LG and high-LG offspring. (B) Preference scores [$T_{sibling}/(T_{sibling} + T_{toy})$] of low-LG and high-LG offspring. (C) Latency (s) to enter the sibling-associated or toy-associated chamber. (D) Preference scores of low-LG and high-LG offspring following administration of vehicle or haloperidol. §$P < 0.1$, $^{*}P < 0.05$.

to spend more time in the sibling-paired chamber ($t_{1,18} = 1.85$, $P = 0.08$). There were no group differences in the percentage of time spent in the center chamber ($P = 0.47$). In this CPP paradigm, low-LG offspring were found to travel a significantly greater distance than high-LG offspring ($t_{1,18} = 2.14$, $P < 0.05$), and distance traveled was therefore used as a covariate in subsequent analyses. Low-LG offspring were found to have a significantly higher preference score for the sibling/cagemate chamber than high-LG offspring ($F_{1,18} = 5.49$, $P < 0.05$; Fig. 6B). Significant group differences (low LG > high LG) were observed in the latency to enter the sibling-paired chamber ($F_{1,18} = 6.46$, $P < 0.05$; Fig. 6C), but not in the latency to enter the toy-paired chamber ($P = 0.97$). An additional CPP test was conducted to determine the effect of haloperidol (a D2/D1 antagonist) on preference for the social vs. toy-associated chamber. Vehicle-treated females showed the maternal LG-associated preferences observed during CPP testing on the previous day (low-LG offspring preferring the social chamber, and high-LG offspring preferring the toy chamber; $t_{1,8} > 3.48$, $P < 0.01$). However, we found no LG-associated differences in preference scores following haloperidol treatment ($P > 0.53$; Fig. 6D). Although haloperidol treatment did reduce total distance traveled ($t_{1,18} = 3.36$, $P < 0.01$) as compared with vehicle, this effect did not vary by LG group, and haloperidol did not increase the amount of time that animals spent immobile ($P > 0.23$). All animals were observed to explore and make entries into each chamber.

Discussion

In the current study, we have demonstrated that natural variation in postnatal maternal care predicts long-lasting variation in the mesolimbic DA system and associated differences in motivated behavior. The density of DA cell bodies within the VTA is elevated by P6 in offspring that experience high levels of maternal LG, and we found elevated expression of Cdkn1c, a transcription factor that promotes DA neuron differentiation, in the VTA of high-LG female offspring at this developmental time-point. The long-term effects of early life maternal care on midbrain DA neuron density do not appear to be associated with altered *Th* mRNA or DNA methylation within the *Th* promoter, but rather with elevations in the expression of Lmx1b, a transcription factor implicated in the maintenance of DA neurons, at P21 and in adulthood in high-LG offspring. At weaning, offspring that have received high levels of LG have elevated DA receptor mRNA levels within the NAc, and we found associated changes in reward preferences. The effects of maternal care on developing DA pathways and reward-directed behavior of female offspring may play a critical role in the behavioral transmission of maternal LG from mother to daughter, and account for individual differences in the mesolimbic DA system.

The effects of maternal care that we observed on offspring TH-immunoreactive cells are highly localised to specific nuclei (i.e. PBP and SNL) that have projection sites and connectivity that are relevant to our understanding of associated reward-directed preferences. DA neurons of the PBP project to the ventral striatum, olfactory tubercle, prelimbic cortex, and infralimbic cortex (Beckstead *et al.*, 1979; Björklund & Dunnett, 2007; Ikemoto, 2007). It has been suggested that the posterior VTA including the PBP, rather than the anterior VTA (including the paranigral nucleus of the VTA and VTAR), mediates the rewarding effects of drugs of abuse, and that PBP connections with the NAc are particularly important for conditioned 'action-arousal' behavioral responses (Ikemoto, 2007). The SNL is likewise functionally relevant for motivated behaviors, and there are projections from this region to the amygdala (Kaelber & Afifi, 1979; Woolf & Butcher, 1982; Björklund & Dunnett, 2007), although there is evidence that these projections are not dopaminergic (Loughlin & Fallon, 1983). The amygdala has been implicated in maternal behavior through projections to the bed nucleus of the stria terminalis, and thereafter the medial preoptic area of the hypothalamus (Fleming, 1986). Although the contribution of the amygdala to reward-directed behaviors was not examined in the current studies, activation of basolateral amygdala neurons projecting to the NAc facilitates reward seeking (Stuber *et al.*, 2011), and lesions to the amygdala have previously been shown to reduce neophobia and enhance maternal behavior (Fleming *et al.*, 1983). Thus, maternal LG-induced differences in TH-immunoreactive cells in the PBP and SNL may be anatomically and functionally relevant for maternal and reward-directed behavioral differences. An important consideration in future studies will be the determination of the effects of maternal LG on other cell populations within the

VTA, such as GABAergic and glutamatergic neurons, which are known to alter the function of midbrain DA neurons (Olson *et al.*, 2005; Yamaguchi *et al.*, 2007; Sesack & Grace, 2010).

