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Genetic variation in the developmental regulation of cortical *avpr1a* among prairie voles

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Early experiences can have enduring impacts on brain and behavior, but the strength of these effects can be influenced by genetic variation. In principle, polymorphic CpGs (polyCpGs) may contribute to gene-by-environment interactions (G × E) by altering DNA methylation. In this study, we investigate the influence of polyCpGs on the development of vasopressin receptor 1a abundance in the retrosplenial cortex (RSC-V1aR) of prairie voles (*Microtus ochrogaster*). Two alternative alleles ('HI'/'LO') predict RSC *avpr1a* expression, V1aR abundance and sexual fidelity in adulthood; these alleles differ in the frequency of CpG sites and in methylation at a putative intron enhancer. We hypothesized that the elevated CpG abundance in the LO allele would make homozygous LO/LO voles more sensitive to developmental perturbations. We found that genotype differences in RSC-V1aR abundance emerged early in ontogeny and were accompanied by differences in methylation of the putative enhancer. As predicted, postnatal treatment with an oxytocin receptor antagonist (OTA) reduced RSC-V1aR abundance in LO/LO adults but not their HI/HI siblings. Similarly, methylation inhibition by zebularine increased RSC-V1aR in LO/LO adults, but not in HI/HI siblings. These data show a gene-by-environment interaction in RSC-V1aR. Surprisingly, however, neither OTA nor zebularine altered adult methylation of the intronic enhancer, suggesting that differences in sensitivity could not be explained by CpG density at the enhancer alone. Methylated DNA immunoprecipiation-sequencing showed additional differentially methylated regions between HI/HI and LO/LO voles. Future research should examine the role of these regions and other regulatory elements in the ontogeny of RSC-V1aR and its developmentally induced changes.

Keywords: differentially methylated region (DMR), DNA methylation, gene × environment interaction, *Microtus ochrogaster*, phenotypic plasticity, polymorphic CpG, retrosplenial cortex, V1aR, vasopressin receptor 1a, zebularine

Environmental experiences during early-postnatal development play a pivotal role in shaping an animal's neuronal and behavioral phenotypes later in life. Early-life experiences such as diet (Georgieff 2007), maternal care (Weaver *et al.* 2004), stress (Lupien *et al.* 2009) and toxin exposure (Kundakovic & Champagne 2011) can drastically change brain and behavior in adulthood. Most studies that use conventional animal models study the effects of early environment while controlling for genetic differences. In nature, however, individuals often differ in their sensitivity and response to environmental experiences (Pigliucci 2001). Genetic differences in environmental sensitivity or 'phenotypic plasticity' (Debat & David 2001) are known as gene-by-environment interactions (Pigliucci 2005), and recent work has identified single-nucleotide polymorphisms (SNPs) associated with variation in developmental risk or resilience (Gillespie *et al.* 2009). However, to better understand the mechanisms of gene-by-environment interactions (G × E), it is important to study this variation in conjunction with the epigenetic modifications that help relay environmental information to genes within the developing brain.

Among epigenetic marks, DNA methylation is the most intensely investigated, and it is often associated with neuronal reprogramming following early-life experiences (Szyf & Bick 2013). The DNA methylation is a stable epigenetic mark that can suppress gene expression by condensing chromatin, disrupting transcription factor, binding or attracting methyl-binding proteins, such as MeCP2 (Bird & Wolffe 1999). Eukaryotic DNA methylation is catalyzed by DNA methyltransferase (DNMT) enzymes, which add a methyl group to cytosines within a CG dinucleotide (Law & Jacobsen 2010). Since DNA methylation occurs almost exclusively at CpG sites, gaining or losing a CpG may alter susceptibility to DNA methylation and sensitivity to the environment. Interestingly, SNPs commonly occur at CpG sites (Tomso & Bell 2003) and such polymorphic CpGs (polyCpGs) have been associated with G × E effects (Parnell *et al.* 2014); nevertheless, their role in mediating plasticity in the developing brain remains largely unexplored. In this study, we investigate the role of polyCpGs in environmental sensitivity and neuronal phenotype by focusing on the vasopressin receptor 1a (*avpr1a*), a gene implicated in the social behavior of male prairie voles (*Microtus ochrogaster*).

Prairie voles are socially monogamous rodents known for their capacity to form enduring pair-bonds (Getz *et al.* 1993). Although prairie voles form bonds, individuals vary in their

sexual fidelity (Ophir *et al.* 2008; Phelps & Ophir 2009). Male fidelity has been linked to individual differences in space-use, and to the expression of vasopressin receptor 1a in the retrosplenial cortex (RSC), a brain region widely implicated in spatial memory (Vann *et al.* 2009). Abundance of *avpr1a* transcripts and vasopressin receptor 1a abundance in the RSC (RSC-V1aR) are strongly predicted by two *avpr1a* alleles, known as 'HI' and 'LO' alleles. These two alleles are defined by four linked SNPs; one of the SNPs (SNP 2170) is a polyCpG located within a putative intron enhancer (Fig. 1).

A variety of data suggests that HI and LO alleles may differ not only in their expression levels but also in their sensitivity to developmental environments. First, the 'HI'/'LO' genotype is a significantly better predictor of RSC-V1aR in uniformly reared lab animals, than in wild-caught animals (Okhovat *et al.* 2015). Second, the LO allele is characterized by a significant excess of polymorphic CpG sites within the intron enhancer; the methylation state of this enhancer is unique among surveyed sites in predicting RSC-V1aR and *avpr1a* expression (Okhovat *et al.* 2015, 2017). Lastly, LO/LO animals seem to be more variable in their phenotypes than HI/HI animals (Okhovat *et al.* 2015, 2017). Based on these data, we hypothesized that LO alleles are sensitive to developmental influences, while HI alleles are constitutively highly expressing.

To test this hypothesis, we first explore the ontogeny of V1aR abundance and *avpr1a* enhancer methylation in the RSC of HI/HI and LO/LO prairie vole pups, identifying when genotype differences in RSC-V1aR first emerge. Next, we manipulate developmental environments using two pharmacological interventions. Oxytocin receptor antagonist (OTA) delivered at postnatal day 1 decreases RSC-V1aR abundance in adulthood (Bales *et al.* 2007); we use this manipulation to determine whether LO/LO voles are more sensitive to silencing than HI/HI animals. To examine consequences of direct alteration of methylation, we compare genotype differences in sensitivity to zebularine, a DNMT inhibitor (Cheng *et al.* 2003). Because there is a strong relationship between *avpr1a* transcript abundance and V1aR protein abundance (Okhovat *et al.* 2015), and because protein abundance is critical to biological function, we use RSC-V1aR as our measure of RSC-*avpr1a* expression. After examining effects of treatments and genotypes on adult RSC-V1aR, we then examine how these treatments affect DNA methylation in the putative intron enhancer. Lastly, we use high-throughput sequencing to explore more distal differentially methylated regions (DMRs) between HI/HI and LO/LO voles, and discuss possible mechanisms that may underlie our findings.

Material and methods

Animal subjects

All animals were lab-reared descendants of prairie voles captured in Jackson County, IL. Breeding pairs heterozygous for the HI and LO *avpr1a* alleles were used to generate homozygous HI/HI and LO/LO siblings. All breeding pairs were kept in 25 × 45 × 60 cm cages in accordance to The Institutional Animal Care and Use Committee (IACUC) regulations and were given food and water *ad libitum*. All sample collection and manipulations were performed during light phase.

In our first study, we examined the natural ontogeny of V1aR abundance and *avpr1a* enhancer methylation. Unmanipulated pups were taken from 7 heterozygous breeding pairs on day 1, 7 or 14. In our second and third studies, developmental manipulations were all performed on postnatal day 1 (P1), and brains were taken on P21. For all three experiments, brains and tail clippings were collected and frozen on dry ice following euthanasia. Frozen tissues were stored at −80°C until further processing.

Neonatal manipulations

At P1, litters from heterozygous breeding pairs received drug or one of two control treatments (saline injection or handling). Treatments were given in randomized orders across breeding pairs. For drug dose calculations we assumed pups weighed on average 3 g at P1. All injections were done intraperitoneally with 30-gauge insulin syringes.

Our first manipulation involved injection of 0.1 mg/kg OTA ([d(CH2)5, Tyr(Me)2, Orn8]-vasotocin, Bachem, Bubendorf, Switzerland) dissolved in 50 μl injectable saline. Control litters were either handled or injected with 50 μl saline vehicle. All pups were returned to their home-cage after treatment, and remained in the cage undisturbed until weaning. At P21, animals were sexed and euthanized. The experiment was set up with 12 heterozygous breeding pairs, but individuals from 2 breeding pairs died before handling control litters were collected, reducing our sample size for the handling-only control.

To manipulate developmental methylation, we administered 400 mg/kg fresh zebularine (1-β-D-Ribofuranosyl-2(1H)-pyrimidinone, Tocris Biosciences, Ellisville, MO, USA) in 50 μl sterile saline. Control animals received 50 μl sterile saline or were handled without injection. The manipulations were conducted on repeated litters from seven heterozygous breeding pairs.

