Neonatal Overexpression of Estrogen Receptor-α Alters Midbrain Dopamine Neuron Development and Reverses the Effects of Low Maternal Care in Female Offspring

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ABSTRACT: **Maternal behavior is dependent on estrogen receptor-alpha (ERα; *Esr1*) and oxytocin receptor (OTR) signaling in the medial preoptic area (MPOA) of the hypothalamus, as well as dopamine signaling from the ventral tegmental area (VTA) to forebrain regions. Previous studies in rats indicate that low levels of maternal care, particularly licking/grooming (LG), lead to reduced levels of MPOA ERα and VTA dopamine neurons in female offspring and predict lower levels of postpartum maternal behavior by these offspring. The aim of this study was to determine the functional impact on maternal behavior of neonatal manipulation of ERα in females that had experienced low versus high levels of postnatal maternal LG. Adenovirus expressing ESR1 was targeted to the MPOA in female pups from low and high LG litters on postnatal day 2–3. Overexpression of ESR1 in low LG offspring elevated the level of ERα-immunoreactive cells in the MPOA and of tyrosine hydroxylase cells in the VTA to that observed in high LG females. Amongst juvenile female low LG offspring, *ESR1* overexpression also decreased the latency to engage in maternal behavior toward donor pups. These results show that virally mediated expression of *ESR1* in the neonatal rat hypothalamus results in lasting changes in *ESR1* expression through the juvenile period, and can "rescue" hormone receptor levels and behavior of offspring reared by low LG dams, potentially mediated by downstream alterations within reward circuitry. Thus, the transmission of maternal behavior from one generation to the next can be augmented by neonatal ERα in the MPOA.** © 2014 Wiley Periodicals, Inc. Develop Neurobiol 75: 1114–1124, 2015

Keywords: **maternal care; estrogen receptor-alpha; medial preoptic area; ventral tegmental area; dopamine**

INTRODUCTION

In humans, early life adversity (abuse, neglect, or disrupted parent–child attachment) predicts an earlier onset of sexual activity (Ellis et al., 1999) and impaired social/emotional development (Manly et al., 2001) whereas nurturing parent–child interactions reduce the incidence of psychiatric dysfunction (Korosi and Baram, 2009; Maselko et al., 2011). These developmental experiences have a lasting impact on brain function and gene expression profiles that have been attributed to epigenetic programming (Gudsnuk and Champagne, 2011). In rats, the impact of mother–infant interactions is no less profound, with low levels of maternal care predicting heightened

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stress reactivity, impaired cognition, and increased sexual receptivity in adult offspring (Meaney, 2001; Cameron et al., 2008). Low compared to high levels of maternal licking/grooming (LG) experienced in infancy is associated with decreased oxytocin receptor (OTR) and estrogen receptor-alpha (ERα) levels in the medial preoptic area (MPOA) of the hypothalamus in female offspring (Francis et al., 2000; Champagne et al., 2001; Champagne et al., 2003; Peña et al., 2013) and decreased tyrosine hydroxylase (TH) -immunoreactive dopamine neurons in the ventral tegmental area (VTA; Peña et al., 2014). These neuroendocrine and reward system changes may account for the low levels of maternal care exhibited by offspring of low LG mothers (Francis et al., 1999; Champagne et al., 2003).

Analyses of the developing female hypothalamus indicate that ERα levels within the MPOA are decreased in offspring of low LG mothers by postnatal day 6 (P6) (Champagne et al., 2006; Peña et al., 2013). Among juvenile female offspring, reductions in ERα mRNA levels are evident at the time of weaning and may account for the reduced maternal responsivity observed in low LG females (Peña et al., 2013). We have hypothesized that reduced hypothalamic ERα leads to reduced estrogen sensitivity and OTR in the adult female brain with functional consequences for LG behavior (i.e. pharmacological OTR antagonism reduces LG; Champagne et al., 2001). However, the functional role of developmental programming of hypothalamic ERα levels by maternal care in the maternal behavior of offspring had yet to be established.

Recently, we have also found reduced levels of dopamine neurons in the VTA at P6 in females reared by low LG dams (Peña et al., 2014). Maternal behavior, a motivated behavior, is dependent on mesolimbic dopamine signaling and is inhibited by 6-OH-DA lesions in the VTA or nucleus accumbens (NAc) and after lesions severing tracts between the MPOA and VTA (Gaffori and Le Moal, 1979; Numan and Smith, 1984; Hansen et al., 1991). The mesolimbic dopamine system is hormonally sensitive and is a direct neuroanatomical target of estrogen-sensitive oxytocin neurons of the MPOA (Morrell et al., 1984; Shahrokh et al., 2010). Reduced dopamine neurons in the VTA in response to low LG that we have observed during development (Peña et al., 2014) may underlie lower levels of NAc dopamine release in adult dams prior to and during pup LG (Champagne et al., 2004), and therefore contribute to the behavioral perpetuation of maternal phenotype in the next generation. The early postnatal emergence of variation in both ERα and dopamine cell numbers suggests that these alterations develop independently in response to maternal care. However, dopamine neurons treated with estrogen increase neurite growth and *TH* mRNA in culture (Reisert et al., 1987; Beyer et al., 2003; Ivanova and Beyer, 2003), and transient levels of ERα have been found in the VTA from embryonic day 17 to P20 in rodents (Raab et al., 1995; Raab et al., 1999), indicating that the VTA may be particularly sensitive to estrogen or ERα during early postnatal development.

