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5

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29

30 Abstract

31 Experiments investigated neuroanatomically localized changes in *de novo* DNA
32 methyltransferase expression in the female Siberian hamster (*Phodopus sungorus*). The
33 objectives were to identify the neuroendocrine substrates that exhibit rhythmic *Dnmt3a* and
34 *Dnmt3b* expression across the oestrous cycle and examine the role of ovarian steroids.
35 Hypothalamic *Dnmt3a* expression was observed to significantly increase during the transition
36 from proestrous to oestrous. A single bolus injection of diethylstilbestrol (DES) and
37 progesterone was sufficient to increase Dnmt3a cell numbers and Dnmt3b immunoreactive
38 intensity in the suprachiasmatic nucleus (SCN). *In vitro* analyses using an embryonic rodent
39 cell line revealed that DES was sufficient to induce *Dnmt3b* expression. Upregulating DNA
40 methylation *in vitro* reduced expression of vasoactive intestinal polypeptide, *Vip*, and the
41 circadian clock gene, *Bmal1*. Together, these data indicate that ovarian steroids drive *de novo*
42 DNA methyltransferase expression in the mammalian suprachiasmatic nucleus and increased
43 methylation may regulate genes involved in the circadian timing of oestrous: *Vip* and *Bmal1*.
44 Overall, epigenetically mediated neuroendocrine reproductive events may reflect an
45 evolutionarily ancient process involved in the timing of female fertility.

46

47 Key words: Rhythmic epigenetics, circadian, neuroendocrine, oestrogen

48

49 Total Words: 7331; Figures: 5; Supplementary Materials: Figure (1) Table (3)

50

51 **Introduction**

52 Gonadotropin-releasing hormone (GnRH) is an evolutionarily conserved neuropeptide
53 that is necessary for sexual reproduction (1). In mammals, the oestrous cycle is characterized
54 by gonadotropin pulses that exhibit low frequency during the luteal phase but increase
55 significantly during the follicular phase (2). On the evening of proestrous, there is a
56 circadian-dependent surge in gonadotropin secretion; after which GnRH pulse frequency
57 slows considerably (3). A fundamental feature of the circadian timing of the proestrous surge
58 is a neural network between the suprachiasmatic nucleus (SCN) and the anterior
59 hypothalamus/preoptic area (AH/POA) (4). Multiple SCN neurons provide tissue-level
60 control for timing the luteinizing hormone (LH) surge. Two neuropeptides highly expressed
61 in the SCN that are consistently implicated in driving GnRH release include vasoactive
62 intestinal polypeptide (*Vip*) (5) and arginine vasopressin (*Avp*) (6).

63 There is now strong evidence that epigenetic modifications affect the timing of puberty
64 (7,8), but whether epigenetic modifications are involved in the neuroendocrine control of the
65 oestrous cycle remains uncharacterized. DNA methylation is one type of epigenetic
66 modification present in diverse taxa and is involved in the control of gene regulation (9);
67 normally associated with transcriptional silencing. DNA methyltransferases (*Dnmt*) are a
68 family of enzymes that catalyse the transfer of methyl-groups to the genome template (10) to
69 maintain the pattern of methylation (imprinting control) or to modify the methylation pattern
70 *de novo*. It has recently emerged that *de novo* DNA methylation is reversible and can exhibit
71 clear oscillations in post-mitotic cells (14). In mammals *de novo* methylation activity is
72 performed primarily by two Dnmt3 enzymes, Dnmt3a and Dnmt3b, encoded by separate
73 genes (11, 12). Dnmt3a and Dnmt3b can induce both CpG and non-CpG methylation and
74 exhibit similar kinetic activity (11). Both Dnmt3a and Dnmt3b express multiple distinct
75 isoforms with some that lack DNA methylation activity (i.e. *Dnmt3b3,* see 11). Dnmt3a and

76 Dnmt3b show highly conserved expression patterns across mammalian evolution with high
77 expression in brain and reproductive tissues (e.g. testes and uterus) (12,13). It has recently
78 emerged that *de novo* DNA methylation is reversible and can exhibit clear oscillations in
79 post-mitotic cells (14). In uterine tissue, expression of Dnmt3a and Dnmt3b in endometrial
80 cells (15-17) appears to be involved with decidualization (18). The prevailing hypothesis is
81 that Dnmt3a-driven changes in uterine function is a critical step for synchronising peripheral
82 reproductive function in mammals (15,16). In the brain, *de novo* DNA methyltransferase
83 expression has been localized in several hypothalamic nuclei, including diencephalic centres
84 critically involved in reproduction, such as the SCN (19,20), the ependymal layer along the
85 3rd ventricle (3^{rd}V) (21) and the AH/POA (22).
86 Siberian hamsters are an important animal model for studying the neuroendocrine control
87 of reproductive physiology (23,24). Prior work in hamsters demonstrated that hypothalamic
88 global DNA methylation was elevated in long-day breeding compared to short-day
89 reproductively regressed hamsters (21). Increased global DNA methylation in the
90 hypothalamus correlated with significantly higher *Dnmt1* and *Dnmt3a* expression (25).
91 Seasonal variation in hypothalamic *Dnmt3a* expression has also been associated with
92 photoperiod induced changes in the timing of seasonal physiology and migratory behaviour
93 in the red-headed bunting (*Emberiza bruniceps*) (26). Seasonal changes in hypothalamic
94 *Dnmt3a/b* expression also correlates with increased methylation in the proximal promoter of
95 a thyroid hormone deiodinase gene (*Dio3*) (21) and is negatively correlated with *Dio3*
96 expression (21,26). These data indicate that rhythmic epigenetic modifications, such as
97 neuroendocrine variation in DNA methylation, is associated with the long-term programming
98 of seasonal reproduction (14).
99 The objectives of the current study were to examine anatomically localised changes and
100 ovarian-steroid dependent regulation of *de novo* DNA methyltransferase expression in the