Genotyping

Genomic DNA (gDNA) was extracted from tail clippings using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All polymerase chain reaction (PCR) amplifications were performed on a BioRad C1000 Thermal cycler (BioRad, Hercules, CA, USA).

We determined sex of weanlings (P21) visually by inspecting anogenital distance. At P1, P7 and P14 we used a PCR assay targeting the SRY locus to determine sex. The PCR assay was validated by correctly predicting sex of six control male and female samples with known sex. We used primers designed to amplify a 214 bp region of the prairie vole SRY locus (FN433505.1, NCBI). Primer sequences were: F:3′-GTGGTCTCGTGATCAGAGGCGCAAG-5′ and R:3′-GGGTCTTGAGTCTCTGTGCCTCTTG-5′. The PCR reactions were set up in 25 ul reactions consisting of GoTaq Hot Start Colorless Master Mix (Promega, Madison, WI, USA), 200 nM of each primer, 1.5 ul gDNA and nuclease-free water. The PCR reaction condition was: 3 min at 93°C, (30 seconds at 93°C, 30 seconds at 63°C and 10 seconds at 72°C) × 32, 10 seconds at 72°C.

All individuals were genotyped for the *avpr1a* HI and LO allele using PCR amplification and allele-specific restriction digestion as described previously (Okhovat *et al.* 2015). Briefly, in a nested PCR assay we amplify 0.8 kb of the *avpr1a* intron, including the 2170 SNP that predicts HI and LO alleles. Next, we digest this amplicon with Bsh1236I restriction enzyme (ThermoFisher Scientific, Waltham, MA, USA) using settings recommended by manufacturer. The 2170 SNP produces a CGCG or CGCT sequence in the LO and HI allele, respectively. Therefore Bsh1236I, which recognizes the CGCG sequence, will only digest the LO allele. Following digestion, we run samples on agarose gel to visualize the banding pattern and determine genotype. Only homozygous subjects (HI/HI and LO/LO) were used for subsequent processing.

RSC-V1aR autoradiography

Frozen brains from homozygous HI/HI and LO/LO animals were sectioned in 20 μm-thick slices at 100 μm intervals and mounted on SuperFrost slides (ThermoFisher Scientific) in four series. The autoradiography procedure has been described previously (Okhovat *et al.* 2015; Ophir *et al.* 2008). In brief, sections were lightly fixed in 1% paraformaldehyde solution following a quick drying step. Slides

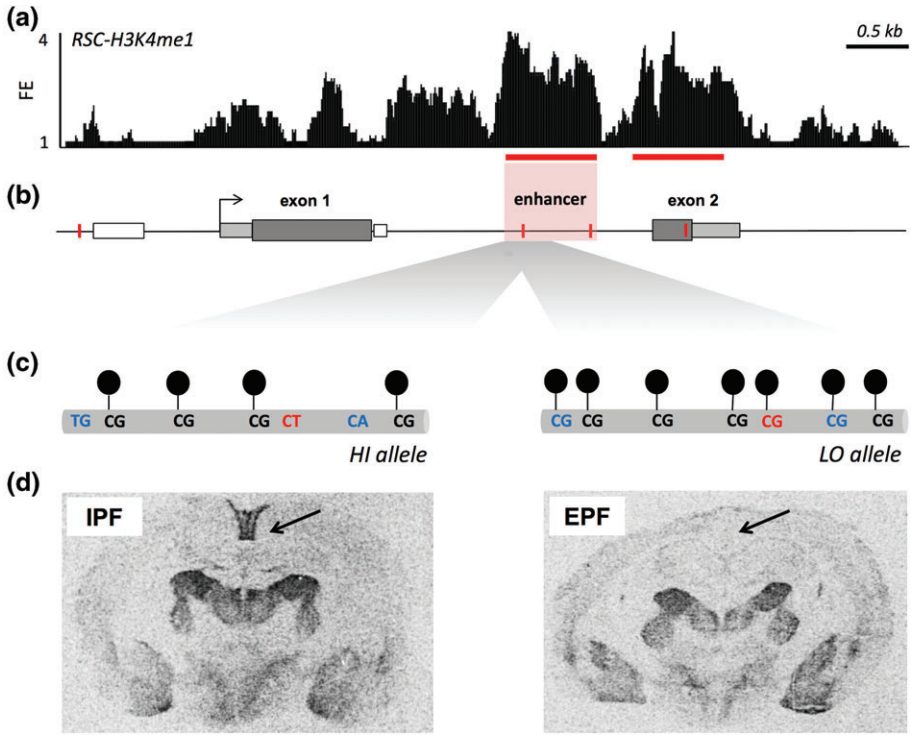

Figure 1: *Avpr1a* genotype differences in enhancer CpG density and susceptibility to DNA methylation. (a) Fold enrichment values for H3K4me1 ChIP-seq on RSC of eight male prairie voles is shown at the *avpr1a* locus. Putative enhancers are marked in red horizontal lines. (b) A schematic view of the *avpr1a* locus. Allele-defining SNPs are marked with red bars. (c) A schema of HI (*left*) and LO (*right*) allele differences in CpG and methylation density within the *avpr1a* putative intron enhancer. Sequence at SNP 2170 is shown in red, other polyCpGs in blue and fixed CpGs are black. Black circles depict 5-methyl at cytosines. (d) V1aR autoradiograms of intra-pair fertilizing (IPF, *left*) and extra-pair fertilizing (EPF, *right*) males. Arrow shows the RSC. (a), (b) and (d) modified from Okhovat *et al.* 2015.

were then washed in Tris and incubated with 50 pM of the radiolabeled ^{125}I-linear arginine vasopressin receptor antagonist (NEX310010UC, Perkin Elmer, Norwalk, CT, USA) for 70 min. Following incubation, slides were washed multiple times in Tris and rapidly dried under hot air. Sections were exposed to film for 68 h, along with radiographic standards. Developed films were digitized using Epson perfection V800 Photo scanner. For each individual, V1aR abundance in the RSC was scored from three sections using FIJI software (Schindelin *et al.* 2012) and averaged. Binding in non-expressing cortical regions of the same section was used to correct for non-specific binding.

DNA methylation

The RSC was dissected from alternative fresh frozen slides. We used the EpiTect Plus LyseAll Bisulfite Kit (Qiagen) to obtain bisulfite-converted gDNA from the dissected tissue. Next, we used a nested PCR approach to amplify two pyrosequencing-compatible fragments of the putative intron enhancer. Primer sequences and PCR settings have been described previously (Okhovat *et al.* 2015). The PCR amplicons were sent to Epigendx (Hopkinton, MA, USA) for bisulfite pyrosequencing.

DNA methylation was measured at four fixed and three polymorphic CpG sites (polyCpGs) and reported to us as %[unconverted C/(unconverted C + converted T)] at each CpG site. To confirm the results of digestion-based genotyping of pups described above, the genotype at polyCpG 2170 was also reported along with the methylation measurements, because this polyCpG is a G/T polymorphism, it is not influenced by bisulfite treatment. Among these reported genotypes, genotypes of 7 (out of 147) individuals differed from our digestion-based results. We interpret these conflicts as incomplete digestion in our digestion-based genotyping assay, but to be conservative, we excluded these subjects from the study.

Calculations of DNA methylation measures have been described previously (Okhovat *et al.* 2017). Briefly, *total %DNA methylation* was calculated by averaging methylation across all seven CpG sites (four fixed and three polymorphic). This measure treats an unmethylable site (e.g. a CG/CT polymorphism) as equivalent to a site that is methylable but unmethylated; thus at the three polymorphic CpG sites in our enhancer, some of the interindividual variation in methylation arises as a direct result of genotype differences. Although such differences are not strictly epigenetic in that they are influenced by sequence variation, they nevertheless provide a view of the overall methylation state of the enhancer that may reflect or influence function. To examine changes in methylation independent of sequence variation, we calculated *%methylation at fixed CpG sites* by averaging methylation at the four fixed (non-polymorphic) CpG sites. Also, we calculated *%methylation at methylable CpGs* by averaging methylation at CpG sites that contained a CG dinucleotide (i.e. all fixed CpG sites and polyCpGs that contain a CG allele). Lastly, we also examined methylation at single fixed CpG sites. Polymorphic CpG sites were not examined on a single CpG resolution basis as the HI allele almost always lacks CpG and methylation at those sites.

Statistical analysis

All data were analyzed in R (https://www.r-project.org). Mixed models were generated using the nlme package (Pinheiro *et al.* 2016). Single tail statistics were only used when direction was clearly predicted and justified based on previous data. Sex was initially included as a fixed effect in all mixed models, if its effect was not significant we repeated the analysis excluding sex effects.

Ontogeny of RSC-V1aR and avpr1a methylation

To characterize the ontogeny of RSC-V1aR at P1, P7 and P14, we generated a mixed model with genotype, age and genotype × age interaction as fixed effects and parentage as a random effect. As the power to detect interaction is generally low, and we had clear *a priori* predictions for interactions, we also examined RSC-V1aR levels between the HI/HI and LO/LO genotype at each age using single-tailed Welch *t*-tests.