These previous findings raised two important questions: (1) Are elevated levels of neonatal ERα sufficient to drive maternal behavior? and (2) Does elevated hypothalamic ERα drive changes within the mesolimbic dopamine system? To determine the impact of developmental changes in hypothalamic ERα on subsequent maternal behavior and mesolimbic dopamine system development in the rat, we injected female offspring that had experienced low versus high levels of maternal LG with an adenovirus expressing the ERα gene (*Esr1*). Direct targeting of the MPOA with this ERα overexpression vector during postnatal development led to sustained increases in ERα, increased VTA dopamine cell counts, and enhanced maternal behavior—abolishing differences in maternal responsivity predicted by the experience of low versus high LG. This study suggests that ERα expression is a mechanism of transmission of maternal behavior across generations.

METHODS

Animals

Long Evans rats (Charles River Laboratories) were maintained on a 12 h light-dark schedule (lights on at 0800h) with food and water provided *ad libitum*. Adult virgin females ($n = 40$) were mated for 1 week and singly housed 1–2 days prior to parturition. At weaning (postnatal day 21–P21) offspring were pair-housed by sex. 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., 2003). Maternal behavior ($n = 35$ dams) was observed for five 60-min observation periods daily during P1–6 [see Fig. 1(A) for study design]. Behavioral observations were made every 3 min during each observation period for a total of 600 observations per litter. Frequency of LG behavior was calculated as the number of observations of LG divided by the total number of observations. Low and high LG dams were defined as engaging in LG frequencies that were either one standard

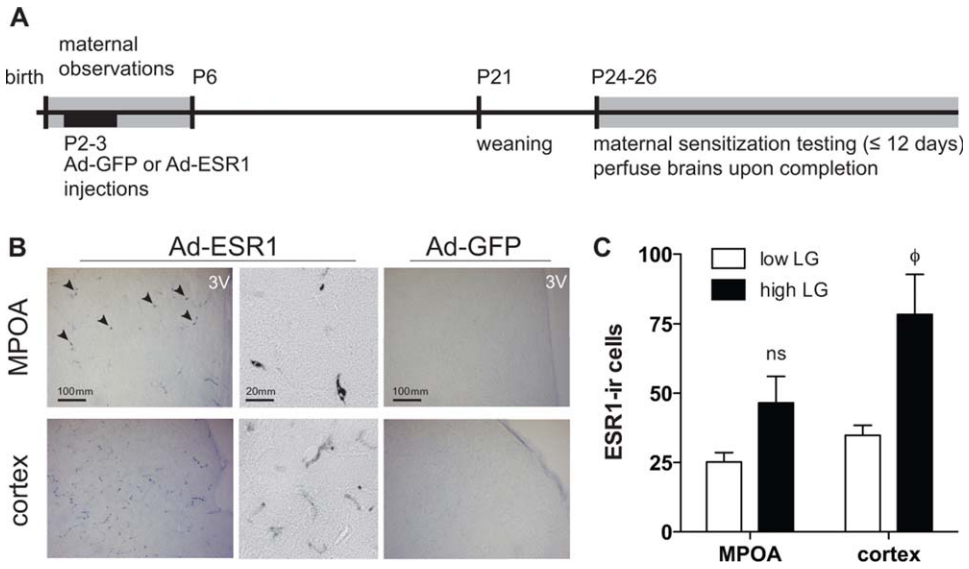

Figure 1 Study design and ESR1 overexpression. (A) Timeline of maternal observations, virus injections, offspring maternal sensitization behavior testing, and brain collection. (B) ESR1-ir was detected in the MPOA and cortex of females that received neonatal injections of Ad-ESR1 (arrow heads), and not in animals that received Ad-GFP control injections. 3V, third ventricle. Higher magnification of Ad-ESR1 staining reveals primarily cell body staining within MPOA, with more tracts stained in cortical regions. (C) ESR1-ir cell counts in low and high LG offspring within the MPOA and cortex. ϕ, $p < 0.1$ (low vs. high).

deviation below (low LG) or above (high LG) the mean LG of the cohort. Mid LG dams engaged in maternal LG frequencies within one standard deviation of the mean LG of the cohort.

Adenovirus

Prepackaged human type-5 adenoviruses containing human *ESR1* (Ad-ESR1, SL100776; NM_000125) or GFP (Ad-GFP, SL100708) under the control of a constitutively active CMV promoter were purchased from SignaGen Laboratories. Both viruses lacked early phase genes E1 and E3 rendering them replication deficient, and had titers of $1 \times 10^{10} \sim 1 \times 10^{11}$ PFU/mL. Adenovirus was chosen over other virus types (e.g., HSV, AAV, lentivirus) due to the rapid onset of expression (~2 days) after introduction to a host and lasting *in vivo* expression (~9 months; Hu et al., 2010; Rahim et al., 2011). Pilot testing confirmed that expression could be detected 6 weeks following injection into the neonatal brain.