101 female hypothalamus. Using Siberian hamsters, we investigated *Dnmt3a* and *Dnmt3b* mRNA
102 during proestrous and oestrous to understand if expression changes prior to -or after the LH
103 surge. Then, we ovariectomised females and administered either a single bolus injection of
104 oil or an ovarian steroid mixture of the highly potent estrogen agonist diesthylstilbestrol
105 (DES) and progesterone (P) compared to vehicle control to identify hormone-dependent
106 effects on Dnmt3a and Dnmt3b protein expression in the hamster hypothalamus. Next, we
107 cultured a rodent embryonic cell line derived from mouse hypothalamus (mHypoE N36/1) to
108 explore the ability of DES to regulate the expression of *Dnmt3a* and *Dnmt3b in vitro.* We
109 also tested the impact of DES administration on the expression of neuropeptides implicated in
110 timing the gonadotropin surge during proestrous, including *Vip*, vasopressin (*Avp*) and
111 kisspeptin (*Kiss1*). To identify a functional role of DNA methylation for timing reproductive
112 physiology, we then used the mHypoE N36/1 cells to investigate whether epigenetic
113 modifying drugs influence the expression of *Vip, Avp* and *Kiss1*. Furthermore, we examined
114 the impact of increased DNA methylation on circadian clock genes: brain and muscle ARNT-
115 like 1 (*Bmal1*), period 1 (*Per1*) and clock (*Clock*). Our findings suggest that ovarian steroids
116 control the expression of *de novo* DNA methyltransferases in distinct regions of the
117 hypothalamus and increased DNA methylation may influence multiple genomic targets
118 involved in the neuroendocrine regulation of the oestrous cycle.

119

120 **Methods**

121 *Siberian hamster colony and ethical approvals*

122 These studies used female hamsters derived from a colony maintained at the
123 University of Aberdeen. Hamsters were housed in polypropylene cages illuminated for
124 16h/day (lights-on 800h). Harlan food and tap water were provided *ad libitum* and each cage
125 was provided cotton-nesting material. All procedures were approved by the University of

126 Aberdeen Animal Welfare and Ethics Committee and Home Office (PPL 70/7017). All
127 procedures were in accordance with the Arrive Guidelines for ethical research involving
128 animals.
129

130 Study 1 – Characterization of *Dnmt3a* and *Dnmt3b* expression during the hamster proestrous
131 and oestrous stages

132 *Animals*:

133 Adult female (3-8 months, n=19) were taken from the University of Aberdeen
134 breeding colony and group housed. Animals were kept in LD (16L:8D) and samples were
135 collected between 1500h-1700h to capture the proestrous surge in prolactin (15, 27). The age
136 range of female hamsters was equivalent across both experimental groups. Hamsters were
137 then sacrificed using cervical dislocation and brains were collected and frozen on powdered
138 dry ice before being transferred to -70°C. The reproductive state of the hamsters was
139 published previously (15). We investigated RNA expression in hypothalami from proestrous
140 (n=11) and oestrous (n=8) hamsters.
141

142 *Anatomical localization and dissection of the Siberian hamster anterior hypothalamus*

143 Brains were placed ventral side facing upward into a cold mouse brain matrix (Alto
144 matrix; CellPoint Scientific). The Allen Mouse atlas was used to determine the anatomical
145 localization of an anterior tissue punch that included the SCN, anteroventral-periventricular
146 (AvPv) and POA, and a posterior punch that comprised the arcuate nucleus (Arc) and
147 dorsalmedial hypothalamus (DMH). The posterior extent of the optic chiasm provided a
148 reliable anatomical landmark to separate the anterior-posterior hypothalamic divide. A
149 second coronal section was performed 1mm in the anterior direction (AH) and a 2mm coronal
150 section in the posterior direction (PH). Tissue sections were then placed on a cold microscope

