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Title: Epigenetic control of the vasopressin promoter explains physiological ability to regulate vasopressin transcription in dehydration and salt loading states in the rat

Short title: Epigenetic control of vasopressin transcription

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The authors declare no conflict of interest.

Abstract

The synthesis of arginine vasopressin (*Avp*) in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus is sensitive to increased plasma osmolality and decreased blood volume, and, thus is robustly increased by both dehydration (increased plasma osmolality and decreased blood volume) and salt loading (increased plasma osmolality). Both stimuli result in functional remodelling of the SON and PVN, a process referred to as functional-related plasticity. Such plastic changes in the brain have recently been associated with altered patterns of DNA methylation at CpG residues, a process thought to be important for the regulation of gene transcription. In this regard, the proximal *Avp* promoter contains a number of CpG sites and is recognised as one of four CpG islands for the *Avp* gene, suggesting that methylation may be regulating *Avp* transcription. We show here that, in immortalised hypothalamic cell line 4B, the proximal *Avp* promoter is highly methylated, and treatment of these cells with DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine, to demethylate DNA, dramatically increases basal and stimulated *Avp* biosynthesis. We report here no changes in expression of DNA methyltransferases, *Dnmt1* and *Dnmt3a*, but decreased expression of the demethylating enzyme ten-eleven-translocation 2, *Tet2*, in the SON by dehydration and salt loading. We found higher methylation of the SON *Avp* promoter in dehydrated but not salt loaded rats. By analysis of individual CpG sites, we observed hypomethylation, hypermethylation and no change in methylation of specific CpGs in the SON *Avp* promoter of the dehydrated rat. Using reporter gene assays, we show that mutation of individual CpGs can result in altered *Avp* promoter activity. We propose that methylation of the SON *Avp* promoter is necessary to co-ordinate the dual inputs of increased plasma osmolality and decreased blood volume on *Avp* transcription in the chronically dehydrated rat.

Introduction

The neuropeptide hormone arginine vasopressin (AVP) is synthesised in magnocellular neurons of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus. Increases in plasma osmolality are detected by osmosensitive neurons in circumventricular organs that provide direct excitatory inputs leading to increased *Avp* synthesis by magnocellular neurons of the SON and PVN and AVP secretion from the posterior pituitary (1). An increase in plasma osmolality of only 1% is sufficient to drive increased AVP synthesis and secretion (2). Vasopressin synthesis and secretion is also sensitive to non-osmotic cues, including changes in blood volume and pressure (3-7). A decrease in blood volume (hypovolemia) is detected by the cardiac right atrium, again resulting in increased AVP synthesis and secretion (8). In this regard, changes in blood volumes greater than 8% are necessary to facilitate this response (3, 5, 9, 10). A population of smaller AVP expressing parvocellular neurons is also found in the PVN, which are important in co-ordinating responses to stress (11).

The two osmotic stimuli of dehydration and salt loading both robustly increase *Avp* mRNA levels by approximately 2-fold in the SON and PVN, with parallel increases in the secretion of AVP (5, 9, 12). Notably, dehydration also decreases blood volume in rats, with >20% reductions in volume by 3 days (5, 9, 13); thus dehydration can be considered as both an osmotic and hypovolemic stimulus. The prolonged exposure to either of these stimuli causes functional remodelling of both brain nuclei as a consequence of persistent neuronal activation, a process referred to as function-related plasticity (14). The visible outcomes of prolonged hyperosmotic stimulation of the PVN and SON are increased volumes of magnocellular neurons and a retraction of glial processes, which is reversed upon cessation of the stimulus (14, 15). The

hypertrophy of magnocellular neurons is recognised to be due to the large increase in transcription and protein synthesis under hyperosmotic stimulation. In this regard, we, as well as others, have reported catalogues of differentially expressed genes in the SON and PVN in response to both dehydration and salt loading consistent with increased levels of transcription (16-18). These lists include upregulated expression of a wide array of transcription factors that through their interaction at the promoters of target genes are important for this wave of increased transcriptional activity.