Adenovirus Injections

Neonatal female pups received stereotaxic injections of either Ad-GFP or Ad-ESR1 on P2–3 [see Fig. 1(A)]. Prior to injection, pups were cryoanesthetized on wet ice for 15 min, until nonresponsive to foot pinch. Ten microliter of Hamilton syringes dedicated to either Ad-GFP or Ad-ESR1 were used to deliver 0.4 μL microinjections of virus per animal. Bregma (visible through the skin at this age) was used to guide needle placement. Injections of Ad-GFP or Ad-ESR1 were aimed at the ventral third ventricle to avoid bilateral placement errors that would be difficult to detect after several weeks of rapid growth. Pilot testing with Ad-GFP revealed staining within the MPOA and ventricle walls following injection. The needle was centered at Bregma and lowered to −5 mm over the course of 30–40 sec. Virus was infused via MicroSyringe Pump Controller (World Precision Instruments) at a rate of 4 nL/sec. After a rest period of 60 sec, the needle was slowly removed and the pup was transferred to a warming pad to recover (up to 30 min). All injected pups survived the procedure. Pups were returned to the home cage and remained unmanipulated, except for weekly cage changes, until weaning. Inclusion in the overexpression group was determined by presence of hESR1 immunostaining.

Juvenile Maternal Sensitization

Offspring were tested for maternal behavior as juveniles while adenovirus was known to be expressed, and prior to onset of puberty, using a maternal sensitization paradigm. Virgin maternal sensitization latency has previously been shown to correlate strongly with LG frequency observed after parturition (Champagne et al., 2001), and thus maternal sensitization among the juvenile virgin offspring in this study is proposed as a proxy of subsequent maternal LG behavior. Maternal sensitization involves exposing test subjects to neonates daily until they display maternal behavior

(Champagne et al., 2001). Animals were tested daily between 1500 h and 1700 h beginning on P24–26 [see Fig. 1(A)]. Each day, three recently fed, 2–5 day-old pups from a donor litter were placed in three quadrants in the cage (one pup per quadrant) away from the nest site. Test animals were observed for 1 h for indices of maternal behavior. Donor pups were left in the cage for 23 h. On test days 2–12, pups from the previous session were removed and replaced with a new set of recently fed pups, thereby commencing another 1-h test session. Testing continued for 12 consecutive days or until a female displayed full maternal behavior (i.e., retrieved all three test pups, grouped them in the nest, and crouched over them) within the 1-h test session.

Immunohistochemistry

On completion of maternal sensitization testing, animals were terminally anesthetized, transcardially perfused with PBS followed by 4% paraformaldehyde, and brains removed and post-fixed overnight in 4% paraformaldehyde at 4°C. Brains were, then, cryoprotected in 30% sucrose in PBS at 4°C until isotonic, and stored at −80°C. Coronal sections, 35 micrometer in thickness, were sliced and floated into PBS. Hypothalamic and ventral midbrain sections (10–16 per animal) were washed in PBS and blocked with normal serum before incubating in primary antisera at 4°C overnight. In one series of staining, all sections were dual-labeled using fluorescent markers for GFP and ERα (chicken-anti-GFP, 1:1000, Aves Labs; rabbit-anti-ERα, 1:3000, Santa Cruz Biotechnology). Sections were, then, washed and incubated in a secondary antisera at 4°C overnight (Alexa Fluor 488 goat-anti-chicken and Alexa Fluor 546 donkey-anti-rabbit, 1:1000, Life Technologies). Sections were washed, mounted on gelatin coated slides, and coverslipped with ProLong Gold Antifade Reagent with DAPI (Life Technologies). In a second series of staining, sections were stained for human ESR1 protein (mouse-anti-hESR1, 1:3000, Life Technologies #49–1002). Signal was amplified with biotinylated goat-anti-mouse (Vectastain) followed by chromogen visualization (Vector SG, Vector Labs). After washing, sections were dehydrated in a series of ethanol washes, cleared with xylene, and coverslipped with DPX. For clarity, total ERα will be referred to as "ERα" while virally overexpressed human ERα will be referred to as "ESR1." In a third series of staining, 8–11 slices per animal containing the ventral midbrain were immunofluorescently labeled for TH, an enzyme in the dopamine synthesis pathway and marker of dopamine neurons (primary antibody: rabbit-anti-TH, 1:10,000, Santa Cruz Biotechnology; secondary antibody: Cy2 conjugated donkey-anti-rabbit, 1:2000, Santa Cruz Biotechnology).

Imaging and Cell Count

Slides were imaged on an Olympus light microscope fitted with fluorescent filters. Anterior–posterior position of each section was noted. Cell counts (immunoreactive cells, -ir) were determined using MCID Core software (InterFocus Imaging Ltd, UK). An observer blind to condition outlined each region of interest for nuclei-specific analysis. Within the ventral midbrain, subnuclei of the VTA and substantia nigra (SN) were distinguished as previously reported (Peña et al., 2014) according to Paxinos and Watson (2005). Region area and density of immunoreactive cells were also recorded. Statistics were performed on an animal's mean total cell count per region across slice sections.

