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*Research*

# Maternal licking regulates hippocampal glucocorticoid receptor transcription through a thyroid hormone–serotonin–NGFI-A signalling cascade

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Variations in parental care direct phenotypic development across many species. Variations in maternal pup licking/grooming (LG) in the rat regulate the development of individual differences in hypothalamic–pituitary–adrenal responses to stress. The adult offspring of mothers that show an increased frequency of pup LG have increased hippocampal glucocorticoid receptor (GR) expression and more modest pituitary–adrenal responses to stress. This parental effect is mediated by the epigenetic programming of a GR exon 1 promoter (exon $1_7$) through the binding of the transcription factor nerve growth factor-inducible factor A (NGFI-A). In this paper, we report that: (i) the association of NGFI-A with the exon $1_7$ GR promoter is dynamically regulated by mother–pup interactions; (ii) this effect is mimicked by artificial tactile stimulation comparable to that provided by pup LG; (iii) that serotonin (5-HT) induces an NGFI-A-dependent increase in GR transcription in hippocampal neurons and NGFI-A overexpression is sufficient for this effect; and (iv) that thyroid hormones and 5-HT are key mediators of the effects of pup LG and tactile stimulation on NGFI-A binding to the exon $1_7$ GR promoter in hippocampus. These findings suggest that pup LG directly activates 5-HT systems to initiate intracellular signalling pathways in the hippocampus that regulate GR transcription.

**Keywords:** maternal care; depression; serotonin; NGFI-A; thyroid hormone; glucocorticoid receptor

## 1. INTRODUCTION

Phenotypic plasticity is the capacity of the organism to exhibit variations in genotype–phenotype relations in response to environmental signals [1]. Variations in parental signals are a prominent source of environmentally induced phenotypic plasticity. Such parental effects are defined as sustained influence of the parental phenotype on that of the offspring [2,3]. There is evidence for parental effects on multiple phenotypic outcomes, including growth, reproductive tactics and defensive responses. Although parental effects are widely studied, there is little understanding of how a parental signal might stably alter the phenotype of the offspring. The 'environmental epigenetics' hypothesis [4,5] suggests that environmental events reliably activate specific intracellular signalling pathways, including transcription factor complexes, which then target chromatin remodelling enzymes that reconfigure the epigenome, resulting in stable alterations in transcriptional activity. One challenge for researchers interested in parental effects is that of clearly establishing the relation between a specific parental signal and the intracellular pathways that initiate chromatin remodelling.

Parental signalling in mammals is a major determinant of the early life environment for neonates, and differences in parent–offspring interactions have developmental consequences for the offspring. For example, variations in maternal care associate with sustained differences in the hypothalamic–pituitary–adrenal (HPA) response to stress in the offspring in primates and rodents [6,7]. The difference in behaviour between rat mothers is the primary source of variation to the early life environment for the offspring living within a nest site. The maternal behaviour that shows the greatest degree of variation between mothers and that remains the most stable over time and even multiple litters is that of licking/grooming (LG) of offspring [7]. These differences in LG represent qualitative differences in the early life environments of offspring, and as such also represent a stable variation in environmental stimulation. The environmental epigenetics hypothesis predicts that such environmental

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conditions would have consequences for gene expression through effects on chromatin.

Indeed, the adult offspring of mothers that exhibit increased levels of pup LG (High-LG mothers) over the first week of life show increased hippocampal glucocorticoid receptor (GR) expression, enhanced glucocorticoid feedback sensitivity over hypothalamic corticotrophin-releasing factor (CRF) synthesis and more modest adrenal glucocorticoid responses to stress in comparison with those reared by Low-LG mothers [8–13]. Infusion of a GR antagonist directly into the dorsal hippocampus eliminates the maternal effect on HPA responses to stress [14]. The results of cross-fostering studies suggest direct effects of maternal care at the level of both gene expression and stress responses [8,10]. Moreover, within-litter variation in the frequency of pup LG directed towards individual offspring predicts hippocampal GR expression in adulthood [12]. Predictably, manipulations that increase the frequency of pup LG in lactating rats associate with increased hippocampal GR expression and more modest HPA responses to stress [8,13,15].

Maternal care alters hippocampal GR expression in adult offspring through epigenetic mechanisms, specifically through effects on DNA methylation in the exon $1_7$ GR promoter. Exon $1_7$ GR mRNA is enriched in neuronal cell populations, and expressed to a greater degree in hippocampi from adult offspring of High- compared with Low-LG mothers [5,10,11]. The maternal effect on the methylation of the exon $1_7$ GR promoter is reversed with cross-fostering [10] and appears to be mediated by enhanced serotonergic activity. A broad range of *in vivo* and *in vitro* studies suggest that pup LG or postnatal handling, which increases the frequency of pup LG in lactating rats, increases GR gene transcription in the offspring through a thyroid-hormone-dependent increase in 5-HT activity at 5-$HT_7$ receptors and the subsequent activation of cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinase A (PKA) [16–23]. *In vivo* manipulations that affect maternal care affect 5-HT turnover in the hippocampus and temporary lesions of the 5-HT system on postnatal day 2 reduce levels of GR binding in adulthood [23]. Treatment with the 5-HT receptor antagonist, ketanserin, blocks the stimulatory effect of neonatal handling on PKA activity and GR expression later in life [18], as well as the effect of 5-HT on GR expression in cultured hippocampal neurons [21]. Ketanserin also blocks a stress-induced upregulation of NGFI-A [24], suggesting a broad relation between the activation of 5-HT systems and NGFI-A. Furthermore, the 5-HT-induced increase in GR expression can be mimicked by the 5-HT agonist 5-carboxamidotryptamine in a methiothepin-dependent manner, suggesting that the 5-HT effect is mediated by the 5-$HT_7$ receptor [21], which has been shown to be highly expressed in the neonatal rat hippocampus [25]. Moreover, the effect of various 5-HT agonists on the activation of cAMP in cultured hippocampal neurons is highly correlated with the effect on GR, and the 5-$HT_7$ receptor is positively coupled to adenylyl cyclase [19,20].

Both the *in vitro* effects of 5-HT and the *in vivo* effects of variations across lactating rats in pup LG on GR mRNA expression are accompanied by an increased hippocampal expression of NGFI-A transcription factor. The exon $1_7$ GR promoter contains a consensus sequence for NGFI-A [26]. Splice variants of GR mRNA transcripts containing the exon $1_7$ sequence are found predominantly in the brain, and hippocampal expression of exon $1_7$-bearing GR mRNA transcripts is increased with manipulations that increase maternal LG [18] as well as in the offspring of High- compared with Low-LG mothers [16]. Postnatal handling increases hippocampal expression of both the transcription factor NGFI-A and GR, and these effects are eliminated by thyroid hormone synthesis inhibitors or a 5-$HT_{2/7}$ receptor antagonist [18,21].

Further investigation of the effects of maternal LG on transcription factor activity revealed an increased association of NGFI-A with the exon $1_7$ GR promoter in pups of High- compared with Low-LG mothers [16]. These differences are accompanied by epigenetic changes at the exon $1_7$ GR promoter that likely affect gene expression: there is reduced methylation and increased histone 3 lysine 9 (H3K9) acetylation at this genomic site in hippocampi from the offspring of High- when compared with Low-LG mothers [10,16]. These epigenetic changes are associated with an open chromatin structure and hence a permissive environment for gene expression [27,28]. Serotonin (5-HT) induces demethylation of the exon $1_7$ promoter and increases GR expression in cultured hippocampal neurons; both effects are blocked by an antisense targeting NGFI-A, while an NGFI-A expression plasmid mimics the 5-HT effect on an exon $1_7$ promoter construct [16].

These studies suggest that variations in maternal care, notably the frequency of pup LG, produce a thyroid hormone-dependent increase in hippocampal 5-HT activity, thus initiating a signalling cascade that in turn induces the expression of NGFI-A and enhances GR transcription through epigenetic events around the exon $1_7$ GR promoter. However, this model is based largely on correlational studies and there is currently no evidence for a direct effect of variations in pup LG on the proposed signalling pathways, nor on NGFI-A expression or NGFI-A association with the exon $1_7$ GR promoter region. The studies described here were designed to directly examine (i) the effect of pup LG and tactile stimulation derived from pup LG on the intracellular signals thought to regulate the epigenetic state of the exon $1_7$ GR promoter, (ii) the role of 5-HT and thyroid hormones in the regulation of NGFI-A association with the exon $1_7$ GR promoter and (iii) the importance of NGFI-A in the regulation of hippocampal GR transcription.

## 2. MATERIAL AND METHODS

### (a) *Animals*

Adult Long-Evans dams (Charles River; St-Constant, Quebec, Canada) were mated in our animal colony and their postnatal day 4 (P4) male offspring used in experiments. Females were singly housed after mating on 10 L : 14 D cycle (lights on at 09 : 00 h) in polycarbonate cages containing bedding, with ad

libitum access to food and water. Litters were undisturbed over the period of the study.

### (b) *Assessment of maternal behaviour*

Maternal behaviour was assessed using a procedure adapted from that previously described [22]. The frequency of maternal LG behaviour was scored on postpartum days 1 through 4. Observers were trained to a high level of inter-rater reliability (greater than 0.90). Dams were observed in their home cage and undisturbed for the duration of the observation period. Daily observations occurred during five 75-min sessions: three occurring during the light phase (10.00, 13.00 and 17.00 h) and two during the dark phase (07.00 and 20.00 h) of the light cycle. Within each observation session, the behaviour of each mother was scored 25 times (one observation/3 min) for pup LG (including both body and anogenital licking). Thus, the frequency score of pup LG for each mother was based on a total of 425 observations (25 observations/session $\times$ 5 session $d^{-1}$ $\times$ 3.4 d = 425 observations/mother) and was expressed as percentage occurrence (number of occurrences/425 $\times$ 100%).