A previous study has suggested that brain plasticity is dependent upon epigenetic mechanisms that result in stable modulation of gene expression (19). Indeed, a study by Guo et al. (2011) (20) suggested that increased neuronal activity could promote vast methylation changes throughout the genome. The epigenetic modification receiving most attention is methylation of CpG (Cytosine-phosphate-Guanine) sites. The notion that the methylation of genomic DNA was stable in adult life is now discredited. The adult brain expresses high levels of DNA methyltransferases (*Dnmt*) that are involved in the active maintenance and *de novo* methylation of CpG residues in genomic DNA (21, 22). In addition, the ten-eleven-translocation (*Tet*) genes are also abundant in the brain and act to facilitate active demethylation at CpG residues (23). Increased methylation is commonly associated with transcriptional inhibition though in some cases transcriptional activation has also been reported (24). Thus, we proposed that changes in methylation may contribute to the plastic response in the hypothalamus in response to dehydration and salt loading.

We focused our attention on the *Avp* promoter. The *Avp* gene has been the subject of a number of methylation studies in both the rat and mouse hypothalamus and other brain regions (25-28). The methylation status of the mouse *Avp* gene has been comprehensively described in the PVN, where early life stress results in hypomethylation at CpGs sites in a putative enhancer within the intergenic region between the *Avp* gene and the gene encoding the closest related hormone oxytocin, which was shown to be consistent with increased *Avp* expression in adult animals (27). A later study showed that the methylation patterns of these CpG sites were also altered by age, suggesting that alterations in methylation signatures within the *Avp* gene could be influenced by events later in life (28).

Four CpG islands have been identified for the *Avp* gene, and the so named CpG island 1 spans the proximal promoter region of the *Avp* gene (27, 29). The proximal portion of the *Avp* promoter includes a number of potential transcription factor binding sites, including activator proteins 1 and 2 (AP-1 and AP-2), cAMP responsive element (CRE) sites, E-box and G-box elements (30, 31). We recently showed that CREB3L1 is a transcription factor of the *Avp* gene and, by chromatin immunoprecipitation, showed that it binds within the first few hundred bases upstream of the transcriptional start site (30). The immediate early genes c-Fos and c-Jun, and CREB have also been implicated in induction of *Avp* transcription via interactions with this segment of DNA (31-33). It has been shown that methylation can inhibit transcription factor interactions with DNA either directly or through the recruitment of methyl binding proteins (34). Thus, we reasoned that the proximal promoter may be subjected to methylation changes in the hypothalamus in response to hyperosmotic stress.

We examined the methylation status of the proximal *Avp* promoter in the SON in response to the hyperosmotic stressors of dehydration and salt loading. The SON was the region of choice, as it contains only magnocellular neurons, compared to the PVN which is more heterogenous in its neuronal phenotypes and physiological functions. We found that dehydration induces hypermethylation of the proximal *Avp* promoter, whereas methylation status was not altered by salt loading.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 275–300 g were used in this study. Rats were maintained under a 14:10 light dark cycle (lights on at 0500) with food and water ad libitum for at least 1 week prior to experimentation. Animal experiments were performed between 9am – 11am. To induce hyperosmotic stress either water was removed for 3 days dehydration or replaced by 2% (w/v) NaCl in drinking water for 7 days as a protocol for salt loading. All rats were humanely killed by striking of the cranium (stunning) and then immediately decapitated with a small animal guillotine (Harvard Apparatus, Holliston, MA). Brains were rapidly removed from the cranium and frozen on dry ice (within 3 minutes after stunning) before being stored at -80°C. All experiments were performed under a Home Office UK licence held under, and in strict accordance with, the provision of the UK Animals (Scientific Procedures) Act (1986); they had also been approved by the University of Bristol Animal Welfare and Ethical Review board.

Dual extraction of DNA and RNA from the same sample

Frozen brains were sliced into 60 µm coronal sections in a cryostat. Sections were mounted on glass slides and stained with 0.1% (w/v) toluidine blue, then visualised on a light microscope until magnocellular neurons of the SON were visible. SON samples (24 punches) were collected from twelve coronal slices using a 0.35 mm sample corer (Fine Scientific Tools) using the optic chiasm as a reference. Punches were dispensed into 0.5 ml tubes kept on dry ice within the cryostat. Cortex samples (4 punches) were collected from two consecutive sections after SON collection using a 0.8 mm sample corer to obtain similar quantities of tissue (Fine Scientific Tools). Total RNA and genomic DNA were extracted from each sample using ZR-Duet DNA/RNA MiniPrep kit (Zymo research). The punch samples (SON and cortex) were removed from dry ice and rapidly resuspended, by vortexing, in 400 µl DNA/RNA Lysis Buffer. The following steps were performed following the manufacturer's protocol.