Offspring Group Composition

Maternal LG data from the first two postnatal days were used to anticipate which litters would be low LG or high LG and pups from these initial litters were selected for injection. Up to four females per litter were injected: two with Ad-GFP and two with Ad- ESR1. Breeding generated a total of 35 litters and 20 of these litters were selected for injection as potential low or high LG. A total of 69 pups were injected with virus. Of the 20 litters, analysis of the full week of maternal observations revealed 5 litters to be low LG and 5 litters to be high LG. Two female offspring from 2 low LG litters that did not receive injections were included as low LG controls. No significant difference was found among noninjected and Ad-GFP injected low LG offspring on behavior or ERα-ir cells ($p > 0.8$) and all pups were considered control. Final group sizes were further constrained based on the number of donor pups available for maternal sensitization testing. Resulting offspring group sizes were as follows: 12 low LG (7 control/Ad-GFP, 5 Ad-ESR1), 14 mid LG (6 Ad-GFP, 8 Ad-ESR1), and 18 high LG (7 Ad-GFP, 11 Ad-ESR1). Offspring of mid LG litters showed behavior and ERα cell counts that were intermediate between those of low and high LG litters. However, offspring from these litters began behavioral testing an average of two days after low and high LG offspring and this delay was found to have a significant effect on outcome measures in the analysis. Mid LG offspring were, therefore, only included in correlation analysis using dam LG frequency as a continuous variable. Offspring were categorized as control or Ad-ESR1 based on the presence of ESR1 staining in the brain, such that only animals that showed ESR1 staining in the brain at any level were included in the Ad-ESR1 group.

Statistics

All statistics were performed using SPSS (PASWS, IBM, version 18.0). Litters were not standardized for size or male/female ratio and these variables were used as covariates in analyses. Main effects and interactions were determined using ANOVA with independent and dependent variables and covariates as noted. Two-tailed student's *t*-test was used for single comparisons between high and low LG animals or between control and overexpression animals. Two-tailed Pearson correlation coefficients were calculated between ERα-ir and maternal behavior or TH-ir. All significance thresholds were set at $p < 0.05$. Analyses

were rerun using litter as a covariate to account for potential litter effects and all reported effects were confirmed even when controlling for this factor.

RESULTS

ESR1 Overexpression

At the level of the MPOA, ESR1-ir was restricted to 2 mm on either side of the third ventricle, indicating that the virus was taken up by the tissue surrounding the injection site but that spread in the immediate vicinity was moderate and within previously reported range [Fig. 1(B); (Rahim et al., 2011)]. There was no significant difference in ESR1-ir cells counted in the MPOA among low and high LG female offspring [$p = 0.17$; Fig. 1(B,C)]. ESR1 staining was also detected in the posterior regions of the hypothalamus, the lateral edge of the ventricles, and within the neocortex [Fig. 1(B)]. Cortical staining was likely due to adenovirus absorption by dividing cells of the ventricular zone followed by cortical migration. Cortical staining within images at the level of the hypothalamus [Bregma +.24 to −1.92, (Paxinos and Watson, 2005)] was observed in somatosensory and cingulate cortices and to a lesser degree in the piriform cortex. There was a trend in the average cortical ESR1-ir cells counted among low and high LG female offspring [$p = 0.06$; Fig. 1(C)]. However, there was not a significant correlation between offspring behavioral measures and ESR1-ir count in the MPOA ($p = 0.97$) or cortex ($p = 0.40$).

ERα Immunoreactivity in the MPOA

We found a main effect of maternal LG [$F(1,29) = 5.85$, $p < 0.05$)] and trend for an interaction between maternal LG and Ad-ESR1 injection [$F(1,29) = 3.02$, $p < 0.1$, Fig. 2(A,B)] on ERα levels in the MPOA. Among control females, there were elevated levels of ERα-ir in high compared to low LG females [$t(1,12) = 4.95$, $p < 0.001$]. No significant difference in ERα-ir was found between low and high LG ESR1-injected females ($p > 0.95$). ESR1-injected low LG females had significantly elevated levels of ERα-ir compared to control low LG females [$t(1,10) = 3.15$, $p < 0.01$]. This effect was not observed in high LG females ($p > 0.37$). Among control females, there was a significant correlation between maternal LG frequency experienced from PN1–6 and ERα-ir in the MPOA [analyses included mid LG offspring; $R(14) = 0.86$, $p < 0.001$]. This correlation was not significant in ESR1-injected females