Mothers were designated as High or Low LG dams on the basis of the pup LG frequency score relative to the mean $\pm$ 1 s.d. for the cohort ($\approx$60–80 mothers/cohort). High-LG mothers were defined as females for which the LG frequency scores were greater than 1 s.d. above the cohort mean. Low LG mothers were defined as females for which the LG frequency scores were greater than 1 s.d. below the cohort mean.

### (c) *Cell culture*

Primary dissociated cell cultures were performed as previously described [20,21] using E20 embryos removed by laparotomy. Hippocampi were isolated by gross dissection under sterile conditions. Cells were mechanically dissociated by trituration after incubation for 15 min at 37°C in 0.25 per cent trypsin (Invitrogen; Carlsbad, CA, USA) and seeded onto poly-D-lysine-coated 60 mm plates at a concentration of roughly $10^6$ $ml^{-1}$ media. Media consisted of Minimal Essential Medium Alpha (MEMα; Invitrogen no. 12561-056) supplemented with 10% v/v heat-inactivated foetal bovine serum (FBS; Invitrogen no. 10082-139), 0.5% w/v glucose, 15 mM HEPES and 20 mM KCl (Sigma-Aldrich). Media was changed the day after seeding and every 2–3 days thereafter with media containing a 1 : 1000 dilution of penicillin/streptomycin (Invitrogen) and 20 μM uridine/5-fluorodeoxyuridine to prevent glial proliferation. Previous characterizations of cultures generated with this protocol reveal more than 95 per cent neuronal composition [21].

### (d) *Western blotting*

Hippocampi were dissected from two littermates on P4 immediately after killing the animals, pooled and flash-frozen, and then stored at −80°C until further processing. Protein from nuclear extracts was quantified by BCA assay (Pierce) and 20 μg of each sample was loaded into 4–12% Bis-Tris gels (Invitrogen) and run at 125 V. Proteins were transferred onto nitrocellulose membranes (GE-Amersham, Buckinghamshire, UK) according to the method of Towbin [29] for 90 min at 25 V. After blocking for 1 h at RT in 5 per cent powdered skim milk in TBS-T (Tris-buffered saline: 0.15 M Tris, 0.8% w/v NaCl, 0.1% Tween-20; pH 7.5), membranes were rinsed briefly in TBS-T and incubated overnight with primary antibody at 4°C (except tubulin, which required only 1 h at RT). Primary antibodies were used at empirically optimized concentrations (1 : 250–1 : 2500 dilutions in TBS-T) and were as follows: CBP (Cell signalling 4772; Beverly, MA, USA), NAB1 (Santa Cruz Biotechnology sc-22813; Santa Cruz, CA, USA), NAB2 (Santa Cruz sc-22815), NGFI-A (Rockland Immunochemicals 600-401-693; Gilbertsville, PA, USA), Sp1 (Santa Cruz sc-17824) and tubulin (Sigma T9026). Membranes were rinsed 3 $\times$ 5 min in TBS-T and incubated with the appropriate horseradish peroxidase-linked secondary antibody (GE-Amersham) diluted 1 : 2500 in TBS-T. Protein was visualized with ECL reagent (GE-Amersham) and Hyperfilm-ECL (GE-Amersham) and developed manually using commercial developer and fixer (Kodak; Rochester, NY, USA). Relative optical densities (RODs) of bands were quantified using a digital camera and computer-assisted densitometry software (MCID 4.0; Imaging Research). Target bands were normalized to α-tubulin from the same lanes and quantified in the same manner.

### (e) *Thyroid hormone measures*

Measurements of triiodothyronine (T3), thyroxine (T4) and thyroid-binding globulin (TBG) were performed from P4 or maternal plasma using enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay RIA kits (ALPCO (Salem NH) 25-FT3HU-E01, 25-FT4HU-E01 and 14-HD-53.1, respectively), according to manufacturer's instructions. For ELISAs, absorbance was quantified using a SpectraMax 384+ plate reader (Molecular Devices) with dedicated software. Radioactivity in RIA assays was quantified using beta-counting (Beckman-Coulter). Deiodinase activity was measured in homogenate of inter-scalpular brown adipose tissue with an excess (20 mM) of T4. ELISA measurements of T3 levels were made before and after 1 h of incubation at 37°C. Data are reported as amount of T3 $\mu g^{-1}$ of protein per hour.

### (f) *Chromatin immunoprecipitation*

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [10]. Animals (P4) were cryoanaesthetized and perfused with 4 per cent paraformaldehyde to cross-link protein–DNA complexes and then stored at −80°C until dissection. Hippocampi were dissected and chromatin was immunoprecipitated using rabbit polyclonal antibody to NGFI-A (Santa Cruz sc-189) or normal rabbit IgG non-immune antibody (from Santa Cruz sc-2027). One-tenth of the lysate was kept before immunoprecipitation and used to quantify DNA levels (input). The rat GR exon $1_7$ [25] of the un-crosslinked DNA was subjected to quantitative PCR (qPCR) amplification as shown later (forward primer sequence (fwd) CT CCCGAGCGGTTCCAAG, reverse primer sequence (rev) TTTAGTTTCTCTTCTCCCAG GCTCCC). Results are expressed as the amount of DNA detected

in immunoprecipitated fraction minus the amount of DNA in negative control normalized to input DNA.

### (g) *qRT-PCR*

Cultures were rinsed two times in PBS and harvested with a cell scraper before centrifugation, and storage of the pellet at $-80^{\circ}$C. RNA was extracted using TRIzol (Invitrogen) coupled with DNase digestion (QIAGEN; catalogue no. 79254). The overall quality and yield of the RNA preparation was determined using A260/280 measures taken with a SpectraMax 384+ plate reader. cDNA synthesis was completed using reverse transcriptase AMV (Roche Applied Science no. 10109118001). Quantitative real-time PCR was performed with a LightCycler 480 (Roche Applied Science), and reference genes ($\beta$2M, GAPDH) from same sample were amplified to control for potential loading errors. The results were identical using both controls and the results are expressed relative to β2M. Primer sequences were as follows: GR Coding fwd CTGCTTTGCTCTGATCTGA, rev TTCAT AGGATACC TGCAATCTTTG; GR $1_7$ transcript fwd AGGGAGCCTGGGAGAAGAGAAACTAA, rev GCCTGGGAGGGAAACCGAGT; GR $1_{10}$ transcript fwd ACTGTTGACTTCCTTCTCCGTGAC, rev CC ACCGCAGCCAGATAAACAAGT; GR $1_{11}$ transcript fwd TGCGGGCTTGTAGGGTGGATT, rev GGC ACGCCCACTTCTAGCAGATAA; NGFI-A fwd TC AGTCGTAGTGACCACCTTACCA, rev GGTATG CCTCTTGCGTTCATCAC; β2M fwd CCGTGAT CTTTCTGGTGCTT, rev AAGTTGGGCT TCCC ATTCTC.

### (h) *Production of recombinant lentiviral vectors*

Viral vectors were derived from the human-immunodeficiency-virus-based lentiviral backbones (generously supplied by S. D. Andrews and M. Szyf, McGill University). For overexpression vector, NGFI-A cDNA [30] was ligated into the pLenti6/V5-Topo vector plasmid (Invitrogen). The resulting expression plasmid contains a cytomegalovirus (CMV) promoter driving expression of NGFI-A or a random sequence (empty vector). For the short interfering RNA (siRNA)-containing vector, NGFI-A siRNA was acquired commercially (Open Biosystems) and subcloned into a pLVTHM plasmid (TronoLab) along with a GFP (green fluorescent protein) expression tag.

Virus was generated by transient co-transfection of the expression plasmid (15 μg), envelope plasmid (pMD2.G; TronoLab; 5 μg) and the packaging plasmids (for overexpression, 10 μg of third-generation packaging plasmids pRSVrev and pMDLg pRRE (TronoLab) were used; for siRNA, 10 μg of second-generation packaging plasmid psPAX2 (TronoLab)) into a 150 mm plate of 90 per cent confluent HEK293T cells by calcium phosphate precipitation. Medium was collected 48 and 72 h after transfection, cleared of debris by low-speed centrifugation and filtered through 0.45 μm filters. High-titer stocks were prepared by ultracentrifugation for 1 h at 138 000*g*. Viral pellet was resuspended in sterile PBS and stored at 80°C. After concentration, typical titres ranged from $10^7$ to $10^8$TU ml$^{-1}$. Sufficient virus was added to cultures to provide multiplicities of infection of 10.

### (i) *Statistical analysis*

All statistical analyses were conducted using PRISM. v. 4.0. Simple comparisons between two groups (High- and Low-LG) were analysed by independent Student's *t*-tests. For analyses involving factorial designs (maternal care and licking bout condition), the primary analysis conducted was a two-way ANOVA. Significant main effects and interactions were interpreted using Tukey's *post-hoc* tests.