151 slide, the lateral and dorsal extent of the hypothalamus was identified, and cuts conducted to
152 isolate the respective hypothalamic regions (Figure S1).
153
154 *RNA extraction, cDNA synthesis and quantitative PCR (qPCR) assay*
155 AH and PH hypothalamic tissue punches were homogenized in Trizol (ThermoFisher
156 Scientific) and RNA extracted as per manufacturer's guidelines. RNA concentration and the
157 260/280 ratio were measured by NanoDrop (ThermoFisher Scientific). cDNA synthesis was
158 carried out using First Strand cDNA synthesis kit (Invitrogen). To measure mRNA
159 expression, cDNA was assayed using qPCR run on a BioRad CFX96. For each well the
160 qPCR mix consisted of 5µl cDNA template, 10µl SYBR green (PrecisionPLUS qPCR Master
161 Mix with SYBR green) 0.5µl (300nM) forward primer, 0.5µl (300nM) reverse primer and
162 4µl RNase-free H2O to make up to 20µl. Primers were all ordered from Invitrogen,
163 sequences for *Gnrh*, *Rfrp3*, *Kiss1*, *Dnmt1*, *Dnmt3a*, and *Dnmt3b* were optimized and
164 published previously (21, 28) as well and the primer sequences for the reference genes
165 glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine
166 phosphoribosyltransferase 1 (*Hprt*) (15) (Table S1). Melt curve analyses were carried out to
167 ensure specificity for each reaction. PCR Miner (29) was used to determine reaction
168 efficiencies (E) and quantification cycle (Cq). According to MIQE guidelines, samples with
169 efficiency values below 0.8 and above 1.2 were excluded from analyses (30). Fold expression
170 of each target gene was measured in relation to the geometric mean of the Cq for two
171 reference genes (*Gapdh* and *Hprt*) and calculated using 2-(ΔΔCq). The geometric average of
172 the reference genes was stably expression across groups [CT $\bar{x}$±SEM Est (15.4±0.2) and Pro
173 (15.5±0.1)].
174

175 Study 2: Assessment of ovarian steroids on Dnmt3a and Dnmt3b expression in the female
176 hamster hypothalamus.

177 *Animals*:

178 Adult female hamsters (n=18) were ovariectomised and maintained in LD (16L:8D) for
179 8 weeks to allow circulating ovarian hormones to clear from the circulation. In brief,
180 ovariectomies were conducted while hamsters were under deep anaesthesia (5% isofluorane
181 gas). The ovaries were externalized via bilateral incisions to the dorsum (lateral to the spine,
182 caudal to the ribcage). The ovary was localized at the distal end of the uterine horn and
183 ligated with sterile sutures (4–0, nonabsorbable monofilament nylon). The ovary was then
184 excised and the procedure was repeated for the second ovary. The abdominal wall and skin
185 were closed separately with sterile sutures. Hamsters recovered under analgesia and had *ad*
186 *libitum* access to enriched food (sunflower seeds additional standard chow) and water before
187 being transferred back to individual cages. 8 weeks following surgery a reduction in body
188 mass of 7.5g (± 0.9g SEM) on average was observed and a reliable indicator of reduced
189 ovarian steroids (31) and were previously reported (see 15). Hamsters were then randomly
190 allocated into two experimental groups, one group to receive vehicle control (i.e. vegetable
191 oil) and the other to receive an ovarian steroid injection. Diethylstilbestrol (DES, Sigma-
192 Aldrich) is a highly potent estrogen agonist (E_2) with full activity at both the estrogen
193 receptor-α and -β and was administered in conjunction with progesterone (P_4, Sigma-
194 Aldrich). DES and P_4 (denoted as E_2P_4) were dissolved in sterile vegetable oil (Oil) to a final
195 dose of 50μg E_2 and 5mg P_4 per ml vegetable oil. These steroid concentrations were taken
196 from previous work in female hamsters (32). Control hamsters (n=7) were injected with
197 100μl oil and treatment hamsters (n=11) were injected with 100μl E_2P_4 cocktail (5μg E_2 and
198 500μg P_4) at 1700h. Female hamster were killed by cervical dislocation either 12- or 24-
199 hours post-injection and the brain were rapidly extracted and frozen in dry ice.

200

201 *Anatomical localization of Dnmt3a and Dnmt3b expression in the female hypothalami*

202 Brains were then transferred to -70°C until tissue section. Brains were coronally
203 sectioned at 30μm thickness on a cryostat (Leica 3050 series) and mounted onto poly-L-
204 lysine/gelatine coated slides. Sections were taken from the anterior commissure through to
205 the caudal aspect of the 3^{rd}V, the full dorso-ventral extension of the hippocampal pyramidal
206 layer was used as an anatomical marker for hypothalamic localization. Coronal sections were
207 grouped into sets (each set consisted of every 8th section) spanning the entire extent of the
208 hypothalamus. By sectioning in this manner, we were able to encompass the rostrally located
209 POA, SCN, 3^{rd}V and the caudally located Arc. Tissue sectioned slides were stored at -70°C
210 until histological assays.

211

212 *Immunohistochemistry*

213 Immunohistochemistry (IHC) was carried out on slide mounted coronal brain
214 sections. The IHC protocol started with a 10-minute fixation step in ice cold 4%
215 paraformaldehyde followed by three washes in 0.1M phosphate buffered saline (PBS) for
216 5mins each. Antigen retrieval was carried out using 10mM sodium citrate pH 6.0 (Sigma-
217 Aldrich) heated to 95°C for 20mins then after three washes in PBS, slides were incubated in
218 3% H_2O_2 (Fisher Scientific) for 30mins at room temperature. Serum blocking was carried out
219 using a species appropriate serum (either goat or horse serum, Vector labs) at 5% in PBS for
220 1hr at room temperature. Slides were again washed using PBSt (0.1M PBS + 0.1% Triton-X
221 (Sigma-Aldrich)) before being incubated with primary antibody diluted in 5% serum at the
222 optimised concentration (see Table S2) at 4°C for 48hrs. After 3 PBSt washes, sections were
223 incubated with a biotinylated secondary antibody appropriate for the species that the primary
224 antibody was raised in for 1hr at room temperature. In this study, the Tyramide Signal

225 Amplification (TSA) Biotin system was used to amplify the signal (Perkin-Elmer,
226 NEL700A001) (33). Briefly, once slides had been incubated with the respective biotinylated
227 secondary antibody, they were washed in PBSt and then incubated with streptavidin-
228 horseradish peroxidase conjugate for 30mins at room temperature. Slides were then washed
229 in PBSt 3 times for 5mins, and subsequently incubated with tyramide solution for 7mins at
230 room temperature. Slides are washed with PBSt before incubation with avidin-conjugated
231 fluorescein (A-2001, Vector Labs) for 1hr in a darkened room. Slides were then washed in
232 H_2O for 5mins, then cover slipped with DAPI (H-1200, Vector Labs).