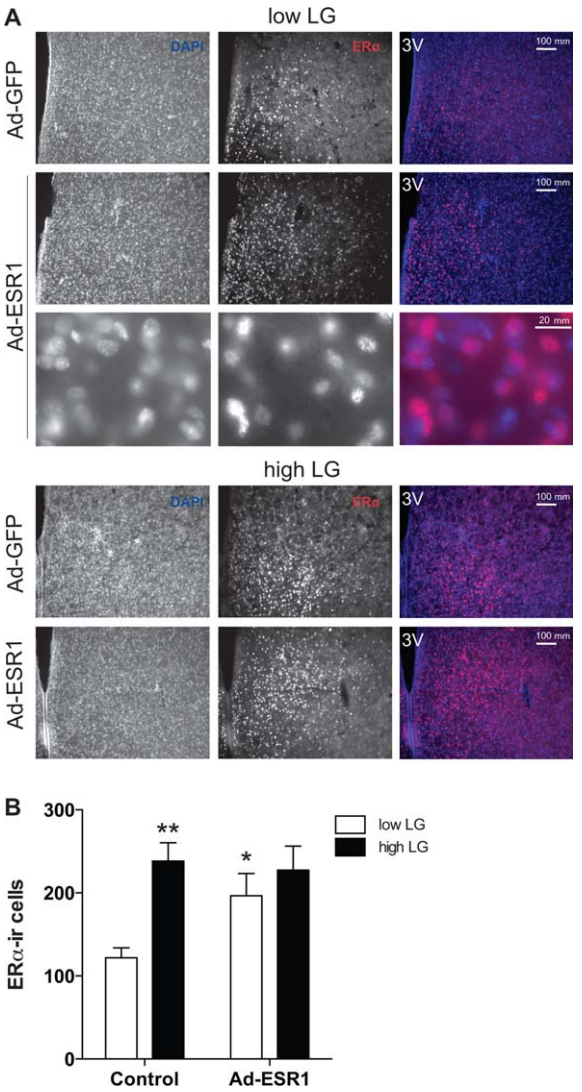

Figure 2 ERα-ir cells in the MPOA of control and Ad-ESR1 low and high LG offspring. (A) Representative images of total ERα-ir in the MPOA of control and Ad-ESR1 low and high LG offspring. 3V, third ventricle. Higher magnification confirms nuclear ERα-ir. (B) Mean ± SEM cells counted expressing ERα protein in the MPOA. *$p < 0.05$ (compared to control) **$p < 0.01$ (low vs. high).

($p = 0.63$). These results indicate that high maternal LG or overexpression of ESR1 is sufficient to increase levels of ERα-ir in the MPOA of females, but that these effects are not additive.

Maternal Sensitization Behavior

Consistent with our previous findings, female offspring that experienced high compared to low levels of postnatal LG were found to have

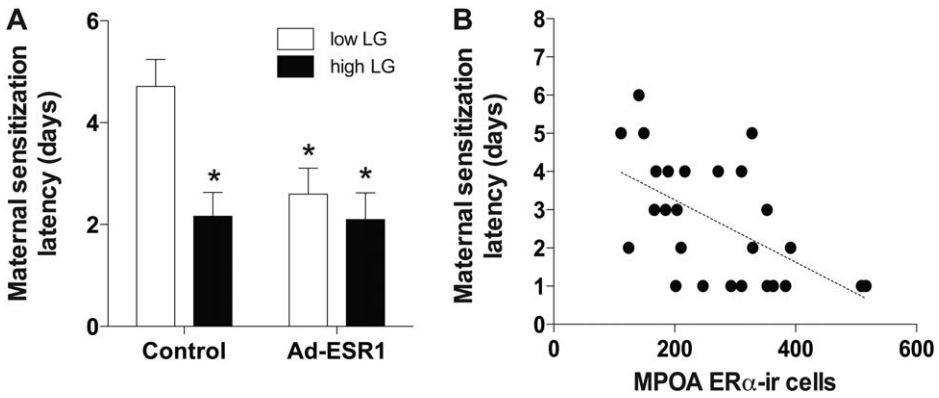

Figure 3 Maternal sensitization latencies of control and Ad-ESR1 low and high LG offspring. (A) Mean ± SEM latency (days) of juvenile females to show full maternal behavior toward donor pups. $^*p < 0.05$ (comparison to low LG control offspring). (B) Correlation between ERα-ir cells in the MPOA with maternal sensitization latency among offspring.

significantly shorter latencies to maternal sensitization [$t(1, 10) = 2.77$, $p < 0.05$; Fig. 3(A)] and, thus, enhanced maternal behavior. This group difference was abolished among females that received neonatal ESR1. Among low LG juvenile females, neonatal ESR1 significantly reduced latencies to maternal sensitization compared to controls [$t(1, 10) = 2.80$, $p < 0.05$; Fig. 3(A)], a difference that was not detected among high LG females ($p > 0.78$). The number of ERα-ir cells in the MPOA was significantly correlated with latency to maternal sensitization behavior [$R(27) = 0.45$, $p < 0.05$; Fig. 3(B)]. These results indicate that high maternal LG or overexpression of ESR1 in the brain is sufficient to decrease maternal sensitization latency.