## 3. RESULTS

### (a) *Characterization of postnatal day 4 offspring*

Previous studies have defined High- and Low-LG mothers based on 6 days of behavioural observations. On the basis of *in vitro* studies showing effects on GR expression in hippocampal neurons following 4 days of exposure to 5-HT [20] and *in vivo* studies showing that the maternal effect on GR expression was apparent at P6 [10], we selected P4 as a time point for study. We then determined whether 4 days of observations were sufficient for an equally valid characterization of individual differences in pup LG. We transformed LG scores from four cohorts of mothers into *z*-scores based on the means and standard deviations for each cohort, and correlated individual *z*-scores after 17 observations (corresponding to the 10 : 00 h observation on P4) to the final scores. Linear regression analysis revealed a strongly significant correlation between the P4 and P6 scores ($p > 0.001$), with an $r^2$-value of 0.7. These findings suggest that P4 characterization of High- and Low-LG mothers closely approximates that of the 6 days of observations used previously.

### (b) *Characterization of maternal behaviour*

Previous studies of the effects of maternal care involved analysis of tissue samples from the pre-weaning offspring of High- or Low-LG mothers without regard to ongoing mother–pup interactions at the time of animal killing. To more closely relate dynamic variations in biological signalling to maternal care, we developed a model [31] in which tissue samples were obtained from animals immediately following a period of active maternal care ('On' condition) and compared with samples obtained following a period of no mother–pup contact ('Off' condition). On the basis of data obtained from previously characterized cohorts, we operationally defined the On condition as the average length of time during an observation where dams were both in continuous contact with the litter and engaged in pup LG behaviour during at least 50 per cent of the given period. Such periods corresponded to nursing bouts. 'Off-bouts' were determined as the average continuous period where dams were away from the nests. The comparison of tissue samples from these conditions allowed for a more precise association between active periods of maternal care with pup LG and biological outcome. As previously reported [22,31,32], we found that the duration of pup LG during the On condition differed

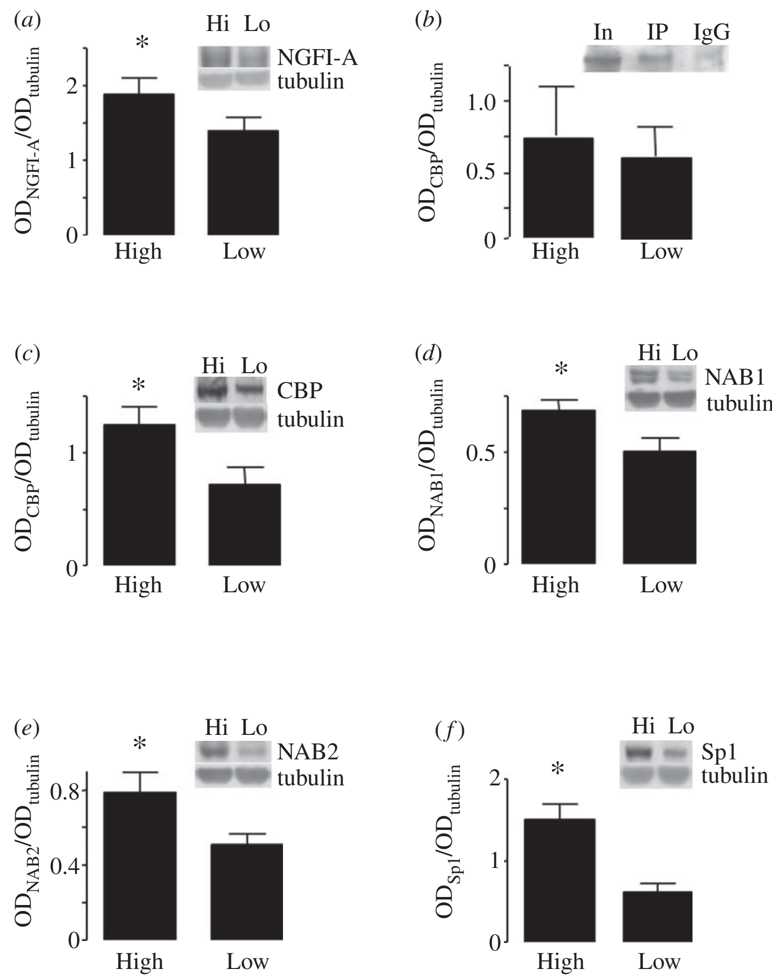


Figure 1. Characterization of NGFI-A and related proteins in nuclear fractions from hippocampus of postnatal day 4 (P4) offspring of High- and Low-LG mothers. Data are presented as mean ± s.e.m. of optical densities (OD) normalized to tubulin for (*a*) NGFI-A, (*c*) CBP, (*d*) NAB1, (*e*) NAB2 and (*f*) Sp1. Representative bands are inset into the respective graphs. (*b*) Mean ± s.e.m. optical densities of CBP co-immunoprecipitated by NGFI-A pulldown from cytosolic fractions from hippocampus from P4 offspring of High- and Low-LG mothers. Representative bands are shown for input (In), NGFI-A-precipitated (Ip) and IgG-precipitated fractions (IgG). $*p \leq 0.05$.

significantly ($H_2 = 5.69$, $p = 0.03$). The duration of bouts of pup LG were longer in High-LG (9.3 ± 1.5 min) compared with Low-LG (4.3 ± 0.7 min) mothers. There were no significant differences between the duration of Off condition in High-, Mid- or Low-LG mothers ($F_{2,74} = 2.27$, $p = 0.1$). The grand mean of the Off-condition duration was 25.9 ± 2.4 min, and accordingly a period 25 min without mother–pup contact was used to define the Off condition (also see [31]).

### (c) *Transcription factor expression*

Previous data from our laboratory have shown that the transcription factor NGFI-A and the histone acetyltransferase CBP play a role in the epigenetic programming of the exon $1_7$ GR promoter [16]. The presence of an Sp1 binding site overlapping the NGFI-A binding site in this promoter [26] suggests that the Sp1 family of transcription factors may also play a role in the regulation of the expression of this gene. We therefore used Western blot analysis to quantify these proteins in hippocampal nuclear fractions prepared from P4 offspring of High- and Low-LG mothers. Blots revealed major bands at the expected molecular weights for the various proteins targeted by the antibodies used. We analysed immunoreactivity as measured by normalized optical density and compared means for hippocampal nuclear fractions from the offspring of High- and Low-LG mothers. As hypothesized, we found statistically significant elevations in hippocampal levels of NGFI-A in the offspring of High- compared with Low-LG dams (figure 1*a*; $t_{22} = 1.71$, $p = 0.05$). Co-immunoprecipitation experiments in hippocampal homogenates confirmed that NGFI-A forms a complex with CBP (figure 1*b*), and Western blot analysis of nuclear fractions of hippocampi also showed that CBP levels in hippocampal nuclear fractions were significantly higher in pups reared by High- compared with Low-LG dams (figure 1*c*; $t_{10} = 2.30$, $p = 0.02$). Likewise, there were increased hippocampal levels of the NGFI-A-binding proteins NAB1 (figure 1*d*; $t_{10} = 2.74$, $p = 0.02$) and NAB2 (figure 1*e*; $t_{10} = 2.31$, $p = 0.02$) in the offspring of High- compared with Low-LG mothers. A significant elevation in the level of the transcription factor Sp1, which shares a binding site with NGFI-A in the exon $1_7$ promoter region, was also apparent in the hippocampal samples from the offspring of High- compared with Low-LG mothers (figure 1*f*; $t_{22} = 4.07$, $p = 0.003$). These differences were