233 The human peptide used to generate the Dnmt3a antibody (catalogue no. 3598; Cell
234 Signalling Technologies, UK) shares 100% sequence homology with the hamster Dnmt3a
235 sequence, whereas an epitope-tagged recombinant mouse Dnmt3b protein was used to
236 generate the Dnmt3b antibody (52A1018; Novus Biologicals, UK) which shares 94%
237 sequence homology to the characterised hamster sequence. Moreover, the antibody has been
238 shown to exhibit cross species reactivity with sheep, rat and humans. We included multiple
239 negative controls in our IHC protocol, including omitting primary antibody, omitting
240 secondary antibody, or omitting TSA amplification to test for non-specific signal generation.
241 In all cases, negligible immunoreactive signal was generated. Pre-adsorption controls were
242 conducted for Dnmt3a using a Dnmt3a blocking peptide (3227BP-50 Cambridge Bioscience).
243 Pre-adsorption controls used 1/500 primary antibody and either 1/100, 1/250 or 1/500
244 blocking peptide concentrations for 3 hours before incubation with hamster tissue. All
245 sections with blocking peptide had the Dnmt3a immunoreactive signal eliminated.

246

247 *Mapping and analyses of hypothalamic Dnmt3a and Dnmt3b immunoreactivity*

248 Images were captured using Axiovert 200M (Zeiss) inverted fluorescent microscope
249 with the Axiovison (version 4.7) software and Hammamatsu camera. Image analysis was

250 carried out using ImageJ software (http://imagej.nih.gov/ij/) with the Fiji bioimaging plugin
251 (34). Photomicrograph images were collected and used to measure immunoreactive Dnmt3a
252 (ir-Dnmt3a) and Dnmt3b (ir-Dnmt3b) cell numbers and signal intensity. Overall, ir-Dnmt3a
253 had a diffuse distribution in the hypothalamus with higher expression levels in the SCN and
254 Arc. The Allen mouse atlas was used as a guide to determine the borders of the SCN, Arc and
255 POA. The ventral and medial boundaries of the SCN and Arc were determined by the base of
256 the hypothalamus and 3^{rd}V, respectively. The expression of ir-Dnmt3a and ir-Dnmt3b cells in
257 the SCN and Arc were within the dorsal and lateral boundaries outlined in the Allen mouse
258 atlas. ir-Dnmt3b stain was localised to the ependymal layer of the 3^{rd}V and SCN. Sections
259 were approximately 240µm apart as recommended by West (35) for stereological counting of
260 cells. Positively stained cells were counted using the Cell Counter plug-in integrated in the
261 Fiji package where only the leading edge of positively stained cells were counted. Images
262 were converted to 8-bit greyscale, then each cell was circled using the circle selection tool on
263 Fiji and the mean greyscale measured. For each section 3, background measurements
264 (adjacent brain region without positive cells) were taken to determine non-specific
265 background fluorescence. The background was then subtracted from fluorescence intensity of
266 each cell to allow for normalised signal intensities to be compared across images.
267 Immunoreactive positive cell counts and signal intensity was measured for ir-Dnmt3a in the
268 SCN and Arc, and ir-Dnmt3b in the SCN and 3^{rd}V.
269

270 Study 3 - *in vitro* analyses of DES and hyper- and hypo-DNA methylation on neuropeptides,
271 circadian clock genes and *de novo* DNA methyltransferase expression.
272 *Cell culture*
273 The mHypoE N36/1 neuronal cell line, derived from an embryonic mouse
274 hypothalamus (36) was used to examine the impact of hyper-methylation and Dnmt inhibition

275 in conjunction with hormonal regulation of mRNA levels. The mHypoE N36/1 neuronal line
276 was selected as these cells provide an optimal *in vitro* model to complement the hypothalamic
277 oestrous analyses conducted in Study 1. Furthermore, as *de novo* DNA methyltransferase
278 enzymes are expressed across multiple hypothalamic nuclei (19-22); these cells provide a
279 model to identify the effect of DES and hyper- and hypo-DNA methylation agents on select
280 neuroendocrine targets. Cells were grown in a humidified incubator at 37°C in 5% CO_2 in
281 Dulbeccos Modified Eagle Media (DMEM - high glucose; 4,500mg/L glucose, L-glutamine
282 and sodium bicarbonate (D5796, Sigma-aldrich), supplemented with fetal bovine serum
283 (FBS, 10%, ATCC), penicillin (10units/ml), and streptomycin (10µg/ml) (Pen/Strep 100x,
284 ThermoFisher) in T75 flasks (FisherScientific). Cells grown to 80% confluency before
285 passage with trypsin/EDTA (ThermoFisher) then split and reseeded into a clean T75 flask.
286 mHypoE N36/1 cells were used for a maximum of 25 total passages after which fresh cells
287 were revived from frozen stocks. For experiments, cells were trypsinised and resuspended in
288 growth media to quantify cell density and viability using trypan blue and a haemocytometer.
289 Cells were seeded at a density of $5x10^5$ cells/well in growth media in 24-well plates
290 (Corning) and allowed to adhere and grow for 24 hr in a humidified incubator.