Epigenetic regulation of gene expression induced by early environmental experiences has emerged as a critical mechanistic pathway within the developing brain, and maternal care has been demonstrated to alter promoter DNA methylation within the glucocorticoid receptor gene (*Nr3c1*) (Weaver *et al.*, 2004), the glutamic acid decarboxylase gene (*Gad1*) (Zhang *et al.*, 2010), the metabotropic glutamate receptor gene (*Grm1*) (Bagot *et al.*, 2012), and the estrogen receptor-α gene (*Esr1*) (Champagne *et al.*, 2006), and may account for the long-term suppression of the activity of these genes in response to low levels of LG. Here, we examined DNA methylation within the *Th* promoter, in a region previously associated with chromatin remodeling and moderate CpG methylation variation (He *et al.*, 2011), and containing regulatory sequences for AP-1 and SP-1 transcription factor binding (Lenartowski & Goc, 2011). Within the CpG sites examined, we found that *Th* DNA methylation increased over developmental time, but did not vary as a function of the experience of postnatal maternal LG, consistent with the finding of no LG-associated changes in *Th* mRNA in the VTA. Although it may be the case that methylation within other regions of the *Th* promoter do vary in response to maternal care, it is also possible for TH-immunoreactive cells to vary independently of epigenetic regulation or transcriptional activation of *Th*. In VTA sections assessed for *Th* mRNA by *in situ* hybridisation and simultaneously immunostained for TH, not all TH-immunoreactive cells show elevated levels of *Th* mRNA (Seroogy *et al.*, 1989). Post-transcriptional and post-translational modifications can alter the translation or stability of TH independently of the level of transcription (Tank *et al.*, 2008; Lenartowski & Goc, 2011), and these processes may contribute to sustained differences in TH immunoreactivity.

Factors that contribute to the differentiation, survival and maintenance of DA neurons projecting from the VTA may be critically involved in the observed effects of maternal care on TH. The transcription factors Nurr1 and Cdkn1c ($p57^{kip2}$) have been shown to interact to induce cell cycle arrest and DA neuron differentiation (Joseph *et al.*, 2003; Perlmann & Wallen-Mackenzie, 2004), and increased apoptosis and delayed differentiation have been observed in *Cdkn1c*-knockout mice (Yan *et al.*, 1997). The reduced levels of *Cdkn1c* expression among low-LG offspring at P6 may thus delay development of the VTA and contribute to the reduced number of DA cells. Similar effects are observed following neonatal 6-hydroxydopamine lesions (Frohna *et al.*, 1997). Although we did not observe any effects of maternal care on Nurr1, increased TH immunoreactivity resulting from prolonged membrane polarisation has been demonstrated in cell culture in the absence of altered Nurr1 immunoreactivity or *Nurr1* mRNA levels (He *et al.*, 2011). This effect has been attributed to an increased proportion of Nurr1-expressing cells committing to a TH-positive phenotype, owing to altered Nurr1–Cdkn1c interactions and activation of the *Th* promoter (Lenartowski & Goc, 2011). The maintenance of these TH-expressing neurons may involve the transcription factors Lmx1b and Pitx3. In *Lmx1b* mutant mice, although TH-expressing neurons are generated, these neurons do not survive beyond embryonic day 16 (Smidt *et al.*, 2000). The level of Pitx3, which is widely expressed in development, but in adulthood is restricted exclusively to the VTA and SN, is also diminished in *Lmx1b* mutant mice (Smidt *et al.*, 1997; Smidt, 2004). Lmx1b and Pitx3 probably alter DA neuron differentiation and maintenance, independently of Nurr1 and Cdkn1c, as suggested by the normal expression levels of *Lmx1b* in *Nurr1* mutant mice (Smidt *et al.*, 2000; Prakash & Wurst, 2006). Our results suggest that maternal care may alter the developing mesolimbic DA system through both of these pathways, promoting the generation of DA neurons through *Cdkn1c* during the early postnatal period, and the maintenance of these neurons through activation of *Lmx1b* in the pre-weaning period, effects that are sustained into adulthood and perhaps complemented by increased levels of neurotrophic factors (e.g. BDNF) in the adult VTA of high-LG offspring.