To characterize the relationship between early postnatal RSC-V1aR and *avpr1a* methylation we performed a simple linear regression between RSC-V1aR and all three methylation measures in the putative enhancer. Next, we examined the effects of age and genotype on *avpr1a* methylation by generating a mixed model with age, genotype and age × genotype interaction as fixed effects and parentage as a random effect. We compared DNA methylation measures between

HI/HI and LO/LO genotypes at each age by single-tailed Welch *t*-tests. Similar mixed models and *post hoc t*-test comparisons were performed at a single CpG resolution for all four fixed CpGs, as well.

Neonatal manipulations

We examined the effects of two different neonatal manipulations on the RSC-V1aR of HI/HI and LO/LO animals. The two control groups (saline and handling only) did not show any statistical difference in any cases and were thus combined into a single control group (CON) within each study.

First, we assessed effects of P1 OTA injections on the RSC-V1aR at P21. We built a mixed model with sex, genotype, treatment and genotype × treatment as main effects and parentage as a random effect. Effects of OTA treatment were further examined within genotypes using mixed models with sex and treatment as fixed effects and parentage as a random effect.

Next, we examined effects of neonatal zebularine administration on P21 RSC-V1aR. Weight measures at P21 were used to test long-term cytotoxicity of zebularine. No significant effect of zebularine treatment was detected on weight, suggesting that long-term cytotoxic effects of zebularine are negligible at weaning. This observation is consistent with previous studies that show minimal cytotoxicity for zebularine (Cheng *et al.* 2003). We then generated a mixed model for RSC-V1aR with genotype, treatment and their interaction as main effects and parentage as a random effect. Next, we split the data set based on genotype and for each genotype we generated a mixed model with treatment as main effect and parentage as random effect.

For subjects in both the OTA and zebularine study, we examined the association of *avpr1a* intron enhancer methylation measures and RSC-V1aR using a linear regression. For each study, we built a mixed model for *total %DNA methylation* with genotype, treatment and genotype × treatment as main effects and parentage as random effect. For consistency, we next split each data set based on genotype and generated mixed models with treatment as main effect and parentage as a random effect. We also built models with genotype, treatment and genotype × treatment interaction as main effect and parentage as random effect for *%methylation at fixed CpGs* and *%methylation at methylable CpGs*. We did not further split the data as no significant effect was detected in these models. We also generated mixed models for methylation at each of the four single fixed CpG sites with genotype, treatment and genotype × treatment interaction as main effect and parentage as random effect.

Methylated DNA immunoprecipitation validation

In order to explore methylation differences outside the targeted enhancer, we validated and used a methylated DNA immunoprecipitation (MeDIP) assay for gDNA from the vole RSC. First, gDNA was extracted using a standard phenol:chloroform procedure and incubated with RNase A (ThermoFisher Scientific) for 45 min at 37°C to remove all traces of RNA. A 20 ug aliquot of the gDNA was brought to 450 ul with 1xTE and sheared into 200–700 bp fragments on ice, using Q125 sonicator [(5 seconds pulse, 5 seconds rest) × 15 at 60% power, Qsonica, Newton, CT, USA]. A 10% aliquot was taken from each sample as INPUT and the rest were used in MeDIP similar to previously described protocols (Mohn *et al.* 2009). Briefly, sheared DNA was denatured at 98°C for 10 min and immediately transferred to ice for another 10 min. We then added anti-5mC antibody (ab10805, Abcam, Cambridge, MA, USA) in a 1:1 mass ratio to DNA, and 50 ul of × 10 IP buffer (100 mM Na-Phosphate pH = 7.0, 1.4 M NaCl, 0.5% Triton X-100) and allowed samples to rotate for 2 h at 4°C. Following incubation, we added 40 ul of cleaned Dynabeads M-280 Sheep Anti-Mouse IgG (Life technologies, Gaithersburg, MD, USA) in 0.1% phosphate-buffered saline-bovine serum albumin to each tube and incubated for another 2 h at 4°C with rotation. Next, beads were collected on a magnetic stand and washed three times with × 1 IP buffer. Cleaned beads were incubated with Proteinase K (Life Technologies) in 50 mM Tris pH = 8.0, 10 mM ethylenediaminetetraacetic acid and 0.5% sodium dodecyl sulphate at 50°C for 3 h with rotation. The DNA was purified from beads using standard phenol:chloroform extraction and EtOH precipitation protocol.

To assess the sensitivity of the MeDIP assay we compared enrichment of native and *in vitro* methylated gDNA at the 5′UTR (untranslated region) of *β-actin*, the promoter of *gapdh* and within the *avpr1a* intron. Prior to MeDIP, a 1 μg aliquot of the fragmented gDNA was *in vitro* methylated using CpG Methyltransferase (M.SssI, New England Biolabs, Ipswich, MA, USA) according to manufacturer's protocol and cleaned using ZymoResearch Clean and Concentrator kit (Zymo Research, Irvine, CA, USA). Total amount of *in vitro* methylated gDNA was calculated based on concentration measures from nanodrop 2000 (ThermoFisher Scientific) and an equal amount of the native fragmented gDNA was taken. A 10% INPUT aliquot was set aside from both native and *in vitro* methylated gDNA and the remaining were immunoprecipitated in parallel according the MeDIP protocol described above. The MeDIP outputs were examined using qPCR with following primers: $F_{\beta\text{-}actin}$: 5′-GGAGCGGCGGAGAAAGAGC-3′, $R_{\beta\text{-}actin}$: 5′-GCGAGGCAGGTGAGTGAGC-3′; F_{gapdh}: 5′-GCCCAACCAGTCCCAG CAC-3′, R_{gapdh}: 5′-ACGAGAGAGGTCCAGCTACTC-3′ and F_{avpr1a}: 5′-G CCTCACACAGTTCCTCATGTTG-3′, R_{avpr1a}: GTCACCTAAGCCCATCCT GAATTTC-3′. All qPCR amplifications were carried out on ViiA Real Time PCR system (Life Technologies) in 10 ul reactions consisting of KAPA SYBR FAST qPCR master mix (ROX low, Kapa Biosystems, Woburn, MA, USA), 200 nM of each primer and 1 ul DNA. Amplifications were performed in triplicate using the following settings: 1 min at 95°C, (1 second at 95°C, 20 seconds at 60°C) × 40. To adjust data based on amount of DNA input, we calculated %INPUT enrichment. Our qPCR technical replicates were used to calculate means and error bars in Fig. 5a, as well as Welch *t*-tests comparisons between enrichment levels of native and *in vitro* methylated DNA.

To assess specificity of the MeDIP assay, we examined specific and non-specific antibody binding to DNA standards provided in the hMeDIP kit (Active Motif, Carlsbad, CA, USA). The DNA standards consisted of unmethylated, fully hydroxymethylated (5-hmC) or fully methylated (5-mC) amplicons of the human *APC* locus. We spiked equal aliquots of sheared vole gDNA with each of the *APC* standards, set aside 10% as INPUT and performed MeDIP according to the protocol described. To confirm absence of background pull down and non-specific qPCR amplification, we also carried out two parallel MeDIPs; one with unspiked vole gDNA and 5-mC antibody, the other with unspiked vole gDNA and IgG antibody. For all assays, INPUT and MeDIP outputs were qPCR amplified in triplicates using *APC* primers provided in the hMeDIP kit (Active Motif). To normalize qPCR results for amount of starting material, we calculated %INPUT enrichment. Technical replicates were used to calculate means and error bars for Fig. 5b and to perform pair-wise Welch *t*-tests between 5-mC spiked MeDIP and each negative control assay.

MeDIP-sequencing on the RSC of HI/HI and LO/LO voles

Genomic DNA was extracted from RSC of four HI/HI and four LO/LO sexually naive adult males. Each sample was treated with RNase, sheared and cleaned up as described above. The DNA concentrations were then measured on a nanodrop 2000 (ThermoFisher Scientific). We combined equal amounts of gDNA from each of the four HI/HI and four LO/LO individuals to generate one HI/HI and one LO/LO gDNA pool. From each of the two pools, 5 μM DNA was end repaired, adenylated, adaptor-ligated and size-selected (250-700 bp) using a KAPA LTP library prep kit (KAPA Biosystems, Woburn, MA, USA) according to manufacturer's instructions. We stored 10% of each sample in −20°C as INPUT, and proceeded with the MeDIP process as described above. The MeDIP outputs were PCR amplified for five cycles using KAPA LTP library prep kit (KAPA Biosystems). The INPUT and MeDIP libraries were submitted for 50 bp single-end sequencing on the Illumina HiSeq 4000 platform at genomic sequencing and analysis facility at University of Texas at Austin (Austin, TX, USA).

Sequencing reads from INPUT and MeDIP were aligned to the modified prairie vole draft genome assembly (Okhovat *et al.* 2015) using *bwa* (Li & Durbin 2009) with default single-end settings. Alignments were improved by *stampy* (Lunter & Goodson 2011) with default settings. Non-unique and low quality alignments (mapping quality ≤20) were filtered out using samtools (Li *et al.* 2009). We used MACS2 (Zhang *et al.* 2008) to normalize MeDIP-sequencing

(MeDIP-seq) signal of each genotype against the corresponding INPUT signal and generate fold enrichment (FE) tracks. The FE values at the *avpr1a* locus for the two genotypes were subtracted to visualize DMRs in the UCSC genome browser (https://genome.ucsc.edu/). Within each DMR the site with maximum FE difference between genotypes was considered the DMR summit.