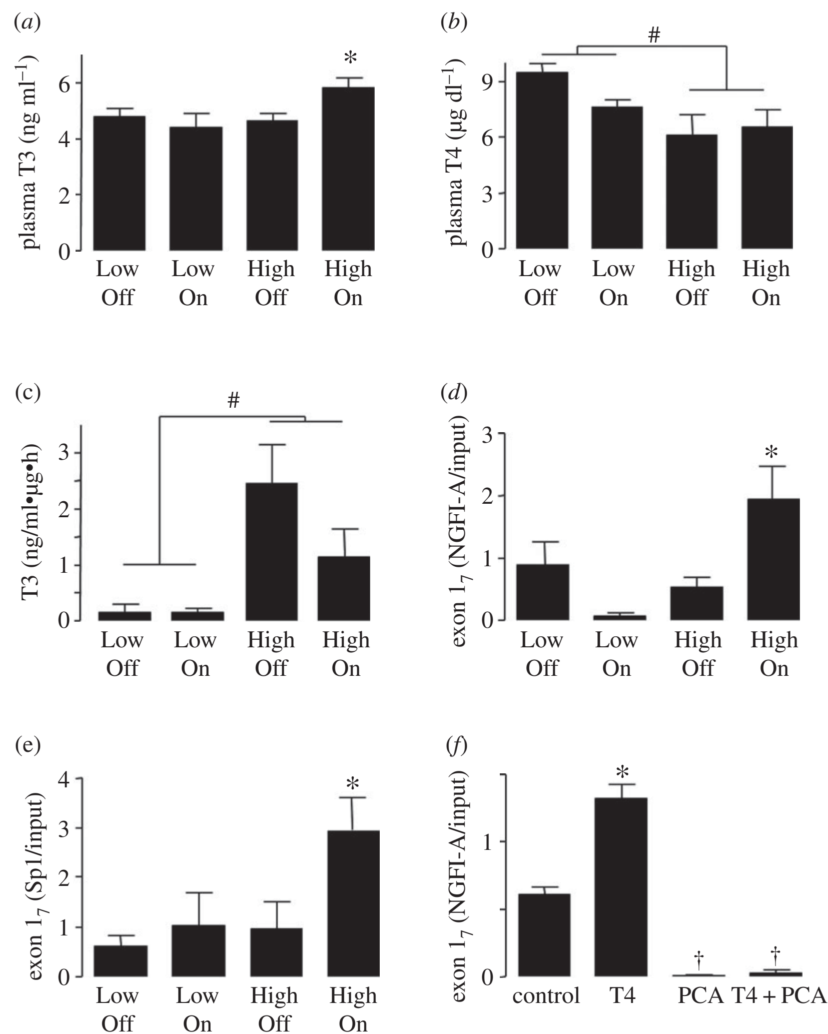


Figure 2. Dynamic effects of variations in maternal care on thyroid hormone levels and transcription factor binding to exon $1_7$ GR promoter. (*a*) Mean ± s.e.m. plasma levels of the active thyroid hormone T3 in the P4 offspring of High- and Low-LG mothers in plasma samples obtained after an active period of mother–pup interactions (On condition) or following an extended period without mother–pup contact (Off condition). (*b*) Mean ± s.e.m. plasma levels of the T3 precursor T4 in the same conditions as in (*a*). (*c*) Mean ± s.e.m. T3-to-T4 conversion capacity of brown adipose tissue from P4 offspring of High- and Low-LG mothers obtained following On or Off conditions. (*d,e*) Mean ± s.e.m. levels of exon $1_7$ promoter transcripts amplified from an NGFI-A (*d*) or Sp1 (*e*) immunoprecipitated fraction minus that from the control IgG-precipitated fraction normalized to an input fraction from hippocampal tissue obtained from the P4 offspring of High- or Low-LG mothers following the On or Off condition. (*f*) Mean ± s.e.m. levels of exon $1_7$ promoter transcripts amplified from an NGFI-A or Sp1 immunoprecipitated fraction minus that from the control IgG-precipitated fraction normalized to an input fraction from hippocampal tissue obtained from the P4 offspring of High-LG mothers treated with saline alone, T4 alone, PCA alone or T4 + PCA. $^{*}p < 0.05$ vs all other groups, $^{\#}p < 0.05$ vs Low, $^{\dagger}p < 0.05$ vs control.

not present in cytoplasmic fractions, nor were there differences in the amount of Sp3 in hippocampal nuclear fractions (data not shown).

### (d) *Dynamic effects of maternal licking/grooming on thyroid hormone*

We previously showed that thyroid hormone manipulation in early life affects hippocampal GR protein levels, and that the handling of pups can induce increases in thyroid hormone that drive hippocampal 5-HT signalling and NGFI-A levels [17,18]. We therefore analysed levels of triiodothyronine (T3) and its precursor, thyroxine (T4), in plasma derived from trunk blood from the offspring of High- and Low-LG mothers after the On or Off condition, as described already. We found a significant interaction effect on plasma T3 levels between maternal phenotype (i.e. High- versus Low-LG) and nesting condition (i.e. On versus Off; figure 2*a*; $F_{1,19} = 5.02$, $p = 0.04$). *Post-hoc* analysis revealed a significant increase in T3 in the offspring of High- compared with Low-LG mothers that was apparent only in the On condition (i.e. immediately following an active period of mother–pup interaction). Similar analysis of T4 levels demonstrated no interaction effect ($F_{1,20} = 1.31$, $p = 0.3$), but did show a main effect of maternal phenotype (figure 2*b*; $F_{1,20} = 4.86$, $p = 0.04$), reflecting increased plasma T4 levels in the offspring of Low- compared with -High-LG mothers, regardless of condition.

Because these ELISAs measure total plasma levels of their respective thyroid hormones, we quantified plasma TBG levels by RIA assay. There were no significant differences in TBG levels between the

offspring of High- and Low-LG mothers ($t_9 = 0.49$, $p = 0.6$; data not shown). We also measured T3 levels in maternal plasma and found no significant differences between High- and Low-LG mothers ($t_{10} = 0.15$, $p = 0.9$; data not shown), suggesting that differences in thyroid hormones in offspring do not originate from maternal circulation.

The elevated T4 plasma concentrations in the offspring of Low- compared with High-LG mothers and the specificity of the increase in T3 to the On condition (figure 2*a*) suggested differences in the conversion of inactive T4 into active T3. We therefore evaluated deiodinase activity through a measure of T4-to-T3 conversion over time in extracts of brown adipose tissue (BAT), a major neonatal source of plasma T3, from the P4 offspring of High- and Low-LG mothers in samples obtained from the On or Off condition. ELISA measurements of T3 concentrations before and after incubation demonstrated that BAT from the offspring of High-LG mothers converted significantly more T4 into T3 regardless of On or Off conditions (figure 2*c*; interaction $F_{1,11} = 1.87$, $p = 0.20$; maternal phenotype $F_{1,11} = 12.01$, $p = 0.005$). Although preliminary analyses of type II deiodinase levels in BAT by qRT-PCR did not suggest differing levels of expression between the offspring of High- and Low-LG dams (data not shown), there are alternative potential mechanisms for this increased T4-to-T3 conversion capacity. The absence of any difference at the mRNA level does not necessarily predict that protein levels will be equivalent between the groups, and does not reveal anything of the post-translational modifications that might modify the enzymatic activity of the deiodinase type I and II proteins. However, these data clearly suggest a stable effect of maternal care on the overall capacity for T4-to-T3 conversion in BAT.

### (e) *Maternal regulation of transcription factor binding to the exon $1_7$ GR promoter*

We previously characterized overlapping binding sites for the transcription factors NGFI-A and Sp1 in the exon $1_7$ GR promoter region [26], and showed that the offspring of High-LG dams exhibit an increased association of NGFI-A with its cognate site in hippocampal tissue at P6 compared with the offspring of Low-LG dams [10]. Furthermore, this site must be intact for NGFI-A to exert its effects on the methylation status of an exon $1_7$ GR promoter construct [16]. We therefore used ChIP assays to examine whether maternal care dynamically regulates the binding of the transcription factors NGFI-A and Sp1 to their overlapping response elements in the exon $1_7$ GR promoter in hippocampal samples from P4 offspring of High- and Low-LG mothers.

There was a significant interaction effect of maternal care with nesting condition on the association of NGFI-A with the exon $1_7$ promoter (figure 2*d*; $F_{1,17} = 5.85$, $p = 0.03$). *Post-hoc* analysis showed significantly ($p < 0.05$) increased NGFI-A association with the exon $1_7$ promoter in hippocampal samples obtained following the On condition when compared with the Off condition in the offspring of High-LG mothers; there were no differences as a result of On and Off conditions in the offspring of Low-LG mothers. There was no effect of maternal care on NGFI-A association with the exon $1_7$ promoter in samples obtained following the Off condition. We also quantified the binding of Sp1 to the exon $1_7$ GR promoter in separate fractions from the same tissue. Analyses revealed a trend for an interaction effect (figure 2*e*; $F_{1,14} = 2.10$, $p = 0.1$) and a significant main effect of maternal care ($F_{1,14} = 4.33$, $p = 0.05$), reflecting increased Sp1 association with the exon $1_7$ GR promoter in hippocampus of the P4 offspring of High- compared with Low-LG mothers. *Post-hoc* tests following the interaction trend did reveal significantly ($p < 0.05$) greater Sp1 association with the exon $1_7$ GR promoter in hippocampus obtained following the On condition of the offspring of High-LG mothers than in samples from the remaining groups. Additionally, Sp1 binding to a cognate response element with a different sequence in the GR exon $1_{11}$ promoter was evaluated, but levels from the specific fraction were comparable to levels from the IgG-precipitated fraction, indicating that Sp1 binding to this region is negligible (data not shown).

Maternal care dynamically regulates both circulating thyroid hormone and NGFI-A association with the exon $1_7$ GR promoter. Previous studies show that thyroid hormones regulate NGFI-A expression [33]. We thus examined whether (i) increased thyroid hormone levels were sufficient to increase NGFI-A binding to the exon $1_7$ GR promoter and (ii) whether such effects were 5-HT-dependent. Pups from uncharacterized mothers were given a single subcutaneous injection of saline or the 5-HT transporter-dependent neurotoxin p-chloroamphetamine (PCA; 7.5 $\mu g\, g^{-1}$ bw) at P0 to lesion 5-HT terminals, as previously described [34]. This treatment was followed by daily subcutaneous injections of T4 (2.5 $\mu g\, g^{-1}$) until P4, at which time they were perfused and hippocampi collected by gross dissection for ChIP assays of NGFI-A association with the exon $1_7$ GR promoter. Plasma was collected from pups processed in parallel, and ELISA results confirmed that T4 treatment elevated T3 levels compared with saline-injected pups (data not shown). We found a significant interaction effect (figure 2*f*; $F_{1,12} = 35.2$, $p < 0.0001$), indicating a greater association of NGFI-A with the exon $1_7$ GR promoter in T4-treated saline controls with no effect among PCA-treated animals. *Post-hoc* analysis showed significantly greater NGFI-A association with the exon $1_7$ GR promoter in T4-treated control animals than each of the other groups ($p < 0.05$). These results suggest that thyroid hormone signalling induces NGFI-A binding to the exon $1_7$ GR promoter, and that this effect requires 5-HT activity. There was also a highly significant effect of PCA treatment, suggesting that detectable NGFI-A binding to the exon $1_7$ GR promoter requires intact 5-HT innveration.