291 The treatment groups were divided across 4 experiments. In the hypermethylation
292 experiment, cells were exposed to 3mM 3-aminobenzamide (3AB, Sigma-Aldrich) or vehicle
293 control (DMSO). 3AB is a well-characterized drug that induces hyper-methylation via the
294 inhibition of poly(ADP-ribosyl)ation (37) and has previously been shown to increase
295 hypothalamic DNA methylation in hamsters (21). In the Dnmt inhibition experiment, cells
296 were incubated with 100µM zebularine (ZEB, Selleckchem) or control (saline). We examined
297 *Kiss1*, *Avp* and *Vip* expression in response to 3AB and ZEB as these neuropeptides are
298 involved in the control of reproductive physiology and previously shown to be regulated by
299 DNA methylation (38-40). To test the influence of ovarian steroids, cells were incubated with

300 1nM diethylstilbesterol (DES, Sigma-Aldrich) or vehicle control (ethanol). Finally, a
301 hormone positive control experiment was run using cells exposed to 10mM
302 dihydrotestosterone (DHT, Sigma-Aldrich), or ethanol (vehicle control). Prior to hormone
303 treatments, cells were subjected to serum starvation for 24hr using serum-free growth media,
304 followed by a saline wash. Serum-free media was used for DES and DHT experiments to
305 minimize any confounding effects of serum steroid hormones (41). Cells used in the 3AB,
306 ZEB and DTH experiments were incubated with 1ml of respective treatment media for either
307 9hr or 24hrs in a humidified chamber with 5% CO_2 at 37°C. Cells treated with DES were
308 incubated with 1ml of respective media for only 24hrs. All samples were lysed *in vitro* using
309 RLT buffer supplemented with 1% v/v β-mercaptoethanol from the Qiagen RNeasy Mini kit
310 and stored at -70°C.
311
312 *RNA extraction and cDNA synthesis*
313 RNA from cells was isolated using the RNeasy Mini Kit (Qiagen). According to the
314 manufacturer's protocol using 33ul nuclease-free H2) to elute the purified RNA from the spin
315 column. The concentration of eluted RNA was determined using a NanoDrop (ThermoFisher)
316 and the RNA was then stored at -70°C until further processing. cDNA was synthesized using
317 oligo dT primers (Precision nanoScript2 Reverse Transcription kit, Primer Design) according
318 to the manufacturer's directions.
319
320 *qPCR assay for Dnmt3a, Dnmt3b and reproductive neuropeptides*
321 qPCRs were run on a BioRad CFX96 Real time PCR machine in a 20µl reaction.
322 Primers were supplied by Invitrogen and the primer sequences are provided in Table S3. Both
323 *Dnmt3a* and *Dnmt3b* express multiple isoforms in many human, mouse and rat tissues. The
324 hamster *Dnmt3a* and *Dnmt3b* primers are not designed to distinguish between the various

325 *Dnmt3a* and *Dnmt3b* isoforms described in rodents (i.e. mice or rats). Therefore, the qPCRs
326 for *Dnmt3a* and *Dnmt3b* provide an overall assessment of transcript levels. All samples were
327 run in duplicate in a 96-well plate format under the following cycling conditions; i) initial
328 denaturing at 95°C for 5min, then 39 cycles of ii) 95°C for 10secs, iii) 30 secs at annealing
329 temperature dependent on gene of interest, then iv) an extension step of 72°C for 30secs. For
330 each qPCR assay no-template H_2O controls (NTC) were included. Melt curve were examined
331 to confirm specificity of the amplification reaction. PCR Miner (25) was used to determine
332 reaction efficiencies (E) and quantification cycle (Cq). According to MIQE guidelines,
333 samples with efficiency values below 0.8 and above 1.2 were excluded from analyses (30).
334 Fold expression of each target gene was measured in relation to the geometric mean of the Cq
335 for two reference genes (*gapdh* and *βactin*) and calculated using 2-(ΔΔCq). The reference
336 gene was stable across treatment groups and drug treatments [CT $\bar{x}$±SEM Control (19.6±0.1)
337 and 3AB (19.5±0.1); [CT $\bar{x}$±SEM Control (22.1±0.1) and DES (22.1±0.1)]; and [CT $\bar{x}$±SEM
338 Control (17.9±0.2) and ZEB (18.2±0.7)].
339
340 *Statistical analyses*
341 For Study 1, a two-tailed t-test was conducted to determine whether the expression of
342 hypothalamic *Dnmt1*, *Dnmt3a* and *Dnmt3b* was different between proestrous and oestrous
343 females. Since *Dnmt3a* expression increased during oestrous, we conducted one-tailed t-tests
344 to examine statistical significance in the E2P4 driven changes in the number of ir-Dnmt3a
345 and ir-Dnmt3b cells and signal intensity. Moreover, one-tailed t-tests were also conducted on
346 DES-driven mRNA expression in Study 3. Data were log-transformed in the event a violation
347 of normality was observed. Statistical analyses were performed using SigmaPlot 13.0 and
348 significance was determined at p≤0.05.
349