TH Immunoreactivity in the Ventral Midbrain

TH-ir cells were counted within nuclei of the VTA and SN of control and Ad-ESR1 females [Fig. 4(A)]. Two-way ANOVA indicated a significant main effect of maternal LG [$F(1,29) = 4.90$, $p < 0.05$; Fig. 4(B)] and of virus [$F(1,29) = 5.70$, $p < 0.05$] on TH-ir cells in the VTA as a whole. This was primarily driven by differences in the parabrachial pigmentosa nucleus of the VTA (PBP), as there were no significant effects observed for any other VTA nucleus. We found a significant interaction between maternal LG and virus [$F(1,29) = 6.00$, $p < 0.05$; Fig. 4(B)] on TH-ir cells counted in the PBP. Further analysis indicated a significant difference between low and high LG control animals in TH-ir cells in the PBP [$t(1, 12) = 4.17$, $p < 0.01$] and entire VTA [$t(1, 12) = 4.68$, $p < 0.01$], such that a greater number of TH-ir cells were found among high LG females. Among low LG females, a greater number of TH-ir cells were found in the PBP of Ad-ESR1 compared to control animals [$t(1, 10) = 3.34$, $p < 0.01$]. These differences were not detected among High LG females ($p > 0.39$). There was also a positive linear correlation between ERα-ir cells in the MPOA and TH-ir cells in the PBP [$R(29) = 0.44$, $p < 0.05$; Fig. 4(D)]. Within the SN, we found elevated TH-ir in the SN-pars compacta dorsal tier (Paxinos and Watson, 2005) among high LG control females [$t(1, 12) = 2.64$, $p < 0.05$] and low LG females that received ad-ESR1 [$t(1, 10) = 2.38$, $p < 0.05$] compared to low LG control females. We did not find a significant effect of maternal care or virus for any other SN nucleus. Finally, the number of TH-ir cells in the PBP was correlated with latency to maternal sensitization behavior [$R(29) = 0.38$, $p < 0.05$]. Together, these findings suggest that there is an effect of maternal LG and of neonatal ERα on midbrain dopamine neurons.

DISCUSSION

Our results provide the first demonstration that increased ERα in the MPOA during postnatal development is sufficient to facilitate maternal behavior. In previous studies, the experience of high compared to low levels of postnatal LG by females was found to predict higher levels of ERα-ir and mRNA in the MPOA, higher TH-ir in the VTA, shorter latencies to maternal sensitization, and increased frequencies of postpartum LG toward pups (Champagne et al., 2001; Champagne et al., 2003; Champagne et al., 2006; Peña et al., 2013; Peña et al., 2014). Suppression of ERα in the MPOA of adult mice has previously been found to inhibit maternal care, including

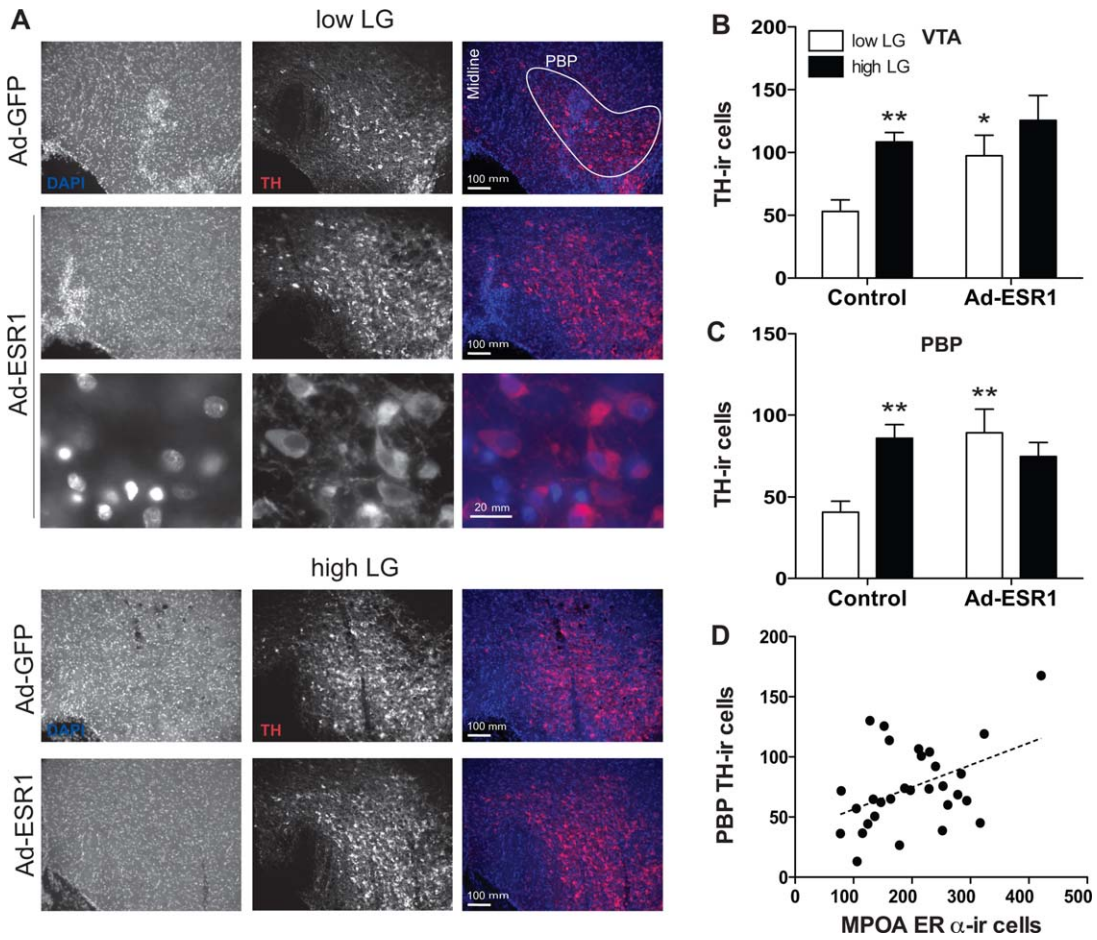

Figure 4 TH-ir cells in the ventral midbrain of control and Ad-ESR1 low and high LG offspring. (A) Representative images of total TH-ir in the VTA and SN (right hemisphere shown) of control and Ad-ESR1 low and high LG offspring. Higher magnification confirms cytoplasmic TH-ir. Mean ± SEM TH-ir cells counted in the (B) VTA as a whole and (C) PBP of the VTA. $*p < 0.05$, $**p < 0.01$ (comparison to low LG control offspring). (D) Correlation between TH-ir cells in the PBP with ERα-ir cells in the MPOA among offspring.