### (f) *NGFI-A is required for serotonin induction of glucocorticoid receptor expression* **in vitro**

Previous studies showed that chronic 5-HT treatment over 4 days can induce increased levels of GR in

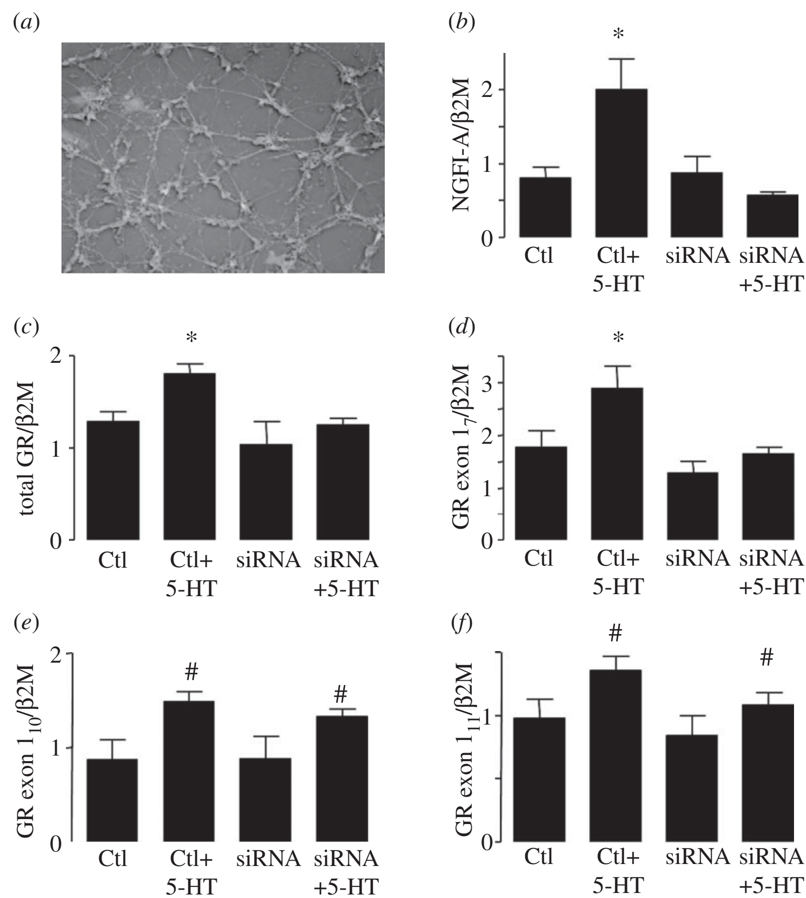


Figure 3. 5-HT induction of GR expression in cultured hippocampal neurons is NGFI-A dependent. (*a*) A photomicrograph of cultured hippocampal cells expressing a lentiviral vector containing an NGFI-A siRNA/GFP expression construct. Transduction rates approached 95%. (*b–f*) Mean ± s.e.m. levels of (*b*) NGFI-A, (*c*) total GR and (*d*) the exon $1_7$, (*e*) exon $1_{10}$ and (*f*) exon $1_{11}$ glucocorticoid receptor promoters transcripts in primary hippocampal cell cultures transducing either an empty or an siRNA-containing vector and treated with control (media alone) or 100 nM 5-HT in media. $^*p < 0.05$ vs all other groups, $^{\#}p < 0.05$ vs Ctl.

primary dissociated cultures of hippocampal neurons [20,21]. Interestingly, the addition of thyroid hormone to culture media has no effect on GR expression (J. B. Mitchell & M. J. Meaney 1992, unpublished data). We then examined whether this effect of 5-HT on hippocampal GR expression is NGFI-A dependent using a lentiviral vector that transduced cells with cDNA coding for either an siRNA against NGFI-A or a non-silencing siRNA-like sequence (empty vector) and a GFP tag. Figure 3*a* shows a representative fluorescent micrograph of an infected primary hippocampal cell culture reflecting transduction rates near 95 per cent based on quantification of GFP-positive cells. Cells from both conditions were treated with control media or media supplemented with 100 nM 5-HT at 3 days following viral infection. This concentration was selected on the basis of previous studies, including concentration–response studies [20,21]. Cells were harvested after 4 days of 5-HT treatment and extracted RNA converted to cDNA for qRT-PCR analysis.

Analysis of NGFI-A cDNA levels revealed a significant interaction effect between 5-HT and siRNA treatments (figure 3*b*; $F_{1,11} = 8.32$, $p = 0.02$) such that there was an elevation of NGFI-A expression in the 5-HT-treated neurons expressing the non-silencing siRNA sequence that was absent in the siRNA-expressing cells. There was no effect of the siRNA in control-treated cultures. Analysis of GR coding transcripts from these cells showed significant main effects of both vector ($F_{1,11} = 12.07$, $p = 0.005$) and 5-HT treatment ($F_{1,11} = 4.74$, $p = 0.05$). *Post-hoc* analysis showed that GR transcript levels in cells transduced with the non-silencing siRNA were significantly (figure 3*c*; $p < 0.05$) higher in 5-HT-treated compared with media-alone (control) cells. There was no such effect among cells treated with the NGFI-A siRNA. There was a similar pattern for exon $1_7$ GR transcript levels (figure 3*d*): there were significant main effects of both vector ($F_{1,11} = 8.49$, $p = 0.01$) and 5-HT ($F_{1,11} = 6.12$, $p = 0.03$). *Post-hoc* analysis revealed that exon $1_7$ GR transcript levels in hippocampal cells transduced with the non-silencing siRNA were significantly (figure 3*c*; $p < 0\ 0.05$) higher in 5-HT-treated compared with media-alone (control) cells, with no 5-HT effect among cells treated with the NGFI-A siRNA.

Interestingly, the same analyses performed on two GR exon 1 promoter sequences that do not contain an NGFI-A response element revealed a different pattern of 5-HT effects (figure 3*e*,*f*). Analysis of the expression of exon $1_{10}$ GR promoter showed only a main effect of 5-HT ($F_{1,10} = 11.43$, $p = 0.007$). *Post-hoc* analysis revealed a significant effect ($p < 0.05$) of 5-HT among cells transduced with the

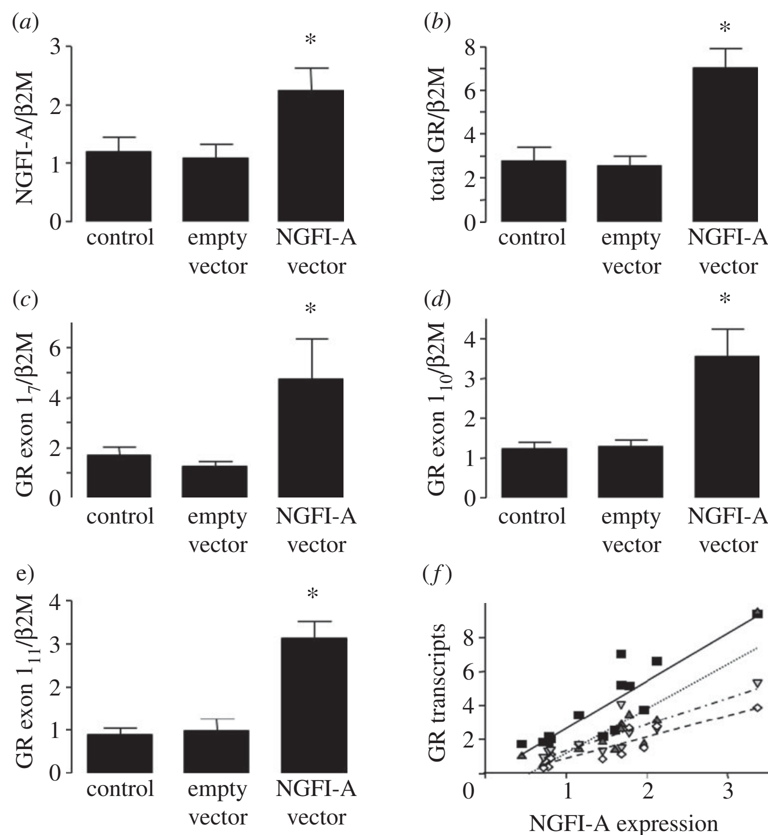


Figure 4. NGFI-A overexpression regulates GR expression in primary hippocampal cell cultures. (*a–e*) Mean ± s.e.m. normalized transcript levels for (*a*) NGFI-A, (*b*) total GR mRNA, (*c*) the exon $1_7$ GR promoter, (*d*) the exon $1_{10}$ GR promoter and (*e*) the exon $1_{11}$ GR promoter. *$p < 0.05$. (*f*) Results of linear regression analyses showing highly significant correlations (all $p < 0.001$) between NGFI-A expression and GR transcript levels (squares, GR coding; triangles, GR $1_7$; inverted triangles, GR $1_{10}$; diamonds, GR $1_{11}$).

non-silencing siRNA and a trend ($p < 0.10$) for a 5-HT effect among cells treated with the NGFI-A siRNA. Importantly, the difference in exon $1_{10}$ transcript levels between 5-HT-treated cells transduced with the non-silencing or NGFI-A siRNA was not significant, suggesting an increased 5-HT expression despite a reduction in NGFI-A levels. A similar analysis of exon $1_{11}$ transcripts also showed no interaction effect ($F_{1,11} = 0.27$, $p = 0.6$) and no main effect of vector ($F_{1,11} = 2.68$, $p = 0.1$), but a main effect of 5-HT ($F_{1,11} = 5.92$, $p = 0.03$).