350 **Results**

351 Hypothalamic *Dnmt3a* expression varies between proestrous and oestrous stages

352 The precision of the tissue punches targeting the AH and PH was confirmed by *Gnrh, Kiss1*
353 and *Rfrp3* mRNA expression. *Gnrh* was only identified in the AH, *Kiss1* was localized to the
354 AH (i.e. AvPv/POA) and PH (i.e. Arc), and *Rfrp3* was expressed at higher levels in the PH
355 (i.e. DMH) (Fig S1C-E). *Dnmt3a* expression was significantly higher in the AH tissue-
356 punches during oestrous (P<0.05), but neither *Dnmt1* (P>0.20) nor *Dnmt3b* (P>0.34) differed
357 between these stages (Fig. 1). *Dnmt1, Dnmt3a* and *Dnmt3b* expression in the PH did not vary
358 across the oestrous stages (P>0.05 for all comparisons). *Dnmt3a* expression was significantly
359 higher in the AH, whereas *Dnmt1* and *Dnmt3b* expression was significantly greater in the PH
360 (data not shown).

361

362 Ovarian steroids stimulate ir-Dnmt3a and ir-Dnmt3b in hypothalamic nuclei.

363 To determine the impact of ovarian steroids, adult hamsters were ovariectomized
364 (OVX) and after 8 weeks were treated with E_2P_4 or vehicle control (oil), and ir-Dnmt3a/b
365 immunoreactivity was examined histologically either 12h or 24h later. There was no
366 significant effect of time on ir-Dnmt3a nor ir-Dnmt3b expression in any nuclei examined
367 (P>0.18 for all comparisons). Therefore, we combined the data from both treatment groups.
368 E_2P_4 was found to significantly increase the number of ir-Dnmt3a cells in the SCN (Fig. 2A-
369 B; P=0.05), without influencing their fluorescence intensity (Fig. 3A; P>0.05). In the Arc, ir-
370 Dnmt3a cell numbers were not affected by E_2P_4 (Fig. 2C-D; P>0.05), nor was their
371 fluorescence intensity (Fig. 3B; P>0.05). Furthermore, E_2P_4 treatment failed to alter the
372 number of ir-Dnmt3b cells or signal intensity in the 3^{rd}V (Fig. 2E-F, Fig. 3D, respectively;
373 P>0.49), but caused an increase in ir-Dnmt3b signal intensity in the SCN (Fig. 3C; P<0.05).
374 Collectively, these data indicate that ovarian steroids markedly upregulate ir-Dnmt3a and ir-

375 Dnmt3b expression specifically in the SCN. When compared to the oestrous study data, these
376 results indicate that *de novo* DNA methyltransferase expression in anterior hypothalamic
377 tissues including the SCN changes over the course of the female ovarian cycle; increased
378 *Dnmt3a* expression coincides with, and is likely driven by, elevated ovarian steroid secretion.
379

380 The potent estrogen analogue DES upregulates *Dnmt3b* expression *in vitro.*

381 In order to begin to elucidate the molecular mechanisms by which ovarian hormones
382 affect DNA methyltransferase activity in the HPG axis, we sought to develop a tractable *in*
383 *vitro* model. To accomplish this, we used a well-characterised cell model, the embryonic
384 mouse hypothalamic cell line, mHypoE N36/1 (36) to test whether DES would influence
385 *Dnmt3a* and *Dnmt3b* expression and alter the expression of reproductive neuropeptides.
386 Following incubation with DES, *Kiss1* (P=0.01) and *Vip* (P<0.05; Fig. 4A) were significantly
387 downregulated in mHypoE N36/1 cells, whereas there was no effect on *Avp* expression
388 (P>0.14). *Dnmt3b* expression was significantly upregulated in DES treated cells (P<0.05; Fig.
389 4B), but DES failed to alter *Dnmt1* (P>0.19) or *Dnmt3a* (P>0.36) expression. Collectively,
390 these results indicate that DES signalling via estrogen receptor upregulates *Dnmt3b*
391 expression, without significantly affecting *Dnmt1* or *Dnmt3a* expression, pointing to a degree
392 of specificity in the effects of ovarian hormone secretion on *de novo* DNA methyltransferase
393 induction.

394 This assay was also used to examine whether *Dnmt3a/b* expression can be induced in
395 response to the non-aromatizable androgen, dihydrotestosterone (DHT), which would offer
396 insights into whether, *in vivo*, effects of DES on *Dnmt3a* and *Dnmt3b* can be mediated via
397 androgen receptors. DHT failed to induce *Dnmt1, 3a* or *3b* expression as compared to vehicle
398 treated controls (Fig. 4C; P>0.10, all comparisons), indicating that, in this paradigm,
399 androgens are not sufficient to upregulate *Dnmt* expression, and may indicate that, *in vivo*,