For *in vitro* studies, the culture medium was removed and the cells were lysed in 350 µl Qiazol Reagent (Qiagen). The lysate was mixed with 350 µl absolute ethanol and added directly into the Direct-zol™ RNA MiniPrep columns (Zymo research; R2052) and extraction continued following the manufacturer's protocol. The concentrations of DNA and RNA were determined using a NanoDrop (Thermo Scientific).

Bisulfite conversion of DNA and TA cloning

Genomic DNA from SON and cortex punches (50 ng) and rat hypothalamic 4B cells (200 ng) was bisulfite converted using EZ DNA Methylation-Gold kit (Zymo research). Primers for amplification of bisulfite converted DNA were designed using MethPrimer design software (35).

The converted DNA was amplified with rat *Avp* promoter primers (5'-TTGTTGAGAGTTGTTGAAATGTTTAAT-3' and 5'-TTTATATCTACAAATATTAATACTAAAAAAC-3') using the following cycling conditions; 94°C for 2 min followed by 45 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 2 min. PCRs were performed using Platinum Taq DNA Polymerase (Life Technologies). The PCR products were purified using Qiagen's PCR purification kit, ligated into pGEM-T Easy vector (Promega), and transformed into DH5 α competent *E.coli* cells. Positive clones were selected by blue/white colony selection on liquid broth agar plates supplemented with X-Gal and IPTG. Plasmid DNA was extracted from overnight bacterial cultures using QIAprep Spin Miniprep kit and the presence of inserts was verified by restriction digestion with EcoRI. Twenty independent clones were sequenced per SON sample and 10 independent clones per sample for cortex and hypothalamic 4B cells.

Cell treatments

Rat hypothalamic 4B cells were cultured in DMEM (Sigma; D6546) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 2mM L-glutamine and 100 unit/ml of penicillin-streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% (v/v) CO₂. For chemical treatments, cells were seeded onto tissue culture plates to 60-70% confluence. After 24 h cells were treated with 1, 2.5, 5 or 10 μ M of DNA methyltransferase inhibitor (36), 5-Aza-2'-deoxycytidine (5-Aza; Sigma, A3656) for 48 h. For activation of the cAMP pathway cells were treated with 10 μ M forskolin (Sigma: F6886) or DMSO (vehicle) for 4 h. Stock solutions of 5-Aza (10 mM) and forskolin (10 mM) were prepared in DMSO.

cDNA synthesis and quantitative PCR analysis

For cDNA synthesis, total RNA (50 ng for punch samples, 500 ng for hypothalamic 4B cells) was reverse transcribed using the Quantitect reverse transcription kit (Qiagen). Primers for *Avp* (5'-TGCCTGCTACTTCCAGAACTGC-3' and 5'-AGGGGAGACACTGTCTCAGCTC-3'), heteronuclear *Avp* (*hnAvp*) (5'-GAGGCAAGAGGGCCACATC-3' and 5'-CTCTCCTAGCCCATGACCCTT-3'), *c-Fos* (5'-AGCATGGGCTCCCCTGTCA-3' and 5'-GAGACCAGAGTGGGCTGCA-3'), *Creb3l1* (5'-GCCAACAGGACCCTGCTCCA-3' and 5'-AGTGCCAGTCTGTGTGGCCG-3'), *Dnmt1* (5'-AACCACTCAGCATTCCCGTA-3' and 5'-TGCTGGTACTTCAGGTCAGG-3'), *Dnmt3a* (5'-AAGACCCCTGGAAGTCTGCTAC-3' and 5'-TGCGAAGAACATCTGGAGT-3'), *Tet1* (5'-TGACCCACTCTTACCAGACC-3' and 5'-GATGGGCCATTGCTTGATGT-3'), *Tet2* (5'-TCGGAGGAGAAGAGTCAGGA-3' and 5'-TAGGGCTTGCATTTTCCATC-3'), *Tet3* (5'-ATGGCATGAAACCACCCAAC-3' and 5'-ACTTGATCTTCCCCTCCAGC-3') and *Rpl19* (5'-GCGTCTGCAGCCATGAGTA-3' and 5'-TGGCATTGGCGATTTTCGTTG-3') were synthesised by Eurofins MWG Operon. The optimisation and validation of primers was performed using standard ABI protocols. The cDNA from reverse transcription reaction was diluted 1:4 with ddH₂O and used as a template for subsequent PCRs, which were carried out in duplicate using SYBR green (Roche) on an ABI StepOnePlus Real-Time PCR system (Applied Biosystems). For relative quantification of gene expression the $2^{-\Delta\Delta CT}$ method was employed (37). The internal control gene used for these analyses was the housekeeping gene *Rpl19*.