These results indicate that 5-HT increases hippocampal GR transcription through effects on multiple promoters. NGFI-A does not contribute to 5-HT-induced increases in GR mRNA leader sequences with promoter regions free of the NGFI-A response element (e.g. exons $1_{10}$ and $1_{11}$). However, NGFI-A does contribute to expression of exon $1_7$ and, most importantly, expression of the GR-coding mRNA. Although the effect of 5-HT on the expression of the exon $1_{10}$ and $1_{11}$ transcripts is not abolished by NGFI-A knockdown, this manipulation was effective in abolishing the effect of 5-HT on GR exon $1_7$ and coding mRNA sequences (figure 3*c*,*d*). The reasons for this apparent contradiction are not obvious, but suggest that 5-HT has widespread effects on the GR gene, not all of which are mediated by NGFI-A. In fact, numerous binding sites for a variety of transcription factors lie in the GR exon 1 promoter sequences [26]. However, the data indicate that in hippocampal cell populations, exon $1_7$ promoter activity might be essential for effects on GR-coding mRNA transcription.

### (g) *NGFI-A overexpression induces glucocorticoid receptor expression*

We directly examined the effect of NGFI-A on GR transcription using primary dissociated hippocampal neuronal cultures transduced with lentiviral vectors expressing either NGFI-A cDNA or a scrambled version (empty vector) from a CMV promoter 4 days after plating. Cultures were maintained in otherwise normal conditions for 10 days before harvesting, RNA extraction and cDNA preparation for qRT-PCR analysis. Statistical analysis demonstrated a significant treatment effect (figure 4*a*; $F_{2,11} = 4.69$, $p = 0.03$), such that the NGFI-A overexpression vector induced an increase in NGFI-A expression compared with the untreated controls and cells treated with an empty vector ($p < 0.01$ for all comparisons). Similar analyses showed a significant increase in total GR expression (figure 4*b*; $F_{2,11} = 14.75$, $p = 0.008$), exon $1_7$ expression (figure 4*c*; $F_{2,11} = 4.99$, $p = 0.03$), exon $1_{10}$ expression (figure 4*d*; $F_{2,8} = 8.57$, $p = 0.01$) and exon $1_{11}$ expression (figure 4*e*; $F_{2,8} = 15.70$, $p = 0.002$). In each case, *post-hoc* analyses showed that target transcript levels were significantly ($p < 0.05$) greater in cells transduced with the NGFI-A overexpression construct compared with cells transduced with empty vector or untreated controls.

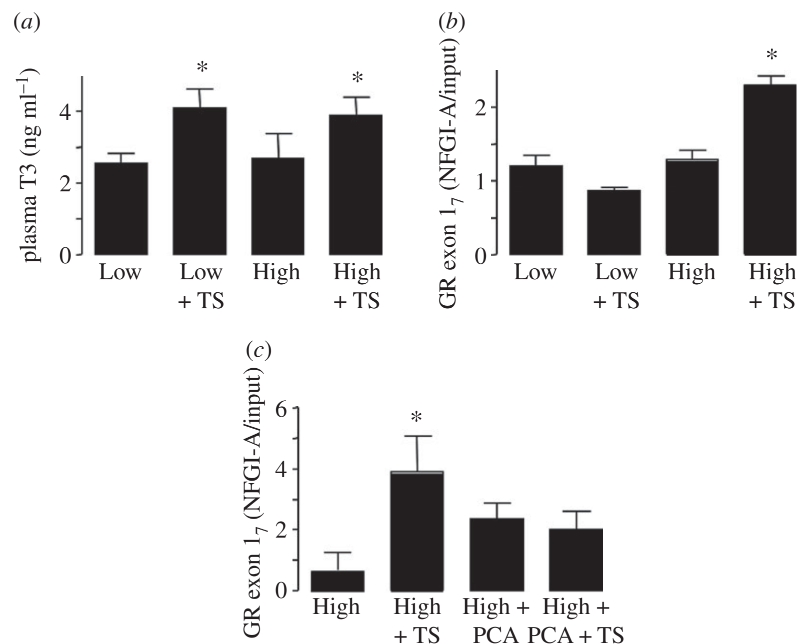


Figure 5. Tactile stimulation regulates plasma thyroid hormone levels and NGFI-A association with the exon $1_7$ GR promoter. Mean $\pm$ s.e.m. levels of plasma T3 (*a*) or exon $1_7$ GR promoter transcripts amplified from NGFI-A immunoprecipitated hippocampal nuclear fractions (*b*) in the P4 offspring of High- and Low-LG mothers after 30 min of maternal deprivation (High/Low) or 25 min of maternal deprivation and 5 min of artificial tactile stimulation (TS). (*c*) Mean $\pm$ s.e.m. level of exon $1_7$ GR promoter transcripts amplified from NGFI-A immunoprecipitated hippocampal nuclear fractions in saline-treated P4 offspring of High-LG mothers after 30 min of maternal deprivation (High) or 25 min of maternal deprivation and 5 min of artificial tactile stimulation (High + TS) or deprivation alone/deprivation + TS following p-chloroamphetamine (PCA; 7.5 $\mu g\, g^{-1}$ bw). $*p < 0.05$.

We also plotted GR transcript levels as a function of NGFI-A expression (figure 4*f*) and performed a linear regression analysis. This analysis revealed significant linear correlations between the expression of NGFI-A and total GR ($r^2 = 0.78$, $p < 0.0001$), exon $1_7$ expression ($r^2 = 0.79$, $p < 0.0001$), exon $1_{10}$ expression ($r^2 = 0.76$, $p = 0.0005$), and exon $1_{11}$ expression ($r^2 = 0.81$, $p = 0.0002$). These results indicate that overexpression of NGFI-A can drive GR transcription through various exon 1 mRNA leader sequences, and that an increase in NGFI-A expression is sufficient to increase GR transcription. We hypothesize that this overexpression of NGFI-A results in higher amounts of the protein binding to its response element and encouraging epigenetic remodelling as seen in previous *in vitro* experiments with NGFI-A overexpression and an exon $1_7$ GR promoter construct [16]. The resultant changes to local chromatin structure would provide a favourable environment for the transcription of adjacent transcripts such as the exon $1_{10}$ and $1_{11}$ sequences. Combined with the data reported in figure 3, we conclude that NGFI-A is sufficient to induce the transcription of the GR coding sequence and exons $1_7$, $1_{10}$ and $1_{11}$; and is necessary to the 5-HT effects on the GR coding sequence and exon $1_7$, but not the 5-HT effects on exons $1_{10}$ and $1_{11}$.

### (h) *Tactile stimulation regulates plasma triiodothyronine- and 5-HT-dependent NGFI-A binding to the exon $1_7$ glucocorticoid receptor promoter*

Pup LG is a source of tactile stimulation with immediate consequences for pup physiology [35,36]. Several immediate and long-term effects of maternal deprivation are reversed with artificial 'stroking' of the pups, which mimics the tactile stimulation afforded by the LG from the mothers [35–37]. We examined the effects of tactile stimulation on plasma T3 levels from P4 pups after 30 min of maternal separation or 25 min of separation followed by 5 min of tactile stimulation (stroking with a horse-hair artist's brush) [35–37]. There was a significant main effect of tactile stimulation on plasma T3 levels (figure 5*a*; $F_{1,12} = 6.90$, $p = 0.02$). *Post-hoc* analyses revealed that tactile stimulation significantly ($p < 0.05$) increased plasma T3 levels in the offspring of High- or Low-LG mothers. These data show that although the pup LG bout of a Low-LG mother is insufficient to produce an elevation in plasma T3 in her offspring (figure 2*a*), the offspring are capable of mounting a T3 response to tactile stimulation. This ability is consistent with the data demonstrating that the offspring of Low-LG dams cross-fostered onto High-LG dams also show increased GR expression [8–10].

To investigate the effects of tactile stimulation on the association of NGFI-A with the exon $1_7$ GR promoter, we obtained hippocampi from perfused P4 offspring of High- and Low-LG mothers after 30 min of maternal separation or 25 min of separation followed by 5 min of tactile stimulation for ChIP analysis as described earlier. Statistical analysis revealed a significant interaction effect between maternal phenotype and tactile stimulation (figure 5*b*; $F_{1,10} = 35.74$, $p = 0.0001$), such that tactile stimulation was associated with increased ($p < 0.05$) NGFI-A association with the exon $1_7$ GR promoter in the offspring of High-, but

not of Low-LG mothers. These data suggest that, like pup LG, tactile stimulation produces a significant induction of NGFI-A binding to its consensus sequence in the exon $1_7$ GR promoter, at least in the offspring of High-LG mothers.

We hypothesized that the repeated elevations in circulating T3 experienced by the offspring of High-LG mothers (and absent in the offspring of Low-LG mothers) lead to epigenetic changes that render the exon $1_7$ GR promoter more accessible to transcription factor binding in hippocampal cells, and that this epigenetic change might be initiated by 5-HT as it is *in vitro* [16]. We tested this hypothesis by examining the role of 5-HT in tactile stimulation-induced NGFI-A binding to the exon $1_7$ GR promoter in the offspring of High-LG mothers. We treated pups on P0 with saline or 7.5 $\mu g\, g^{-1}$ bw of PCA [34] and perfused on P4 following the same tactile stimulation protocol described earlier. We then performed ChIP assays to quantify NGFI-A association with the exon $1_7$ GR promoter. We found a significant interaction effect between PCA treatment and tactile stimulation (figure 5*c*; $F_{1,8} = 5.57$, $p = 0.05$). *Post-hoc* analysis showed that tactile stimulation produced an increase in the association of NGFI-A with the exon $1_7$ GR promoter in the saline-treated offspring of High-LG mothers; this effect was absent among PCA-treated animals. These data show that PCA blocks the ability of tactile stimulation to induce NGFI-A binding to the exon $1_7$ GR promoter in the offspring of High-LG mothers, a finding consistent with the hypothesis that the effects of tactile stimulation derived from pup LG on NGFI-A regulation of GR transcription are mediated by the repeated stimulation of ascending 5-HT innervation into the hippocampus.