Results

Ontogeny of RSC-V1aR and avpr1a methylation

To characterize early postnatal changes in the abundance of vasopressin receptor 1a in the retrosplenial cortex (RSC-V1aR) we measured RSC-V1aR in unmanipulated homozygous HI/HI and LO/LO pups ($n_{males} = 18$, $n_{females} = 22$) at postnatal day 1 (P1; $n_{HI/HI} = 6$, $n_{LO/LO} = 7$), P7 ($n_{HI/HI} = 7$, $n_{LO/LO} = 7$) and P14 ($n_{HI/HI} = 7$, $n_{LO/LO} = 6$). The RSC-V1aR was undetectable at P1 in both genotypes; therefore we assigned zero RSC-V1aR to all P1 individuals. The RSC-V1aR increased significantly with age within the first 2 weeks of life, especially among HI/HI animals. We found significant effects of age and genotype × age interaction on RSC-V1aR (genotype $t_{30} = -0.04$, $P = 0.97$; age $t_{30} = 3.31$, $P = 0.003$; genotype × age $t_{30} = 2.54$, $P = 0.02$; Fig. 2a,b). Our model explained 72% of all the RSC-V1aR variation. Although there was no main effect of genotype, the significant genotype by age interaction suggested this might be due to the lack of expression at P1 in both genotypes. Indeed, we found that HI/HI animals had significantly more RSC-V1aR compared with LO/LO animals at both P7 (LO/LO: 1280 ± 341, HI/HI: 2911 ± 656, mean ± SE; Welch *t*-test: $t = -2.21$, $P = 0.03$) and P14 (HI/HI: 5319 ± 1008, LO/LO: 2757 ± 669; Welch *t*-test: $t = -2.12$, $P = 0.03$) but not at P1 (Fig. 2a,b).

Across ages, we found a negative correlation between RSC-V1aR abundance and (1) *total %DNA methylation* ($r = -0.39$, $P = 0.014$), (2) *%methylation at fixed CpG sites* ($r = -0.41$, $P = 0.008$) and (3) *%methylation at methylable CpGs* ($r = -0.44$, $P = 0.006$). We also assessed effects of genotype, age and their interaction on each of the methylation measures. There was a significant effect of genotype on *total %DNA methylation* but no effect of age or genotype × age interaction (genotype $t_{29} = -18.54$, $P < 0.0001$; age $t_{29} = -0.20$, $P = 0.84$; genotype × age $t_{29} = -1.33$, $P = 0.19$; Fig. 2c). For *%methylation at fixed CpG sites,* we found no genotype or age effect, and only a weak trend was detected in the genotype × age interaction (genotype $t_{29} = -0.29$, $P = 0.78$; age $t_{29} = -0.10$, $P = 0.92$; genotype × age $t_{29} = -1.61$, $P = 0.12$; Fig. 2d). We found similar results when examining *%methylation at methylable CpGs* (genotype $t_{29} = -0.61$, $P = 0.54$; age $t_{29} = -0.14$, $P = 0.89$; genotype × age $t_{29} = -1.61$, $P = 0.12$; Fig. 2e). Examination of methylation at single fixed CpG sites showed no effect of genotype or age, but one site showed significant genotype × age interaction (Table S1, Supporting Information).

Pair-wise genotype comparisons showed that *total %DNA methylation* was higher in LO/LO pups compared with HI/HIs across all ages (Welch *t*-test, P1: $t = 17.36$, $P < 0.0001$; P7: $t = 28.07$, $P < 0.0001$; P14: $t = 16.48$, $P < 0.0001$; Fig. 2c). At fixed CpG sites, however, LO/LO animals had higher methylation at P7 (Welch *t*-test: $t = 2.34$, $P = 0.02$) and P14 (Welch *t*-test: $t = 1.96$, $P = 0.045$) but not at P1 (Welch *t*-test: $t = 0.54$, $P = 0.30$; Fig. 2d). Similarly, genotype comparisons of *%methylation at methylable CpGs* showed that the LO/LO animals had higher methylation compared with HI/HI subjects at P7 (Welch *t*-test: $t = 3.51$, $P = 0.002$) and P14 (Welch *t*-test: $t = 2.19$, $P = 0.03$) but not at P1 (Welch *t*-test: $t = 0.93$, $P = 0.19$; Fig. 2e). A broad similar pattern was observed when analyzing methylation at a single CpG site resolution, however, not all CpG sites showed significant difference in methylation at P7 and P14, and one of the fixed CpG site exhibited significant genotype methylation difference at all three ages (Fig. S1; Raw ontogeny data available at http://dx.doi.org/10.5061/dryad.c5961).

Effects of neonatal OTA and zebularine injections on RSC-V1aR

Among animals in our OTA study ($n_{males} = 24$, $n_{females} = 28$), we found a significant main effect of genotype, treatment and sex on RSC-V1aR following P1 OTA injections (genotype: $t_{36} = 2.70$, $P = 0.01$; treatment: $t_{36} = -2.34$, $P = 0.03$; sex: $t_{36} = -3.09$, $P = 0.004$; Fig. 3a). This model accounted for 52% of the overall variation in RSC-V1aR. The genotype × treatment term was not significant ($t_{36} = 1.31$, $P = 0.20$). Nevertheless, RSC-V1aR levels were significantly lower in LO/LO voles that received OTA compared with control subjects (LO/LO_{OTA}: 669 ± 100, LO/LO_{CON}: 1698 ± 227, mean ± SE; $t_{19} = -2.78$, $P = 0.01$; Fig. 3a), while HI/HI animals were unaffected (HI/HI_{OTA}: 2177 ± 434, HI/HI_{CON}: 2538 + 285; $t_{10} = -0.38$, $P = 0.71$; Fig. 3a). In our *post hoc* models, effect of sex on RSC-V1aR was only found among HI/HI animals, with males showing significantly lower RSC-V1aR compared with females ($t_{10} = -2.63$, $P = 0.03$).

In our zebularine study ($n_{males} = 23$, $n_{females} = 25$), we found an effect of both genotype and treatment, but no genotype × treatment interaction effect (genotype: $t_{39} = 4.52$, $P = 0.0001$, treatment $t_{39} = 1.98$, $P = 0.055$, genotype × treatment: $t_{39} = -0.63$, $P = 0.53$; Fig. 3b). This model explained 50% of the overall variation in RSC-V1aR. Splitting the data by genotype, however, showed a significant main effect of zebularine treatment among LO/LO animals (LO/LO_{ZEB}: 6946 ± 763, LO/LO_{CON}: 5584 ± 1109, mean ± SE; $t_{16} = 2.37$, $P = 0.031$), but not HI/HI subjects (HI/HI_{ZEB}: 12 932 ± 1138, HI/HI_{CON}: 11 464 ± 1373; $t_{20} = 1.09$, $P = 0.29$; Fig. 3b).

Effects of neonatal manipulations on methylation of avpr1a enhancer

Among subjects in the OTA study, we found a significant linear relationship between *total %DNA methylation* within the *avpr1a* enhancer and RSC-V1aR ($r = -0.47$, $P = 0.0004$; Fig. 4a). However, we did not find a significant correlation between RSC-V1aR and *%DNA methylation at fixed CpGs* ($r = -0.14$, $P = 0.30$) or *%methylation at methylable CpGs* ($r = -0.14$, $P = 0.32$).

In the OTA study, we found a significant effect of genotype on *total %DNA methylation* but no effect of treatment or genotype × treatment interaction (genotype:

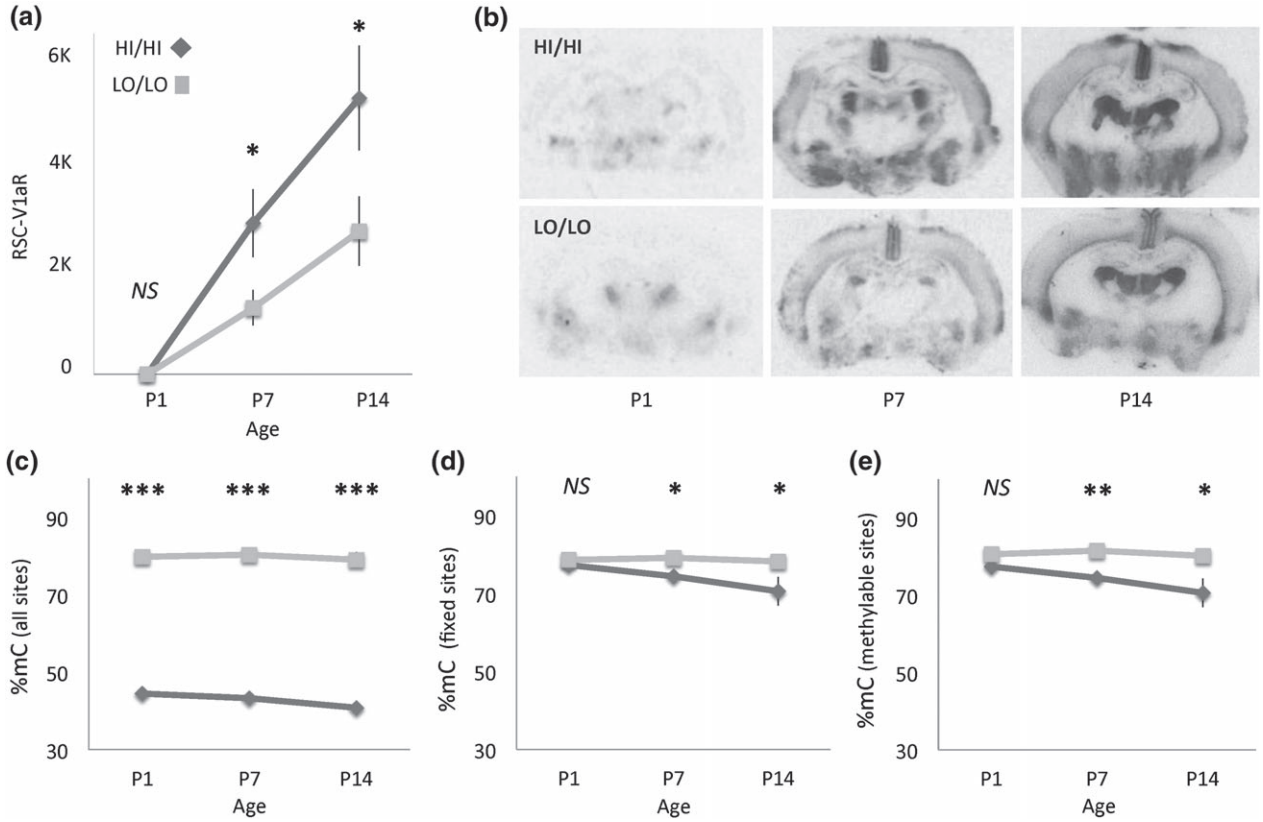

Figure 2: Postnatal genotype differences in RSC-V1aR and intron enhancer methylation. (a) Changes in RSC-V1aR abundance in first two postnatal weeks among HI/HI and LO/LO pups. (b) V1aR autoradiograms in HI/HI and LO/LO pups at P1, P7 and P14. (c)–(e) Ontogeny differences between HI/HI and LO/LO animals in methylation of the putative intron enhancer in developing RSC. Data presented as (c) total %DNA methylation (d) %methylation at fixed CpGs, and (e) %methylation at methylable CpGs. Data points are mean ± SE. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

$t_{37} = -13.19$, $P < 0.0001$; treatment: $t_{37} = 1.038$, $P = 0.31$; genotype × treatment: $t_{37} = -1.19$, $P = 0.24$; Fig. 4b). This model accounted for 88% of the total variation in *total %DNA methylation* in the *avpr1a* enhancer. Effect of treatment on *total %DNA methylation* remained insignificant even after splitting subjects based on genotype (HI/HI: $t_{11} = -1.50$, $P = 0.16$; LO/LO: $t_{19} = 1.11$, $P = 0.28$; Fig. 4b). We found no effect of genotype, treatment or genotype × treatment interaction for either *%methylation at fixed CpGs* (genotype: $t_{37} = 1.40$, $P = 0.17$; treatment: $t_{37} = 0.19$, $P = 0.85$; genotype × treatment: $t_{37} = -1.52$, $P = 0.14$) or *%methylation at methylable CpGs* (genotype: $t_{37} = 0.48$, $P = 0.63$; treatment: $t_{37} = 0.09$, $P = 0.93$; genotype × treatment: $t_{37} = -1.43$, $P = 0.16$). Single CpG methylation analysis also showed no genotype × treatment interaction ($P > 0.1$), however, three of four fixed CpG sites showed a significant genotype effects ($P < 0.05$, Table S2) and one site showed a weak treatment effect ($P = 0.05$, Table S2; Raw data available at http://dx.doi.org/10.5061/dryad.c5961).

Among subjects in the zebularine study, we found a significant linear relationship between *total %DNA methylation* of the *avpr1a* enhancer and RSC-V1aR ($r = -0.60$, $P < 0.0001$; Fig. 4c). We did not find an association between RSC-V1aR and *%methylation at fixed CpGs* ($r = -0.1$, $P = 0.48$), but we detected a trend between RSC-V1aR and *%methylation at methylable CpGs* ($r = -0.24$, $P = 0.09$).

In the zebularine study, we found a significant main effect of genotype and treatment on *total %DNA methylation* but no genotype × treatment interaction (genotype: $t_{39} = -5.65$, $P < 0.0001$; treatment: $t_{39} = 1.99$, $P = 0.05$; genotype × treatment: $t_{39} = -1.59$, $P = 0.11$; Fig. 4d). Our model accounted for 74% of the total variation in *total %DNA methylation* in the *avpr1a* enhancer. After splitting the data by genotype, no effect of treatment was found in either LO/LO ($t_{16} = 1.35$, $P = 0.20$; Fig. 4d) or HI/HI subjects ($t_{20} = -0.86$, $P = 0.40$, Fig. 4d). We found no effect of genotype, zebularine treatment or genotype × treatment interaction on *%methylation at fixed CpGs* (genotype: $t_{39} = 1.39$, $P = 0.17$; treatment: $t_{39} = -0.02$, $P = 0.98$; genotype × treatment: $t_{39} = -0.55$, $P = 0.59$) or *%methylation at methylable CpGs* (genotype: $t_{39} = 0.03$, $P = 0.98$; treatment: $t_{39} = 0.59$, $P = 0.56$; genotype × treatment: $t_{39} = -0.92$, $P = 0.36$). Our single CpG methylation analysis showed no effect of genotype, treatment or genotype × treatment interaction at any of the four

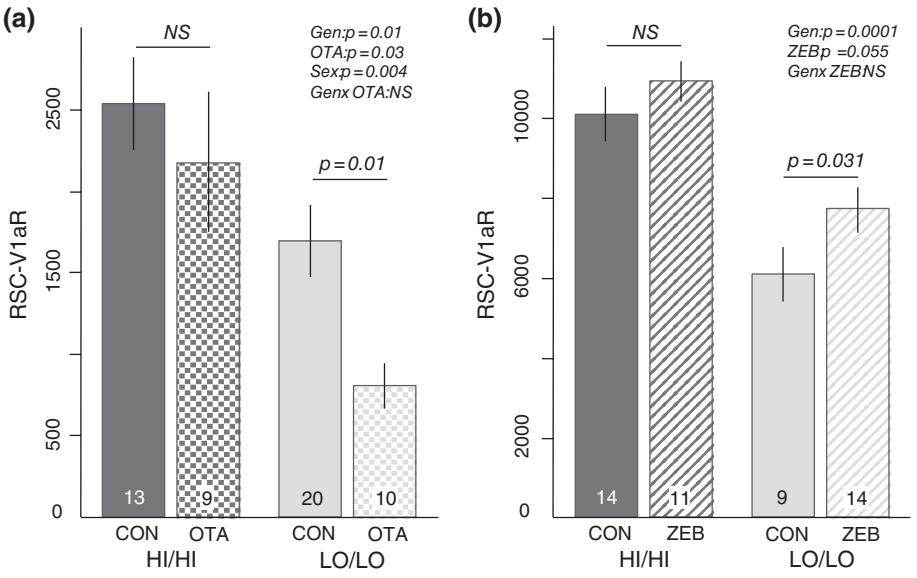

Figure 3: Genotype differences in sensitivity to neonatal manipulation. (a) Abundance of RSC-V1aR at P21 is shown among subjects in OTA study. (b) Autoradiogram RSC-V1aR measures are shown for subjects in zebularine study. Sample sizes are provided on each bar. CON, control, ZEB, zebularine. Bars are mean ± SE.

fixed CpG sites ($P > 0.05$, Table S3; Raw data available at http://dx.doi.org/10.5061/dryad.c5961).

MeDIP validation and MeDIP-seq of HI/HI and LO/LO RSC

We used MeDIP-seq to find differentially methylated regions (DMRs) outside the immediate vicinity of the *avpr1a* locus. First, to validate the technique we performed parallel MeDIPs on *in vitro* methylated and native vole gDNA. The MeDIP output from native gDNA showed low %INPUT enrichments at *β-actin* (2.67% ± 0.28%, mean ± SD) and *gapdh* (1.83% ± 0.03%, mean ± SD), where we expect low methylation levels in native gDNA, but not at the *avpr1a* intron (15.02% ± 0.46%, mean ± SD), where CpGs are highly methylated in native gDNA (Okhovat *et al.* 2017). Compared with native gDNA, MeDIP enrichment of *in vitro* methylated DNA significantly increased at both *β-actin* (Welch *t*-test, $t = -65.76$, $P = 0.0002$) and *gapdh* (Welch *t*-test, $t = -19.00$, $P = 0.002$). In contrast, %INPUT enrichment decreased at the *avpr1a* enhancer (Welch *t*-test, $t = 4.77$, $P = 0.01$), most likely reflecting the higher genome-wide competition for antibody binding following *in vitro* methylation (Fig. 5a). Next, we tested the specificity of our assay by performing MeDIP on unmethylated, hydroxymethylated (5-hmC) or methylated (5-mC) DNA standards. Background precipitation and non-specific amplification was relatively low, with %INPUT measures ranging from only 0.21% to 31.03%. Significantly higher %INPUT enrichment was obtained by performing MeDIP on vole gDNA spiked with 5-mc standard DNA (387% ± 17%, mean ± SD; Welch *t*-test, all $P < 0.001$; Fig. 5b).