pup retrieval (Ribeiro et al., 2012) and compliments findings indicating the critical role of the MPOA and estrogen sensitivity within this region for the expression of mother–infant interactions (Fahrbach and Pfaff, 1986; Ogawa et al., 1998). We have previously speculated that LG-associated changes in hormone receptor expression within the developing female hypothalamus are a critical mechanistic link between the experience of maternal care and changes to the maternal brain and behavior in later life, and the current findings provide direct evidence to support this conclusion. Neonatal upregulation of ERα was capable of mimicking the effects of high levels of maternal LG and shift the neuroendocrine, mesolimbic dopamine, and behavioral development of offspring reared by a low LG mother. Similar to neonatal cross-fostering (Champagne et al., 2003; Champagne et al., 2006), this target-specific intervention was capable of attenuating group differences in behavior. Although LG-associated effects on neuroendocrine systems and subsequent behavior may vary as a function of sex (Kurian et al., 2010) and within-litter variation in LG (Cavigelli et al., 2010), our data are suggestive that LG-induced effects on maternal sensitization in females is significantly regulated by hypothalamic ERα during development.

The hypothalamic neuroendocrine system and the mesolimbic dopamine system are anatomically connected and implicated together in maternal behavior, and we provide evidence that these two systems may be linked at a molecular level by ERα expression during early postnatal development. Estrogen-sensitive oxytocin neurons project from the MPOA and paraventricular nucleus of the hypothalamus to the VTA,

and infusion of oxytocin directly into the VTA induces dopamine release in the NAc (Morrell et al., 1984; Shahrokh et al., 2010). Treatment of ovariectomized females with estradiol increases firing probability in dopamine neurons of the VTA, providing strong physiological evidence for midbrain sensitivity to estrogen (Sakamoto et al., 1993). In both rats and mice, ovariectomy reduces TH-ir cells in the VTA and SN (Johnson et al., 2010). Treatment with estradiol or an ERα agonist normalizes the level of TH-ir cells in the VTA, while ERβ treatment only restores TH-ir cells in the SN of rats (Johnson et al., 2010), which is consistent with our finding that MPOA Ad-ESR1 treatment specifically enhanced VTA TH-ir cells. This hormonal sensitivity of the mesolimbic dopamine system is important for maternal behavior. For example, pups are only able to elicit dopamine release in the NAc of postpartum dams and hormonally primed females (Afonso et al., 2008; Afonso et al., 2009), and pharmacological activation of D1 receptors in the NAc enhances maternal sensitization and pup retrieval in pregnancy-terminated (hormonally primed) females (Stolzenberg et al., 2007). Furthermore, low LG dams have decreased oxytocin neurons projecting from the MPOA and paraventricular nucleus of the hypothalamus to the VTA (Shahrokh et al., 2010). Our findings of elevated VTA TH-ir among low LG offspring after Ad-ESR1 and of a correlation between ERα-ir and TH-ir suggests that VTA dopamine neuron development may be downstream of hypothalamic ERα expression. Elevated VTA dopamine neuron levels, naturally by LG or artificially via Ad-ESR1, likely contribute to the enhanced maternal behavior observed. However, further studies simultaneously reducing TH levels in the VTA would clarify whether sensitization of maternal behavior is dependent on developmental effects induced by ERα within the VTA, or whether elevated MPOA ERα alone is sufficient to promote this behavior. In addition, while our findings imply that elevated MPOA ERα expression is sufficient to facilitate onset of maternal behavior, it has yet to be determined whether exclusively increasing the number of dopamine neurons in the VTA during development would have similar effects on this behavioral outcome.

The mechanism of maternal sensitization in prepubertal versus cycling, hormonally primed, or postpartum females is important to consider. It has been proposed that virgin female rats are not initially maternal due to neophobia toward pups and that continuous pup exposure allows the female to overcome this aversion and show positive-approach responses (Fleming et al., 1999). Interestingly, local hormone receptor levels may still mediate this anxiolytic response. Elevated oxytocin levels are associated with decreased anxiety in addition to increased maternal behavior (Neumann et al., 2000). Thus, estrogen-regulated oxytocin levels may play a role in pup-induced decreases in anxiety (Champagne et al., 2001). Offspring reared by high LG dams have elevated levels of ERα and OTR in the MPOA as juveniles at P21, prior to puberty (Peña et al., 2013). While plasma hormone levels change significantly across juvenile and pubertal development, estradiol and testosterone measured within the hypothalamus are fairly constant from P20 through adulthood (Konkle and McCarthy, 2011), suggesting that the receptor level rather than brain hormone level may be most important for priming a behavioral response. Moreover, experience-dependent maternal behavior has been observed to be estrogen-independent (Stolzenberg and Rissman, 2011), and may involve complex interactions between hormone receptors and dopamine (Afonso et al., 2008). Dopamine has been shown to stimulate ERα (Smith et al., 1993; Olesen et al., 2005), thus providing one mechanism for activation of ERα independent of its traditional ligand estradiol. In cell culture, ERα has also been shown to stimulate TH and other dopaminergic factors (Sabban et al., 2010), so it is possible to have a feed-forward loop between these systems. Thus, prior to puberty and the onset of elevated gonadal hormones, dopamine may play a more significant role in maternal motivated behaviors.