## 4. DISCUSSION

Our findings are consistent with the proposed model for the effects of maternal care on hippocampal GR expression. While the results of earlier studies suggest that variations in specific forms of maternal care in the rat actively regulate NGFI-A expression with associated changes in transcription, such studies are largely correlational [16,18]. This concern applies to both the influence of maternal care and the effect of NGFI-A on GR transcription. We addressed the first issue by comparing hippocampal samples obtained from animals following a period of mother–pup contact and pup LG (On condition) or following a naturally occurring maternal separation from the pups (Off condition). We found an increased association of NGFI-A with the exon $1_7$ GR promoter in the offspring of High-LG mothers, but only in samples obtained in the On condition (figure 2*d*). There was a comparable effect in studies examining Sp1 association with the exon $1_7$ GR promoter (figure 2*e*), which was not detected at other Sp1 sites in the exon $1_{10}$ and $1_{11}$ promoters that do not overlap NGFI-A binding sites (data not shown). Sp1 protein levels in the hippocampi of P4 pups are also regulated by maternal care (figure 1*f*). We previously showed a similar effect of maternal care on NGFI-A association with the GAD1 promoter [31], which also contains an NGFI-A consensus sequence. These findings suggest that the increased interaction of NGFI-A with its consensus sequence within promoters that regulate transcription, such as that for the GR gene, is determined by active periods of mother–pup interaction.

Previous studies implicated thyroid hormones in the regulation of hippocampal 5-HT activity and GR expression [17,18]. The present findings suggest that an increased frequency of pup LG typical of High-LG mothers associates with an increased conversion of the T4 precursor to the more active T3 in BAT (figure 2*c*). Interestingly, pup LG produces a modest but physiologically relevant decrease in pup body temperature [38], which commonly associates with increased sympathetic activity. Sympathetic activation of BAT stimulates the conversion of T4 to T3 [39]. The absence of any difference in deiodinase activity between the On and Off conditions suggests that this effect occurs independent of concurrent maternal contact, implying a sustained effect that might reflect maternal regulation of sympathetic input to BAT. However, we did see increased plasma T3 levels in the offspring of both High- and Low-LG mothers as a result of tactile stimulation (figure 5*a*). This result indicates that the offspring of Low-LG mothers are capable of mounting a T3 response to prolonged tactile stimulation, suggesting that additional mechanisms might control plasma T3 levels in response to maternal contact. It is worth noting that the typical On bout of a Low-LG mother was found to last a mean of 4.3 min (*q.v.*, §3*a*); over this time the mother typically attends to multiple pups within the litter. In contrast, our tactile stimulation protocol lasted 5 min, and pups were stroked for 30 s consecutively of each minute. It might therefore be considered a more prolonged and frequent stimulus than the LG bout of a Low-LG mother. Our data show that Low-LG pups are capable of mounting a T3 response to stroking (figure 5*a*), but the behaviour of the Low-LG mother never elicits this response (figure 2*a*), which is consistent with the ability of High-LG mothers to reduce exon $1_7$ GR promoter methylation and promote GR expression in hippocampi from the cross-fostered offspring of Low-LG mothers [8,10].

Previous research has implicated tactile stimulation and other environmental stimuli as providing impetus for biological signalling within neonates. For example, tactile stimulation of pups can prevent a specific decrease in enzymatic activity associated with maternal separation that could not be reversed by an anaesthetized mother [35,40]. Stroking neonates during isolation suppresses stress-induced elevations of adrenocorticotropin (ACTH) secretion caused by maternal deprivation [41], and differences in the frequency of stroking stimuli administered to artificially reared offspring altered both maternal behaviour and open-field activity in later life [37]. The environmental enrichment provided by the mouse communal nesting paradigm also blunts adult neuroendocrine and behavioural response [40], and though High- and Low-LG mothers have litters of comparable sizes, the increased maternal LG behaviour might represent a similar form of environmental enrichment [23]. Interestingly, a comparable situation has been

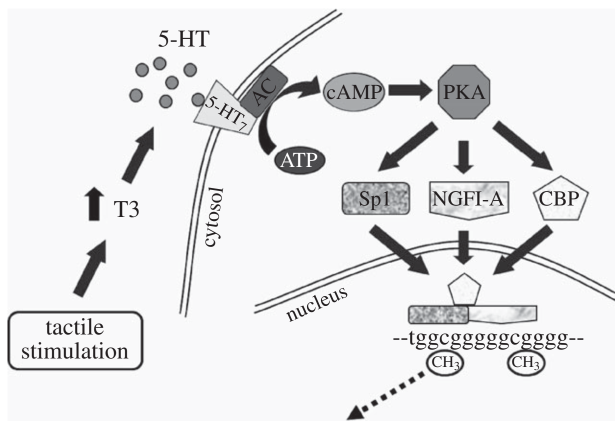


Figure 6. Depiction of the proposed mechanism for tactile stimulation effects on GR gene expression. Tactile stimulation derived from pup LG increases plasma levels of thyroid hormone, which in turn stimulates 5-HT signalling in the hippocampus. Increased 5-HT levels work through the 5-$HT_7$ receptor to stimulate cAMP, which in turn stimulates PKA. Molecules such as the histone acetyltransferase CBP and the transcription factors NGFI-A and Sp1 are activated through the actions of PKA and form a complex that binds to specific genomic regions such as the exon $1_7$ GR promoter. The binding of this complex initiates the demethylation process at this region, and provides a permissive environment for gene expression.

apparent in reviews of the human literature for almost 30 years, noting that tactile, vestibulo-proprioceptive and mutisensory inputs reversed some of the developmental impairments associated with pre-term birth [42]. Massage and passive limb movement of pre-term infants is capable of accelerating growth and maturation of electroencephalograph activity, possibly through activation of the vagal system and the release of growth hormone and insulin-related growth factors [43–46]. These studies all provide important examples of how chronic differences in early life environment can have life-long developmental consequences for the phenotype of neonatal mammals.

Our working model (figure 6) predicts that maternal LG and tactile stimulation regulate GR expression through increases in hippocampal 5-HT that lie downstream of the dynamic T3 signalling events observed after tactile stimulation and the LG bouts of High-LG mothers. T3 administration in the neonatal period results in an increase in GR binding in rats as adults [17], and inhibition of T3 production in neonates by maternal administration of propylthiouracil prevents the effects of handling on 5-HT turnover and NGFI-A expression [18]. The positioning of thyroid hormones within our working model was reflected in the findings that daily T4 administration increases NGFI-A association with the exon $1_7$ GR promoter, and that this effect is blocked by chemical lesioning of the ascending 5-HT system (figure 2*f*). These findings confirm that chronic elevations in T3 are sufficient to drive NGFI-A binding to the exon $1_7$ GR promoter in a 5-HT-dependent manner, consistent with previous reports of thyroid-hormone-induced increases in hippocampal 5-HT activity [18], NGFI-A expression [33], GR binding [17], and the ability of ketanserin to block handling-induced increases in hippocampal NGFI-A levels [18].

This conclusion is buttressed by the experimental finding that the stroking of pups, which mimics the tactile stimulation derived from pup LG, increases the association of NGFI-A with the exon $1_7$ GR promoter (figure 5*b*). Interestingly, this effect was apparent only in the offspring of High-LG mothers. This finding is similar to the results of previous studies using brief, daily postnatal handling of pups, which increases the frequency of pup LG in lactating rats [9]. Repeated handling results in a cAMP- and PKA-dependent increase in hippocampal NGFI-A expression, and the effect of handling can be blocked by either thyroid hormone synthesis inhibition or 5-$HT_7$ receptor antagonism [18]. However, the effects on PKA and NGFI-A are only apparent following repeated daily handling, suggesting a 'sensitization' of the underlying molecular signalling pathways. We suggest that this same process underlies the increased response of the pups of High- compared with Low-LG mothers to an acute episode of experimental tactile stimulation. The offspring of High-LG mothers are subject to longer LG bouts more frequently than are the offspring of Low-LG animals. This difference represents a prolonged and profound environmental difference in the early experience of the respective offspring, affecting gene expression and therefore phenotype through signalling pathways that include endocrine (T3), neural (5-HT) and intracellular (NGFI-A/CBP) signals that are dynamically regulated by maternal LG and stroking.

The ability of tactile stimulation to elicit an increase in T3 without a concomitant increase in NGFI-A binding to the exon $1_7$ GR promoter in the offspring of Low-LG mothers (figure 5*a*,*b*) suggests a sensitization of these pathways in the offspring of High-LG mothers. The chronically elevated levels of pup LG exhibited by High-LG mothers result in repeated elevations in T3 signalling in their offspring over the course of days. This drives NGFI-A binding to the exon $1_7$ GR promoter in a 5-HT-dependent manner, and it is our hypothesis that this NGFI-A binding to specific response elements in the genome initiates changes to local chromatin structure to create a permissive environment for gene expression. Furthermore, we hypothesize that changes of this nature require prolonged influence from the environment, such as the differences between the LG behaviours of High- and Low-LG mothers. Low-LG mothers do not elicit the same repeated T3 increases in their offspring as High-LG mothers, and as such, their offspring would not experience the same chronically elevated increases in NGFI-A activity driving the establishment of this permissive environment for transcription factor binding. As a result, NGFI-A is unable to access its response element in the offspring of Low-LG mothers despite transient increases in T3. Our hypothesis predicts a similar phenomenon underlying the lack of an increase in NGFI-A binding to the exon $1_7$ GR promoter in the PCA-treated offspring of High-LG mothers: although the LG bout should be sufficient to increase T3 levels in the PCA-treated pups, the damage to the serotonergic neurons prevents the repeated increases

in 5-HT-driven activity to initiate changes in chromatin status, rendering them unresponsive to tactile stimulation at the level of NGFI-A binding to the exon $1_7$ GR promoter.