From our HI/HI and LO/LO MeDIP and INPUT high-throughput sequencing, we obtained an average of 85 million (85.0 ± 24.5, mean ± SD), single-end, 50 bp reads. All samples exhibited high-alignment efficiency to the vole draft genome, even after vigorous mapping-quality filtration (%alignment efficiency post filter: 77.0% ± 3.3%, mean + SD). We created methylation fold enrichment (FE) tracks for HI/HI and LO/LO MeDIP libraries relative to the corresponding INPUT libraries. These enrichment tracks represent a measure of relative DNA methylation abundance genome-wide, and can be used to compare methylation levels between genotypes across the *avpr1a* locus. We observed that both *avpr1a* genotypes had low DNA methylation at the *avpr1a* transcription start site (TSS). Methylation levels increase toward the first exon-intron boundary, consistent with earlier descriptions of methylation at the *avpr1a* locus (Okhovat *et al.* 2017). We also found elevated DNA methylation at the transition between the second exon and 3′UTR. In a 25 kb window centered at the *avpr1a* TSS, MeDIP-seq FE ranges were similar between the two genotypes, with LO/LO ranging from 0.00 to 4.08 and HI/HI from 0.00 to 3.53. However, within this window we found that LO/LO animals showed higher methylation levels in the putative intron enhancer compared with HI/HI animals (FE at summit, LO/LO = 1.64, HI/HI = 0.07; Fig. 5c,d). In addition, we found two new DMRs located approximately 0.5 and 8.5 kb upstream of the *avpr1a* TSS. At both DMRs, DNA methylation was higher in the LO/LO sample compared with HI/HI sample ($DMR_{0.5kb}$: LO/LO = 2.72, HI/HI = 0.53; $DMR_{8.5kb}$: LO/LO = 2.76, HI/HI = 0.14; Fig. 5d,e; Raw and processed data available at http://dx.doi.org/10.5061/dryad.c5961).

Discussion

In this study, we characterized the developmental regulation of vasopressin receptor 1a in the retrosplenial cortex (RSC-V1aR), a neuronal phenotype implicated in sexual fidelity and space use of prairie voles in adulthood (Fig. 1; Okhovat *et al.* 2015; Ophir *et al.* 2008). We explored how genetic variation at the *avpr1a* locus interacts with developmental forces to influence the adult expression

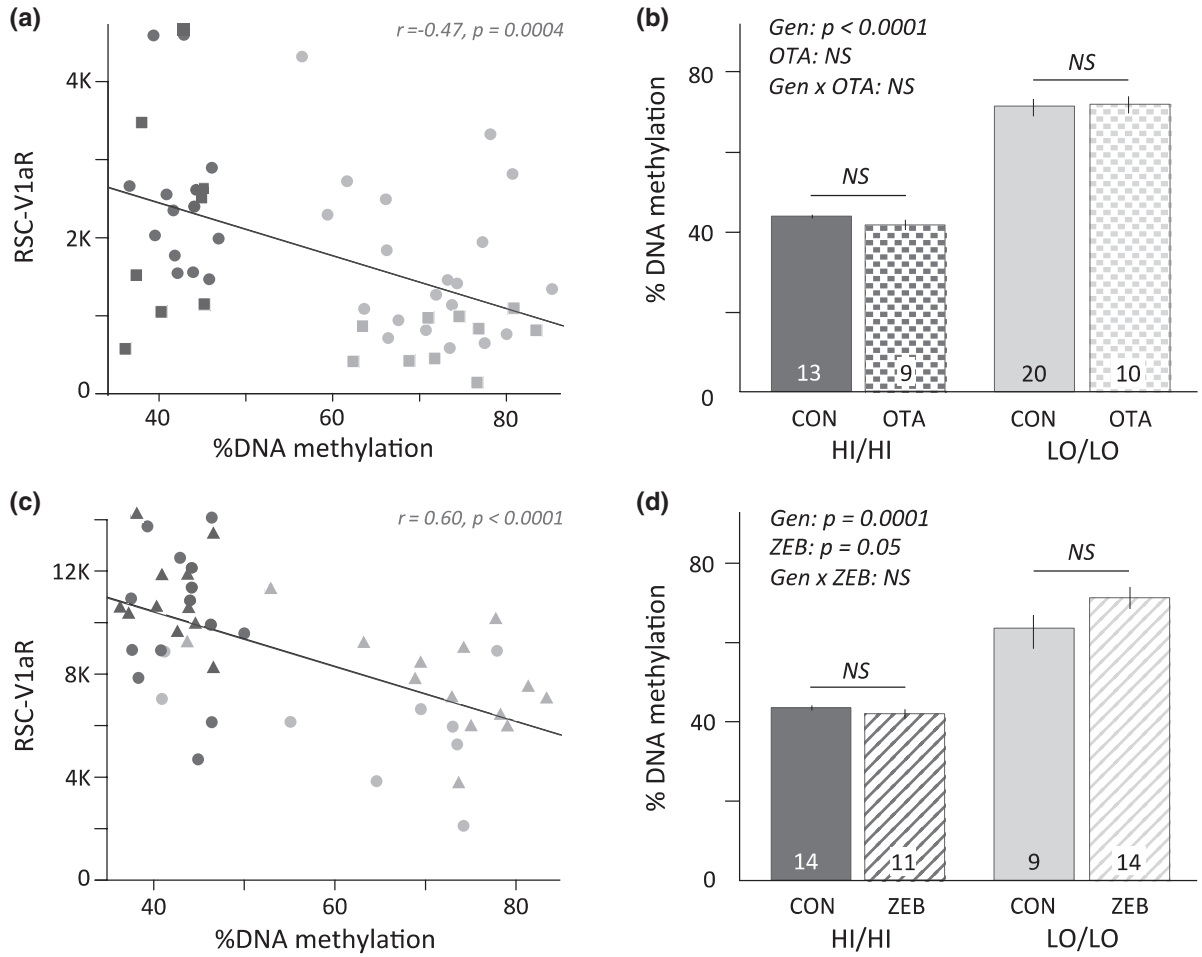

Figure 4: *Avpr1a* enhancer methylation in the RSC of HI/HI and LO/LO voles. (a) Total %DNA methylation in the putative intron enhancer plotted against abundance of RSC-V1aR in HI/HI (dark gray) and LO/LO (light gray) voles receiving OTA (squares) or control treatments (circles). (b) Total %DNA methylation for control and OTA treated HI/HI and LO/LO subjects. (c) Total %DNA methylation plotted against RSC-V1aR of HI/HI (dark gray) and LO/LO (light gray) voles receiving control treatments (circles) or zebularine injections (triangles). (d) Total %DNA methylation of control and zebularine-treated HI/HI and LO/LO voles. Sample sizes are provided on each bar. CON, control, ZEB, zebularine. Bars are mean ± SE.

of cortical vasopressin receptors. Specifically, we asked whether increased abundance of CpG sites within a putative RSC enhancer increases the sensitivity of LO alleles to developmental perturbation.

By visualizing brains of unmanipulated pups on postnatal day 1 (P1), P7 and P14, we found that RSC-V1aR levels changed drastically during the first 2 weeks of life in both HI/HI and LO/LO animals. All pups were born with undetectably low levels of RSC-V1aR that increased rapidly in the following 2 weeks (Fig. 2a,b). These observations were generally in line with previous descriptions of V1aR ontogeny in the RSC of prairie voles (Wang *et al.* 1997). However, we observed that the postnatal rise in V1aR was steeper in HI/HI voles compared with LO/LO animals, as evident by a significant age × genotype interaction in a mixed model for RSC-V1aR. Our *post hoc* comparisons of HI/HI and LO/LO genotypes across age groups showed that although there are no RSC-V1aR genotype differences at birth, HI/HI subjects had significantly more RSC-V1aR than LO/LOs at both P7 and P14 (Fig. 2a). Hence, the *avpr1a* genotype differences in RSC-V1aR exhibited by adult voles (Okhovat *et al.* 2015) emerge sometime during the first postnatal week. We have previously showed that RSC-V1aR abundance measured by autoradiography faithfully and strongly predicts *avpr1a* transcription ($R^2 = 0.75$, $P < 0.0001$; Okhovat *et al.* 2015). Thus, we expect that the observed changes in RSC-V1aR reflect developmental changes in *avpr1a* expression in the RSC.