Construction of *Avp* promoter mutants by overlap extension PCR

A series of mutant rat *Avp* promoter luciferase constructs were generated by overlap extension PCR. Primers 350-F (5'-CGGGGTACCAATGAGACCTGGGGACCCCT-3') and 350-R (5'-

CCCGCTCGAGCCTGAGCGGGCTGGGCTGT-3') were designed to amplify 350 bp of the rat *Avp* promoter, and contained KpnI and XhoI restriction sites, respectively. These primers were used in combination with deletion specific forward and reverse primers to amplify *Avp* promoter fragments using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The PCR products from the initial PCRs were combined and used as template for a subsequent PCR using primers 350-F and 350-R. Mutations (C-A) were performed for 6 separate CpG sites and complete 350 bp fragments were ligated into KpnI and XhoI sites of pGL3-Basic plasmid (Promega). The following primers were used for creation of site directed mutations;

CpG1 (5'-CCCTCAAGTAGGCTCACCTCCC-3' and 5'-GGGAGGTGAGCCTACTTGAGGG-3'), CpG2 (5'-TCACTGTGGAGGTGGCTCCCG-3' and 5'-CGGGAGCCACCTCCACAGTGA-3'),

CpG3 (5'-CGGTGGCTCCAGTCACACGGTG-3' and 5'-CACCGTGTGACTGGAGCCACCG-3'),

CpG4 (5'-CCCGTCACAAGGTGGCCAGTG-3' and 5'-CACTGGCCACCTTGTGACGGG-3'),

CpG6 (5'-TTAGCAGCCAAGCTGTCGCCTCC-3' and 5'-GGAGGCGACAGCTTGGCTGCTAA-3') CpG7 (5'-GCCACGCTGTAGCCTCCTAGCCA-3' and 5'-TGGCTAGGAGGCTACAGCGTGGC-3').

***In vitro* methylation of plasmid DNA**

Methylation of *Avp* promoter constructs was performed using CpG methyltransferase (M0226L; NEB) according to manufacture's instructions. Mock methylation reactions were performed for all plasmids with identical reaction chemistry but with the omission of CpG methyltransferase. The methylation reactions were incubated at 37°C for 8 h followed by 65°C for 20 min. Plasmid

DNA was purified using QIAprep Spin Miniprep kit. To confirm methylation, plasmids were cut with methylation sensitive restriction enzyme PmlI (CACGTG) which recognises CpG site 5 within the *Avp* promoter of the pGL3-*Avp* promoter constructs.

Luciferase assay

Human Embryonic Kidney cells HEK293T/17 (ATTC CRL-11268) were cultured in DMEM (Sigma; D6546) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 2mM L-glutamine and 100 unit/ml of penicillin-streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% (v/v) CO₂. For luciferase assays, 3 x 10⁵ cells/well were seeded in 12-well tissue culture plates in the absence of antibiotics. Next day, plasmids (0.5 µg pGL3-*Avp* promoter and 0.5 µg pcDNA3 or pcDNA3-*Creb3ll* and 0.05 µg pRL-TK vector/well) were transfected with FuGENE HD transfection reagent (Promega). The culture media was replaced with fresh media at 8 h after transfection. Luciferase assays were performed using Promega's Dual-Luciferase® Reporter Assay kit. At 36 h after transfection, culture media was removed and cells were washed with phosphate buffered saline and lysed with 500 µl of the supplied lysis buffer. Luciferase activity was measured using a Lumat LB 9507 Luminometer (Berthold Technologies).

Statistical analysis

One-way ANOVA with Tukey's post hoc test were used to determine the difference between more than two samples with only a single influencing factor. Two-way ANOVA with Bonferonni post hoc test was used to determine interactions between two independent variables on the dependent variable. In some cases statistical differences between two experimental groups were evaluated

using independent-sample unpaired Student's *t* tests. Correlations were performed using Pearson's correlation coefficient. $p < 0.05$ was considered significant.