The exon $1_7$ GR promoter contains an NGFI-A consensus sequence [26] and ChIP assays reveal increased NGFI-A association with the exon $1_7$ promoter in the neonatal offspring of High- compared with Low-LG mothers ([10,16]; figure 3*a*). Extensive sequencing of the exon $1_7$ promoter has not revealed any evidence for a polymorphism or for other genetic variations that might influence gene expression in a *cis*-fashion ([10,11,16]; M. Sokolowski & M. J. Meaney 2007, unpublished data). In concert with the data presented here and the knowledge that the offspring of Low-LG mothers cross-fostered onto High-LG mothers show increased GR expression [8,10], it would appear that differences in the level of NGFI-A association with the exon $1_7$ GR promoter are driven by maternally regulated increases in NGFI-A.

The results of the *in vitro* studies directly link hippocampal NGFI-A and GR expression. Overexpression of NGFI-A in cultured hippocampal neurons produced an increase in GR transcription as well as in GR transcripts bearing the exon $1_7$ sequence, suggesting increased transcriptional activation through this promoter (figure 4*b*,*c*). Indeed, total GR mRNA levels were highly correlated with those of the exon $1_7$ GR promoter (figure 4*f*). Interestingly, the same effect was apparent for GR mRNA transcripts bearing the exon $1_{10}$ and exon $1_{11}$ GR promoter sequences and expression of these promoters was also highly correlated with activity of these promoters (figure 4*f*). *In silico* analysis did not reveal evidence for an NGFI-A consensus sequence within these promoters. However, NGFI-A has widespread transcriptional effects that could produce a downstream transcriptional activation through these sequences. Nevertheless, the findings reflect the direct effect of NGFI-A on GR transcription. The influence of NGFI-A is also apparent in the studies using an siRNA-targeting NGFI-A expression. The transduction of cultured hippocampal neurons with a lentiviral vector bearing an NGFI-A siRNA (figure 3*a*,*b*) completely blocked the effects of 5-HT on total GR mRNA and exon $1_7$ GR promoter transcripts (figure 4*c*,*d*). In contrast, while 5-HT also increased transcripts bearing the exon $1_{10}$ and exon $1_{11}$ GR promoter sequences, the influence of the NGFI-A siRNA on such 5-HT-induced transcription was absent (exon $1_{10}$) or modest (exon $1_{11}$). Importantly, both NGFI-A overexpression and 5-HT treatment represent chronic conditions and 5-HT treatments shorter than 4 days are ineffective in inducing GR expression [19].

According to our hypothesis, a chronic elevation in NGFI-A should correspond to the association with elements capable of chromatin remodelling (figure 6). Previous ChIP studies of neonatal hippocampus also reveal increased CBP association with the exon $1_7$ promoter in the offspring of High- compared with Low-LG mothers [16]. CBP is a histone acetyltransferase [47] and, predictably, assays with the same samples show increased levels of histone 3 lysine 9 (H3K9) acetylation at the exon $1_7$ promoter of the offspring of High- compared with Low-LG mothers [10,16]. This histone post-translational modification is strongly associated with open chromatin and increased transcriptional activity [27,28]. Co-immunoprecipitation assays (figure 1*b*) are consistent with previous studies showing an interaction between NGFI-A and CBP [48], and the increases in both CBP binding and H3K9 acetylation (H3K9ac) at the exon $1_7$ GR promoter depend upon an intact NGFI-A response element [16]. These findings suggest that the transcription factor NGFI-A anchors a complex that includes the chromatin remodelling enzyme CBP. Increased H3K9ac associates with decreased DNA methylation. While this association reflects in large measure the ability of methylated DNA to recruit histone deacetylases (HDACs) to maintain low levels of histone acetylation [49,50], there is evidence for bidirectional effects: HDAC inhibitors increase histone acetylation and can initiate DNA demethylation [51,52]. Intra-hippocampal infusion of an HDAC inhibitor into the adult offspring of Low-LG mothers increases H3K9ac of the exon $1_7$ GR promoter, which in turn associates with demethylation of the promoter sequence and increased GR transcription [10]. Likewise, co-transfection studies show that NGFI-A overexpression results in the demethylation of a previously methylated exon $1_7$ GR promoter construct [16].

Interestingly, at birth, the exon $1_7$ GR promoter sequence shows a comparable level of methylation in offspring of High- and Low-LG mothers. The differences in the level of promoter methylation and in GR transcription emerge over the first week of life [10], which corresponds to the period when High- and Low-LG mothers differ in the frequency of pup LG [22,53]. Studies with cultured hippocampal cells show that treatment with 5-HT or 8-bromo-cAMP results in a demethylation of the exon $1_7$ GR promoter [16]. The effect of 5-HT is blocked by an antisense oligonucleotide against NGFI-A [16]. Taken together with the results of the current study, these findings suggest that the tactile stimulation associated with pup LG increases thyroid hormone metabolism and activates ascending 5-HT pathways to the hippocampus. The increased 5-HT signalling induces an increase in NGFI-A expression and the binding of NGFI-A/CBP complexes to the exon $1_7$ GR promoter. The binding of CBP increases H3K9ac as an initial step in the maternally regulated demethylation of the promoter sequence. By comparing pups in the On and Off conditions, we also found that pup LG dynamically modulated the association of the transcription factor Sp1 to the overlapping NGFI-A/Sp1 response elements in the exon $1_7$ GR promoter (figure 2*e*). This increase in Sp1 binding was absent at another Sp1 site in the exon $1_{11}$ promoter that does not overlap with an NGFI-A response element (data not shown), which suggests that NGFI-A may recruit a number of factors to highly specific genomic sites. *In vitro* studies show that overexpression of Sp1 leads to the remodelling of DNA methylation states [54,55]. Taken together, these findings suggest that the tactile stimulation derived from pup LG regulates peripheral and extracellular signals, notably 5-HT, which then initiate an intracellular cascade that actively remodels

the epigenetic state of the exon $1_7$ GR promoter, including that of cytosine methylation. This complex includes the transcription factors NGFI-A and Sp1, and the histone acetyltransferase CBP. Recent preliminary studies also suggest the active participation of the methylated DNA-binding protein, MBD2, which appears to enhance NGFI-A binding to its consensus sequence on the exon $1_7$ GR promoter. What remains to be identified is the enzyme directly responsible for the altered methylation state. Nevertheless, the data presented here provide support for a direct effect of variations in pup LG in the extracellular and intracellular signals that regulate the epigenetic programming of GR transcription and hypothalamic–pituitary–adrenal responses to stress.

Another important question surrounding the impact of the early environment is the potential to reverse such effects in later life. We have used pharmacological approaches, such as HDAC inhibition and methionine supplementation, to manipulate the epigenetic status of the exon $1_7$ GR promoter and reverse phenotypes established by maternal care [10,11]. The issue of whether a so-called critical period exists for maternal effects has yet to be addressed. Environmental enrichment in adolescence reverses at least some effects of maternal care [56], suggesting that even if this critical period exists, the effects of differences in maternal LG over the neonatal period might be reversible through a variety of mechanisms. The discovery that tactile stimulation regulates many of the same signalling mechanisms induced by maternal care suggests that manipulations of stroking in artificial rearing paradigms will allow the determination of this critical period, whether tactile stimulation of particular anatomical locations are more or less effective in eliciting these responses, and whether a 'dose-dependent' relationship exists between tactile stimulation in early life and effects on gene expression.

Previous studies show that variations in the frequency of pup LG in the rat associate with differences in the methylation of the exon $1_7$ GR promoter, an effect that persists into adulthood [10,16]. Methylation of the exon $1_7$ GR promoter is increased in the adult offspring of Low-LG mothers, and increased methylation reduces the capacity for NGFI-A binding to the exon $1_7$ GR promoter [16]. *In vivo* studies show increased association of NGFI-A with the exon $1_7$ GR promoter sequence in adult hippocampal tissue of the offspring of High- compared with Low-LG mothers, despite comparable levels of NGFI-A expression [10,16]. Intra-hippocampal infusion of a HDAC inhibitor reverses the differences in methylation, as well as those in NGFI-A binding, GR expression and HPA response to stress [10]. Importantly, the difference in methylation is initiated by the increased association of NGFI-A with the exon $1_7$ GR promoter [16]. Likewise, variations in pup LG over the first week of life result in the differential methylation of another NGFI-A-regulated gene, *GAD1*, which encodes for glutamic acid decarboxylase [31]. The results of the present studies suggest that variations in maternal care in the rat initiate a 5-HT-dependent intracellular cascade that regulates the binding of an NGFI-A-anchored complex that leads to the epigenetic remodelling of the exon $1_7$ GR promoter.

All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care with protocols approved by the McGill University Animal Care Committee.

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