Drastic changes in gene expression are common during the first few postnatal weeks in the rodent brain and are often accompanied by dynamic epigenetic changes, especially in DNA methylation (Simmons *et al.* 2013). While genetic variation is clearly important in regulation of RSC-V1aR, previous developmental evidence (Bales *et al.* 2007; Okhovat *et al.*

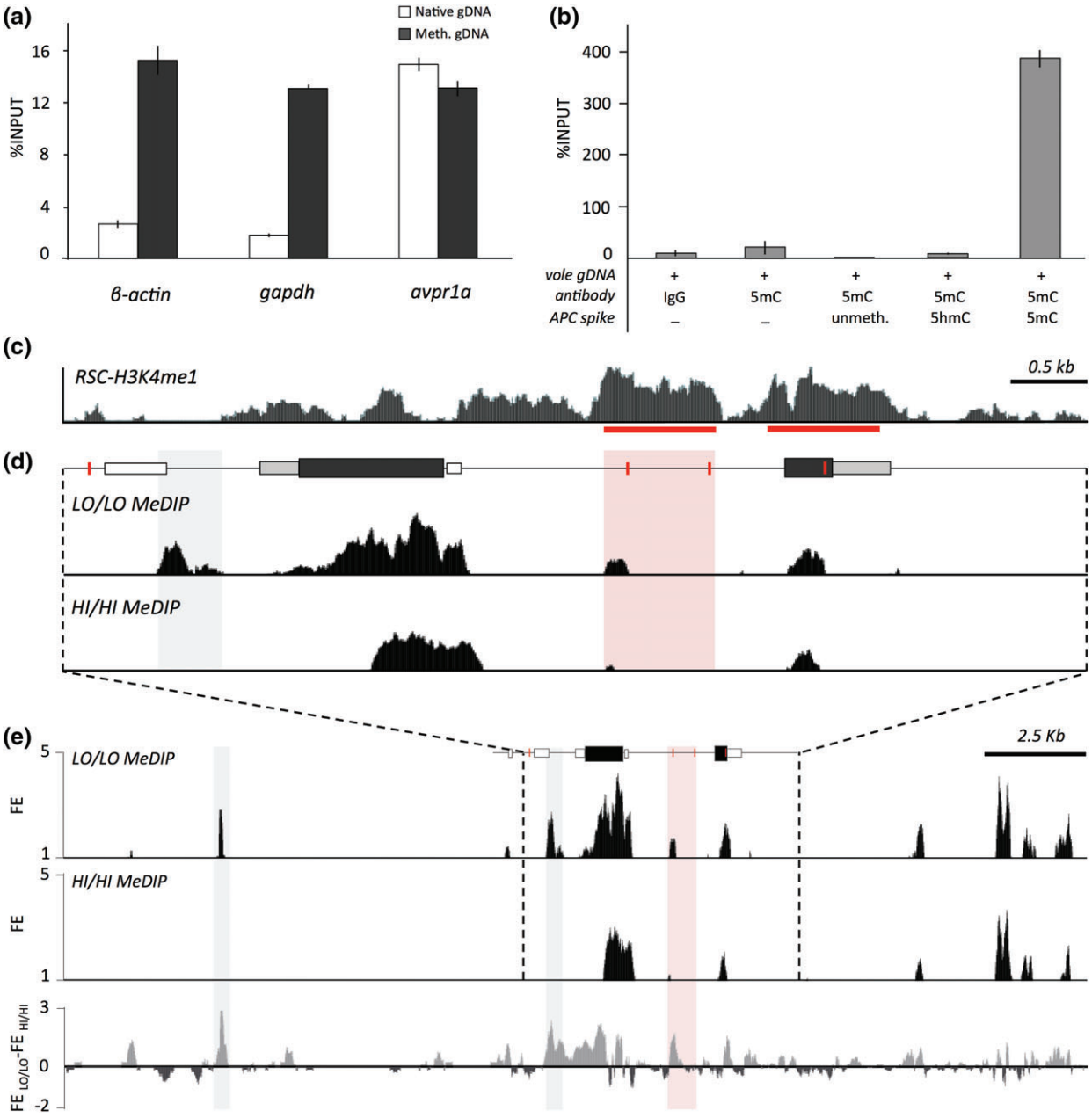

Figure 5: Methylated DNA immunoprecipitation of RSC from HI/HI and LO/LO animals. (a) MeDIP enrichment (%INPUT) of native (white) and *in vitro* methylated prairie vole gDNA (black) at the *β-actin, gapdh* and the *avpr1a* intron enhancer. (b) Ability of MeDIP to detect methylated control spike-in DNA (human *APC* locus, *APC*) in the presence of vole genomic DNA. Treatments from left to right include non-specific IgG antibody (IgG) but no *APC*; 5 mC antibody but no *APC*; 5 mC antibody and unmethylated *APC*; 5 mC antibody + hydroxymethylated *APC* (5 hmC-APC); and 5 mC antibody + methylated *APC* (5 mC-APC). Bars are mean ± SD. (c) FE track for H3K4me1 shown at the *avpr1a* locus. Significant peaks marked with red bars. Data from Okhovat *et al.* 2015. (d) MeDIP FE tracks for RSC of LO/LO (*top*) and HI/HI animals (*bottom*) along the *avpr1a* locus. The putative intron enhancer is shaded pink. (e) MeDIP FE track over a 25 kb window centered on the *avpr1a* transcription start site for LO/LO (*top*) and HI/HI animals (*middle*) and their difference (*bottom*). Differentially methylation regions (DMRs) are shaded.

2015) indicates that this phenotype is also influenced by epigenetic variation. Of note, the unusually high CpG polymorphism in the *avpr1a* putative intron enhancer (Okhovat *et al.* 2017) and the strong correlation between enhancer methylation – at both fixed and polyCpGs – and RSC-V1aR expression (Okhovat *et al.* 2015), suggested that DNA methylation is involved in regulation of RSC-V1aR. Here, we asked whether developmental alterations in RSC-V1aR are also associated with changes in methylation of the putative intron enhancer of *avpr1a*.

Consistent with our previous findings (Okhovat *et al.* 2015; Okhovat *et al.* 2017), we found a significant negative relationship between *total %DNA methylation* and RSC-V1aR among all pups. Although *total %DNA methylation* may be an important contributor to the regulatory state of an element, it is highly influenced by sequence at polymorphic CpG sites (polyCpG). Thus, measures of total methylation are confounded by differences in genotype. We controlled for effects of CpG polymorphisms by calculating *%methylation at fixed CpG sites* and *%methylation at methylable CpGs*. These measurements allowed us to examine developmental changes in methylation. Looking across ontogeny, we again found a negative correlation between these methylation measures and RSC-V1aR. We found that LO/LO pups, which have more CpG sites in the targeted intron enhancer, also had significantly higher *total %DNA methylation* compared with HI/HI throughout the first two postnatal weeks (Fig. 2c). Interestingly, however, *%methylation at fixed CpG sites* and *%methylation at methylable CpGs* was only higher among LO/LO animals compared with HI/HI pups at P7 and P14, and not at birth (Fig. 2d,e). Thus, genotype differences in *total %DNA methylation* in the enhancer are present since birth, presumably due to sequence differences alone. Genotype differences at fixed and methylable CpGs, however, appear sometime during the first postnatal week, which coincides with the emergence of genotype differences in RSC-V1aR abundance. This suggests a genetic interaction with epigenetic processes.

With a few exceptions, analysis of methylation at a single CpG resolution shows a broadly similar pattern (Fig. S1). These results indicate that the first postnatal week represents a critical neurodevelopmental stage for RSC-V1aR and emergence of *avpr1a* genotype differences both in expression and methylation. While the exact timing may differ, such 'critical' developmental stages are common in rodent neurodevelopment, and represent a period when the brain is highly sensitive to environmental or epigenetic perturbations (Roth & Sweatt 2011). In our study, the synchronized emergence of genotype differences in RSC-V1aR and enhancer methylation suggested a role for *avpr1a* enhancer methylation in the development of RSC-V1aR expression.

To investigate whether allelic differences in CpG abundance resulted in differences in sensitivity to developmental silencing, we manipulated development with oxytocin receptor antagonist (OTA), a treatment known to reduce RSC-V1aR (Bales *et al.* 2007). We predicted that LO/LO pups, which have more enhancer CpGs, would be more sensitive to OTA-induced silencing. We injected pups at P1, before genotype differences in RSC-V1aR are established, and measured RSC-V1aR at weaning (P21). Among our subjects, we found a significant main effect of genotype, treatment (OTA vs. control) and sex on RSC-V1aR (Fig. 3a). Interestingly, *avpr1a* genotypes differed in their sensitivity to OTA; LO/LO animals showed significant decrease in RSC-V1aR while HI/HI animals were unaffected (Fig. 3a). While systemic OTA exposure does not model any particular natural experience *per se*, it may mimic variation in parental care (Feldman *et al.* 2010). Interestingly, the presence of paternal care does seem to change RSC-V1aR among prairie voles in adulthood (Prounis *et al.* 2015, but see Ahern *et al.* 2009). Together these results suggest that the developing prairie vole brain – much like other rodents' (Weaver *et al.* 2004) – is influenced by the quality and quantity of early parental care, and that this impact may vary among individuals.