A critical question that has been raised in the context of developmental studies is the mechanism through which the effects of early life experience on brain function and behavior are sustained over time. Steroid hormones are known to have organizational effects on brain development, inducing permanent morphological and molecular changes within a discrete developmental window. In rats, from E18 to P5 there is a critical period for estrogen to masculinize sexually dimorphic brain region morphology, including the MPOA (Rhees et al., 1990; Rhees et al., 1990). While it is a possibility that neonatal ESR1 overexpression induces organizational effects within sexually dimorphic or other downstream brain circuits such as the VTA, it is likely that the enhanced maternal behavior observed is due to the sustained upregulation of ERα levels in the MPOA consequent to Ad-ESR1. Offspring that have experienced high levels of LG during infancy likewise have sustained elevations in ERα gene expression which is attributed to epigenetic modifications within the *Esr1* promoter, including DNA hypomethylation, posttranslational modifications (trimethylation of H3K4 and H3K9),

and increased STAT5b (signal transducer and activator of transcription) binding (Champagne et al., 2006; Peña et al., 2013). Importantly, both Ad-ESR1 and high LG achieve their long-term effects through elevations in MPOA ERα, although MPOA-induced effects on downstream targets, such as the mesolimbic dopamine circuit, are a critical feature of the maternal brain and are also thought to induce variations in maternal behavior (Shahrokh et al., 2010). Moreover, the correlation between ERα and maternal responsivity in the prepubertal female suggests the possibility of nonligand dependent activation of ERα, possibly through dopamine–ERα interactions (Olesen et al., 2005).

Regional specificity of ERα-mediated effects on reproductive behavior is an important consideration within this study, particularly in light of reproductive trade-offs apparent in association with the experience of high versus low levels of maternal care. Although elevated levels of maternal LG induce increased ERα and estrogen sensitivity in the MPOA, low levels of LG have similar effects within other hypothalamic regions, such as the anteroventral paraventricular nucleus, leading to increased hypothalamic-pituitary-gonadal activity, an earlier onset of puberty, and heightened sexual receptivity (Cameron et al., 2008). The targeting of ERα via Ad-ESR1 induced overexpression could provide a valuable tool for determining the region-specific role of ERα in maternal versus sexual behavior and the limited spread of adenovirus from the site of injection argues for the feasibility of this approach. The cortical expression of Ad-ESR1 is consistent with previous reports of P1 Ad5-GFP injections (Rahim et al., 2011) and with the timing of cortical migration of neurons from the subventriclar zone in the developing brain (Doetsch and Alvarez-Buylla, 1996). Although we have previously found sex-specific effects of maternal care on ERα expression in the prefrontal cortex of juvenile mice (increased in females, decreased in males; Kundakovic et al., 2013), cortical ERα expression is reduced significantly by the second postnatal week (Prewitt and Wilson, 2007), and we have not observed differences in ERα between the offspring of low versus high LG mothers within the cortex. Moreover, the role of the cortex in maternal behavior is attributed to the processing of olfactory responses and recognition of offspring rather than pup retrieval and other active maternal responses (Koch and Ehret, 1991; Calamandrei and Keverne, 1994; Ehret and Buckenmaier, 1994).

The transmission of parental behavior across generations has significant implications for the neurobiological and behavioral development of offspring and grand-offspring. In humans, the experience of neglect, maltreatment, or abuse during childhood predicts the occurrence of these behaviors toward the next generation (Pears and Capaldi, 2001; Berlin et al., 2011) and with increased risk of psychiatric dysfunction (Manly et al., 2001). Within the normal range of parenting behavior, infant attachment is predicted by the attachment scores, sensitivity, and intrusiveness of mothers and grandmothers (Benoit and Parker, 1994; Kretchmar and Jacobvitz, 2002). Rhesus macaques deprived of maternal contact display abusive and neglectful maternal behaviors toward offspring (Arling and Harlow, 1967). Abusive or protective parenting among macaques has been found to be consistent across generations, and cross-fostering studies indicate that it is the experience of abusive caregiving rather than genetic or *in utero* factors which mediate this matrilineal transmission (Maestripieri et al., 2007). Rodent studies have permitted the analyses of the cellular and molecular mechanisms involved in the transmission of maternal behavior and have illustrated the epigenetic impact on the maternal brain of neonatal abuse (Roth et al., 2009) and variations in maternal LG (Champagne et al., 2006). Although future studies will need to confirm the postpartum phenotype of females with induced hypothalamic ERα via Ad-ESR1, and the maternal behavior of their offspring, this study contributes significantly to our understanding of the inheritance of variation in parental behavior by suggesting the role of maternally induced transcriptional activity within the brain of offspring rather than DNA sequences in the germline in perpetuating maternal phenotypes from one generation to the next.

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