Reduced DA tone within the mesolimbic DA system, as indicated by increased basal and stimulus-dependent DA release in the NAc, has been associated with anhedonia and depression, whereas elevated DA tone is associated with heightened motivational drive, altered responses to drugs and stress, and positive symptoms in schizophrenia (Wise, 2008; Rodrigues *et al.*, 2011). Elevated TH immunoreactivity in the VTA has been previously associated with elevated DA content in the NAc and increased CPP (Kostic *et al.*, 1997; Shim *et al.*, 2000; Vucetic *et al.*, 2010; Liang *et al.*, 2012). On the basis of the effects of maternal behavior on TH-immunoreactive cell numbers, we predicted altered reward-directed behavior in offspring, and focused on natural rewards: HFD and social interaction. Our finding of decreased CPP for the HFD in low-LG offspring is consistent with the finding of decreased preference for palatable foods and reduced saccharine intake in offspring exposed to an unstable maternal environment (Ventura *et al.*, 2012). This behavioral indication of reduced reward preference, combined with the lower VTA TH immunoreactivity in low-LG offspring, is suggestive of an anhedonic phenotype (Wise, 2008). However, analyses of the preference for social interaction in low-LG offspring vs. high-LG offspring challenges this conclusion. The increased place preference for sibling/cagemate interaction is consistent with a theory of 'play compensation', whereby maternal or social deprivation increases the salience of social stimuli during the juvenile period (Bernstein & Dobrofsky, 1981). Evidence in support of this theory is based on the increased play behavior of juveniles exposed to maternal separation or to social isolation (Goy & Wallen, 1979; Bernstein & Dobrofsky, 1981; Panksepp, 1981; Beatty *et al.*, 1982; Wallen, 1996), and previous studies have documented increased home cage play behaviors among offspring that received low levels of LG (Birke & Sadler, 1987; Moore & Power, 1992; Parent & Meaney, 2008; Parent *et al.*, 2012). The contrasting preferences of low-LG and high-LG offspring suggest the differential integration of upstream or downstream targets of the mesolimbic DA system in LG-induced alterations in motivated behavior. The use of multiple paradigms for assessing motivation in this context will be essential in future studies, in order to provide convergent evidence of divergent reward-directed responses.

Lactating females that engage in elevated levels of LG have previously been shown to have an increased density of D1 and D3 receptors in the NAc shell (Champagne *et al.*, 2004), and in the current study we have demonstrated that the female offspring of high-LG dams have increased DA receptor mRNA (D1, D2, and D3) at the time of weaning. Although it might be expected that increased numbers of DA neurons in the VTA would lead to downregulation of DA receptors in the NAc, in order to maintain homeostasis, studies have also shown that activation of the VTA induces long-lasting enhancement of D1 receptors in the NAc (Hu *et al.*, 2002). This altered receptor mRNA expression within the NAc (and the presumed change in receptor level) may have implications for motivated behaviors. Administration of the mixed D1/D2-like antagonist haloperidol abolishes LG-associated differences in preference for social interaction, and it is the high-LG offspring that are most sensitive to this treatment. Moreover, our findings suggest that

preference for social interactions may be enhanced through reduced mesolimbic DA activity. This finding contrasts with studies of mother–infant interactions, in which haloperidol administration has been shown to inhibit pup retrieval and LG among lactating dams (Stern & Taylor, 1991). Our finding of increased CPP for pups vs. toy in high-LG dams and increased preference for toy vs. social interaction in high-LG offspring suggests a role of DA receptors in these preferences, and, although speculative, it may be that the reduced pup preference is the reproductive cost of enhanced preference for play/social interactions. Adult low-LG and high-LG offspring show a reproductive trade-off between sexual receptivity and maternal behavior that may be programmed through variation in estrogen sensitivity within brain regions implicated in these divergent behavioral responses (Champagne *et al.*, 2003b, 2006; Cameron *et al.*, 2008, 2011). Moreover, haloperidol administration has previously been found to increase sexual behavior in female rats (Grierson *et al.*, 1988), which is similar to the shift in social preference that we have observed in high-LG juvenile females. Thus, variation within the mesolimbic DA system, within the context of maternal LG-associated changes in other neural pathways, particularly those involved in reproductive behavior, may be a critical mechanism for determining reward sensitivity and the transmission of maternal behavior from one generation to the next. The challenge for future studies on the impact of maternal care and other early life experiences will be determining the cascade of molecular and cellular mechanisms through which developmental experiences are encoded within the DA system, and the integration of these signals between interconnected brain regions that will impact on behavioral phenotypes.

Supporting Information

Additional supporting information can be found in the online version of this article:

Table S1. Gene expression and pyrosequencing primer sequences.

Table S2. Experimental design of conditioned place testing (CPP) using reward (neonatal pups, high-fat diet, sibling) and control (familiar toy, standard chow) stimuli.

Fig. S1. TH-immunoreactive (TH-ir) cells in the ventral tegmental area and substantia nigra nuclei of adult females.

Fig. S2. Offspring gene expression in the ventral midbrain.

Fig. S3. Offspring TrkB gene expression in the nucleus accumbens.

Fig. S4. Conditioned place preference for pups vs. toy among lactating low-LG, mid-LG and high-LG dams.

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Disclosure

The authors declare no competing financial interests.

Abbreviations

BDNF, brain-derived neurotrophic factor; CPP, conditioned place preference; DA, dopamine; HFD, high-fat diet; LG, licking/grooming; NAc, nucleus accumbens; P, postnatal day; PBP, parabrachial pigmented nucleus of the ventral tegmental area; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SN, substantia nigra; SNL, substantia nigra pars compacta lateral part; TH, tyrosine hydroxylase; VTA, ventral tegmental area; VTAR, rostral ventral tegmental area.

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