If CpG abundance alters sensitivity to DNA methylation, we hypothesized that drugs that interfere with methylation should promote RSC-V1aR expression in LO/LO animals, but not in HI/HI animals. Zebularine is a low toxicity DNMT inhibitor commonly used to disrupt DNA methylation both *in vitro* and *in vivo* (Cheng *et al.* 2003). As predicted, neonatal zebularine injection increased RSC-V1aR, among LO/LO animals, but not among HI/HI animals (Fig. 3b). Thus for two very different developmental manipulations, LO/LO voles show increased sensitivity to environmental manipulations. Although the results were promising, pharmacological manipulations are inherently difficult to interpret because observed outcomes may result from effects elsewhere in the genome, the brain or both. Thus, to elucidate whether changes in RSC-V1aR were directly mediated by methylation changes at the *avpr1a* intron enhancer, we measured enhancer methylation in subjects from the OTA and zebularine studies.

As expected, in both OTA and zebularine data sets, we found a negative correlation between *total %DNA methylation* and RSC-V1aR abundance (Fig. 4a,c). To our surprise, however, we found that the majority of variation in enhancer methylation was explained solely by genotype. While developmental manipulations of methylation are common in behavioral epigenetics, our unexpected methylation results highlight some shortcomings of this approach. First, it is possible that either pharmacological manipulations or the HI/LO alleles change the cell type composition of the tissue, complicating detection and interpretation of changes in DNA methylation and expression. Because the HI and LO alleles govern expression of a locus not previously implicated in cell proliferation, survival or fate determination, genotype differences in cell composition seem unlikely but possible. Alternatively, our pharmacological manipulations may affect neurodevelopment outside the tissue or locus of interest, leading to unexpected changes in methylation and gene expression later in life. Such global alteration of methylation could change function at numerous distal and proximal enhancers that contribute to regulation of a focal gene. Our complex results led us to explore additional regions with differential methylation between genotypes, which may provide targets for future study.

To examine methylation at distal sites, we first validated the specificity of a MeDIP-seq assay for prairie vole brain

tissue. We found that MeDIP-seq was able to enrich methylated regions of the vole genome. We also show that unlike traditional bisulfite-conversion sequencing methods, such as DNA methylation pyrosequencing, this technique effectively discriminates between methylated and hydroxymethylated cytosines (Fig. 5a,b). We compared methylation levels around the *avpr1a* locus in HI/HI and LO/LO voles (Fig. 5d). The overall patterns of methylation were similar between the two genotypes, but the MeDIP assay suggests at least three DMRs. First, it replicates our findings of differential methylation localized to the intron enhancer (Fig. 5c–e). Second, it shows differential methylation approximately 500 bp upstream of the TSS (Fig. 5d,e) – a somewhat surprising finding, because promoters are often stably unmethylated, even when gene expression is suppressed (Weber *et al.* 2007). While this DMR is likely important to cortical *avpr1a* expression, there are no genetic differences between HI and LO alleles within this region (Okhovat *et al.* 2015), suggesting it is a downstream consequence of regulation at another site. However, the MeDIP showed one additional DMR approximately 8.5 kb upstream of the *avpr1a* locus which also exhibits higher levels of methylation among LO/LO animals (Fig. 5d,e). To determine if this DMR contributes to *avpr1a* regulation and developmental sensitivity, future research should further characterize its genetic and epigenetic variation between HI/HI and LO/LO voles.

Involvement of additional genetic elements in regulation of *avpr1a* is complex and exciting, but perhaps not surprising. Regulation of eukaryotic gene expression is complicated and involves many regulatory regions and transcription factors. Often multiple enhancers regulate tissue-specific gene expression; these enhancers can be quite distal from their target gene (Bulger & Groudine 2010). In fact, genes with G × E interactions are found to have disproportionally high association with distal regulatory loci compared with other gene groups (Grishkevich & Yanai 2013). Similarly, genetic contributions to variation in gene expression are often due to multiple enhancer variants. Each variant may have a modest effect on expression, but effect sizes may change in response to external stimuli, or as variants are inherited together (Corradin *et al.* 2014). Although we focus on a single SNP and linked CpG polymorphisms within an intronic enhancer, additional genetic differences occur within other regulatory regions (Okhovat *et al.* 2015), and genetic variation at more distal regulatory sequences has not been characterized. Hence, it is possible that additional linked variants at distal enhancers contribute to HI and LO differences in *avpr1a* regulation. If so, one or more of those additional sites may contribute to genotype differences in environmental sensitivity. Such effects may interact with CpG polymorphisms in the intron enhancer, or may operate through alternative mechanisms.

In addition to the potential effects of distal enhancers, it is plausible that methylation at the *avpr1a* intron enhancer and our pharmacological manipulations interact in other ways. For example, the methyl-binding protein MeCP2 regulates expression of a variety of genes, including neuropeptides such as vasopressin (*avp*; Murgatroyd *et al.* 2009) and corticotrophin-releasing hormone (*crh*; McGill *et al.* 2006). Phosphorylation of MeCP2 can influence its affinity for methylated DNA, or even convert it into an activator of expression (Zimmermann *et al.* 2015). If our treatments alter MeCP2 phosphorylation – as happens in mice exposed to early life stress, drug treatments and learning paradigms (Zimmermann *et al.* 2015) – the relationship between methylation status and *avpr1a* expression might change in unexpected ways. As the LO allele has higher methylation density, this allele is more likely to interact with methyl-binding effector proteins, such as MeCP2. As a result, we would expect LO/LO voles to be more susceptible to environmentally induced modifications to methyl-binding effector proteins. Alternatively, it is possible that affinity of transcription factors predicted to bind favorably to the LO allele enhancer (Okhovat *et al.* 2017) may be influenced by our pharmacological treatments, thereby causing LO-specific changes in RSC-V1aR. While our observations are consistent with these hypotheses, the exact mechanisms by which OTA and zebularine interact with *avpr1a* sequence variation remain to be determined. These results highlight the challenging complexity posed by GxE interactions, but also suggest that tools like MeDIP, ChIP-seq provide exciting new means to meet this challenge. Also, because correlative DNA methylation studies are inherently unable to determine whether alteration in methylation is a cause or consequence of changes in gene regulation, using modern manipulative tools – such as sequence-targeted effector proteins (e.g. dCas9-MecP2 fusion proteins) – can shed new light on the regulatory interactions between genetic and epigenetic variation.

The complex interplay between developmental processes and genetic variation shapes phenotypic diversity, and is increasingly important for our understanding of animal behavior, evolutionary biology and mental health (Caspi & Moffitt 2006; Grishkevich & Yanai 2013; Manuck & McCaffery 2014). We asked whether an increased frequency of CpG sites would make an allele more sensitive to developmental perturbation. We tested this hypothesis by focusing on allelic differences in enhancer methylation and V1aR abundance within the RSC, an expression pattern associated with complex socio-spatial behaviors in the field. We found that genotype differences in RSC-V1aR abundance are absent at birth but emerge within the first postnatal week, and that these changes are accompanied by differences in methylation of a polymorphic *avpr1a* enhancer. We found that LO/LO animals were indeed more sensitive to developmental manipulations, but that this sensitivity is not simply due to differences in the methylation of our focal enhancer. This work highlights the complexity of interpreting pharmacological manipulations on gene expression, and the many genetic and epigenetic factors that come into play for even a single candidate gene. Lastly, despite the complexity of our results, this work highlights the utility of non-model organisms in better understanding genetic diversity. Such diversity is an essential component of individual and species differences in brain, behavior and evolution.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Supplemental materials (Tables S1–S3 and Fig. S1) describe effects of genotype, ontogeny and developmental manipulation on the methylation state of individual fixed CpG sites. Raw and processed MeDIP-seq data, along with a complete dataset with genotype, sex and breeding information and protein and methylation measures for all individuals is available at Dryad Digital Repositroy doi:10.5061/dryad.c5961, http://dx.doi.org/10.5061/dryad.c5961.

Figure S1: Postnatal genotype differences in CpG methylation at four fixed enhancer CpG sites. (a) Fixed (black) and polymorphic CpG sites (red/blue) within the putative *avpr1a* enhancer are annotated based on their position relative to the translation start site. (b) Ontogeny differences between HI/HI (dark gray) and LO/LO (light gray) animals in methylation of single fixed CpG sites in the developing RSC. Data points are mean ± SE. $*P \leq 0.05$, $**P \leq 0.01$.

Table S1: Effects of genotype, age and genotype × age interaction on methylation at single fixed CpG sites. Data from the ontogeny study was used to generate mixed models for methylation at four fixed CpGs with genotype, age and genotype × age interaction as fixed effect and parentage as a random effect. Significant fixed effects ($P \leq 0.05$) appear in bold text.

Table S2: Effects of genotype, OTA treatment and genotype × OTA treatment interaction on methylation at single fixed CpG sites. Data from the OTA study was used to generate mixed models for methylation at four fixed CpG site with genotype, treatment and genotype × treatment interaction as fixed effect and parentage as a random effect. Significant fixed effects ($P \leq 0.05$) appear in bold text. OTA, oxytocin receptor antagonist.

Table S3: Effects of genotype, zebularine treatment and genotype × treatment interaction on methylation at single fixed CpG sites. Data from the zebularine study was used to generate mixed models for methylation at 4 fixed CpG site with genotype, treatment and genotype x treatment interaction as fixed effect and parentage as a random effect. Significant fixed effects ($P \leq 0.05$) appear in bold text. ZEB, zebularine.