400 estrogen signalling may be the essential pathway by which gonadal hormones activate
401 hypothalamic *Dnmt* expression.
402
403 Functional role of DNA methylation on neuropeptide and circadian clock genes
404 In order to identify potential targets of increased hypothalamic DNA methylation, we
405 used two epigenetic-modifying drugs to either inhibit Dnmt enzymes with ZEB or hyper-
406 methylate DNA using 3AB. Treatments did not affect transcript expression across 9- and 24-
407 hr sampling points (P>0.28); therefore we collapsed the data to simplify the presentation of
408 the results. To eliminate any potential effects of time on clock gene expression; qPCR was
409 conducted using cells treated with 3AB for 24hrs. We identified 2 genes expressed in the
410 SCN that were significantly reduced after exposure to 3AB, *Vip* (Fig 5A; P<0.05) and *Bmal1*
411 (Fig 5B; P<0.05). The 3AB-induced hypermethylation appears to be specific as no
412 differences in expression other reproductive neuropeptides gene (i.e. *Kiss1, Avp*) (Fig. 5A;
413 P>0.16) or other circadian clock genes (i.e. *Per1, Clock*) (Fig. 5B; P>0.27) were observed.
414 However, ZEB treatment reduced DNA methyltransferase but did not significantly alter *Vip*,
415 *Kiss1,* or *Avp* expression (Fig. 5C; P>0.28). The lack of an effect may be due to the relatively
416 short exposure of the cells to ZEB (i.e. <24hrs).
417
418 **Discussion**
419 While the impact of epigenetic modifications has been implicated in timing fertility in
420 peripheral tissues (15-17), a role in the hypothalamus has not been investigated. This study
421 examined the distribution of *Dnmt3a* and *Dnmt3b* across the hamster oestrous cycle and
422 identified that ovarian steroids stimulate ir-Dnmt3a and ir-Dnmt3b expression in the SCN.
423 During proestrus, AH (i.e. SCN) *Dnmt3a* expression is significantly reduced; this effect was
424 anatomically specific, as no effect was observed in the PH, which is also sensitive to ovarian

425 steroid feedback. Together these data reveal a novel neuroendocrine mechanism in which
426 epigenetic modifications driven by DNA methylation in the SCN control fertility timing in
427 mammals. We propose that DNA methylation in the SCN provides transient inhibition on
428 critical genes (i.e. *Vip, Bmal1*) that in turn, promotes cyclic changes in fertility and female
429 sexual behaviour which constitute the oestrous cycle.

430 These data indicate that DNA methylation may regulate SCN gene transcription as
431 females transition from proestrous to the oestrous stage. *Vip* is highly expressed in the SCN
432 and is critical for the synchronization and maintenance of molecular timekeeping (42). VIP
433 immunoreactive neurons in the SCN also project directly onto GnRH neurons, with greater
434 abundance after puberty (43). Male and female *Vipr2*$^{-/-}$ mice exhibit reduced fertility, and
435 females exhibit irregular oestrous cycles (44), consistent with a model in which *Vip*, localized
436 in the core circadian nuclei, functions as an integral part of the molecular machinery,
437 regulating the neuroendocrine output of GnRH neurons. Here, we extend this model to
438 indicate that *Vip* expression is significantly reduced by DES, and this may be mediated by the
439 increased expression of *Dnmt3a/3b* driven DNA methylation. DES was sufficient to stimulate
440 the expression of *Dnmt3b* mRNA; whereas DES and progesterone was required to drive
441 Dnmt3a protein expression. Given the observation that Dnmt3a and Dnmt3b have dissociable
442 effects on the genome (45), it is likely that ovarian steroids exerted independent effects on
443 DNA methylation by selectively recruiting different Dnmt3a/b enzymes throughout the
444 oestrous cycle. Furthermore, as the levels of estrogen receptor-α and -β expression are very
445 low in the SCN (46), it is likely that ovarian steroids act in other hypothalamic nuclei (e.g.
446 Arc, 47) and indirectly regulate *de novo* DNA methyltransferase expression in the SCN.
447 Further examination of how estrogen and progesterone signalling regulate SCN *dnmt3a* and
448 *dnmt3b* expression is warranted.

449 The daily control of female reproductive physiology requires kisspeptin-dependent
450 regulation of GnRH release (2,5, 48). Avp-expressing cells in the SCN project to Kiss1
451 neurons in the anteroventral periventricular and periventricular preoptic nuclei (AvPv/PeN)
452 and may provide SCN-dependent circadian timing of oestrous (6). 3AB hypermethylation of
453 mHypoE N36/1 cells did not affect *Avp* nor *Kiss1* expression. These findings suggest that *de*
454 *novo* DNA methylation may not be involved in the hamster SCN Avp to AvPv/PeN
455 kisspeptin to GnRH neuroendocrine pathway. However, further research using *in vivo*
456 approaches are required to determine whether DNA methylation regulates either Avp or
457 kisspeptin signalling across the oestrous cycle.

458 One pathway through which this novel effect may be mediated is via the circadian
459 clock gene *bmal1*, which is a core component of the circadian clock, and is also a target for
460 Dnmt3a (49). *In vitro* hyper-methylation via 3AB reduced *Bmal1* expression within 24 hrs
461 and thus indicates a functional role of DNA methylation induced changes in circadian clock
462 gene transcription. The lack of an effect on other circadian clock genes (i.e. *Per1*) is likely
463 due to the single 24hr sampling period. The assessment of circadian clock gene expression
464 over several days is necessary in order to determine the downstream effect of increased DNA
465 methylation on the *Bmal1* expression. Daily DNA methylation oscillations in the *Bmal1*
466 promoter likely exerts a critical role on the oestrous cycle by maintaining the functional
467 integrity of the circadian transcriptional-translational feedback loop, which regulates the
468 timing of the luteinizing hormone surge during proestrous. The present data here add to a
469 growing body of work which indicates that, beyond its well-characterised role in
470 embryogenesis and gametogenesis**,** rhythmic *de novo* methylation may be widespread in the
471 adult brain.

472 Rhythmic changes in *de novo* DNA methyltransferase enzymes across seasonal time
473 scales have been reported in the hypothalamus (21,25,27), gonads (15), liver (50) and

474 leukocytes (51). The evidence indicates that *Dnmt3a* and *Dnmt3b* expression are regulated by
475 a combination of environmental and endogenous signals (52). The present study reveals
476 anatomically localized effects of ovarian steroids on *Dnmt3a* and *Dnmt3b* expression and
477 indicates that rhythmic DNA methylation might be a novel component for the timing of
478 female fertility. The availability of the Siberian hamster genome will facilitate the dissection
479 of localized rhythmic DNA methylation across neuroendocrine and peripheral reproductive
480 tissues (53).
481

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632

633 **Figure Legends**

634

635 Figure 1 – Oestrous plasticity in DNA methyltransferase mRNA expression in the anterior
636 hypothalamus. The fold change in *Dnmt3a* mRNA in the anterior hypothalamus
637 had significantly higher expression during oestrous (EST) compared to proestrous
638 (PRO) hamsters. Neither *Dnmt1* nor *Dnmt3b* were observed to be significantly
639 different between PRO and EST stages. Asterisks indicate statistical significance
640 by * P<0.05. Numbers in the bar graph denote sample size.

641

642 Figure 2 – Ovarian steroids stimulate Dnmt3a in the suprachiasmatic nucleus. A single bolus
643 injection of an estrogen analogue diethylstilbestrol (DES) and progesterone
644 (denoted as E_2P_4) was found to have anatomically localized effects on Dnmt3a and
645 Dnmt3b expression in the hypothalamus. Ovariectomized hamsters that received
646 E_2P_4 significantly increased the number of Dnmt3a cells in the suprachiasmatic
647 nucleus (SCN) compared to oil treated controls (OVX) (A). Representative
648 photomicrographs of Dnmt3a immunoreactivity in the SCN (B). There was no
649 effect of E_2P_4 on Dnmt3a immunoreactive cells in the Arc (C, D). There was no
650 significant effect of E_2P_4 on Dnmt3b immunoreactive cells in the ependymal layer
651 along the 3^{rd}V (E, F). White lines denote the boundary of the SCN (B) and Arcuate
652 (D). The white arrows indicate immunoreactive Dnmt3a/b cells. Abbreviations: 3rd
653 ventricle (3^{rd}V), Arcuate (Arc), oestrous (EST), Median eminence (ME),
654 proestrous (PRO) and Suprachiasmatic nucleus (SCN). Asterisks indicate statistical
655 significance by * P<0.05. Numbers in the bar graph denote sample size.

656

657 Figure 3 – Ovarian steroid regulation of *de novo* DNA methyltransferase immunoreactive
658 intensity in the female hamster hypothalamus. Dnmt3a cell intensity in the SCN
659 (A) and Arc (B) was not significantly different between ovariectomized females
660 treated with oil or E_2P_4. Ovarian steroids were found to significantly increase
661 Dnmt3b signal intensity in the SCN (C), but there was no significant effect on
662 immunoreactivity in the 3^{rd}V (D). Asterisks indicate statistical significance by *
663 P<0.05. Numbers in the bar graph denote sample size.

664

665 Figure 4 – Diethylstilbestrol and Dihydrotestosterone effects on neuropeptide and DNA
666 methyltransferase expression. Using mHypoE N36/1 (an immortalized
667 hypothalamic neuronal cell line) neurons were treated with the highly potent
668 estrogen agonist diethylstilbestrol (DES) (A, B). DES caused decreases in
669 vasoactive intestinal polypeptide (*Vip*) and kisspeptin (*Kiss1*) expression (A) and
670 upregulated *Dnmt3b* expression (B). There was no significant effect on vasopressin
671 (*Avp*), *Dnmt1* or *Dnmt3a*. Cells treated with DHT did not show significant
672 variation in *Dnmt1*, *Dnmt3a* or *Dnmt3b* expression (C). Asterisks indicate
673 statistical significance by * P<0.05. Numbers in the bar graph denote sample size.

674

675 Figure 5 - *In vitro* analyses of DNA methylation on neuropeptides and circadian clock gene
676 expression. Using mHypoE N36/1 neurons were treated with either the
677 hypermethylation compound 3-aminobenzimide (3AB) or the DNMT inhibitor
678 zebularine (ZEB). 3AB inhibited *Vip* (A) expression and *Bmal1* (B) mRNA levels,
679 but not vaopresson (*Avp*), kisspeptin (*Kiss1*), Clock (*Clock*) or period1 (*Per1*)
680 expression. DNMT inhibition via ZEB application did not significantly affect *Vip,*
681 *Avp* or *Kiss1* expression (C). *P<0.05 vs. control. Numbers in the bar graph denote
682 sample size.

683