Results

CpG sites in the proximal *Avp* promoter

The *Avp* gene is comprised of 3 exons and 2 introns (Fig. 1A). Computational studies mapping the distribution of CpG residues throughout the *Avp* gene have described the location of a CpG island in the proximal *Avp* promoter region (27). We focused our attention on 7 CpG residues in this CpG island (Fig. 1B). This region of the *Avp* promoter contains a number of transcription factor binding motifs.

Demethylation of the *Avp* promoter dramatically increases *Avp* transcription

We firstly looked at *Avp* promoter methylation in an immortalised rat hypothalamic cell line 4B. Hypothalamic 4B cells are derived from embryonic day 19 rat hypothalamus and have a neuronal phenotype expressing corticotropin releasing hormone, *Avp* and glucocorticoid receptors similar to parvocellular neurons of the hypothalamus (38). We found high methylation of the *Avp* promoter region in hypothalamic 4B cells (Fig. 2A). Treatment of hypothalamic 4B cells with the DNA methyltransferase inhibitor 5-Aza dramatically increased *Avp* expression (Fig. 2B). We treated cells with 5-Aza in the presence or absence of forskolin (Fig. 2C). Forskolin alone increased *Avp* synthesis and further enhanced responses to 5-Aza treatment, suggesting that methylation regulates cAMP induced *Avp* synthesis.

Decreased expression of Tet2 in SON of dehydrated and salt loaded rats

We used a micro punch to isolate SON samples to minimise contamination from surrounding brain regions (Fig. 3). Our data replicate previous findings that *hnAvp* expression is robustly induced in the SON by the two osmotic stimuli of dehydration and salt loading (Fig. 3A) (5). As expected, using immediate early gene *c-Fos* (Fig. 3A), a marker of neuronal activity, we confirmed comparable increases in neuronal activity in both dehydrated and salt loaded rat SON. We have recently identified transcription factor CREB3L1 as a putative transcriptional regulator of the *Avp* gene and confirm here that *Creb3l1* is induced by dehydration and salt loading (Fig. 3A; (30)). To see if changes in methylation may underlie increased transcription of *Avp*, we looked at the mRNA expression of *Dnmt* and *Tet* genes in the SON (Fig. 3A). We found no changes in expression of *Dnmt1*, *Dnmt3a*, *Tet1* or *Tet3* in the SON of dehydrated or salt loaded rats compared to control. In contrast, *Tet2* expression was significantly decreased in SON by both stimuli. We chose to examine gene expression in an unrelated brain region, cortex (Fig. 3B). In this brain region *Avp* expression was undetectable, and there were no differences in the expression of any of the genes examined compared to control, with the exception of increased *c-Fos* in salt-loaded animals.

Change in methylation status of the *Avp* promoter in the dehydrated rat

We examined the methylation profile of the *Avp* promoter within the SON by sequence analysis of bisulfite converted DNA (Fig. 4). Using primers spanning the proximal *Avp* promoter (-325 to -24) we investigated the methylation status of this cluster of 7 CpG sites. Analysis of the methylation pattern of CpGs in single clones from individual control, dehydrated and salt-loaded animals are depicted in Fig. 4A. These data showed that all CpGs in this region have some

degree of methylation in all experimental conditions. Analysis of the overall methylation of the *Avp* promoter for the SON revealed increased methylation in dehydrated compared to control and salt loaded animals (Fig. 4B). In comparison, methylation was not significantly affected by dehydration or salt loading in the cortex (Fig. 4B).

We next compared the methylation profiles of the three experimental groups. Of the 7 CpGs analysed, 5 showed increased methylation (CpGs 2, 3, 4, 6 and 7), 1 decreased methylation (CpG1) and 1 no change of methylation (CpG5) in SON of dehydrated compared to control animals (Fig. 4C). By contrast, methylation of the *Avp* promoter was not altered in the SON by salt loading. To see if these changes were specific to the SON we used the same method to look at *Avp* promoter methylation in the cortex of these animals (Fig. 4D). Of note, the pattern of methylation displayed across these CpGs was remarkably similar in the SON and cortex of controls, suggesting that this pattern was not unique to the SON. In contrast to the SON, dehydration only significantly influenced methylation of CpG7 in the *Avp* promoter in the cortex. However, salt loading also had no effect on methylation in the cortex.

We performed correlation analyses, comparing the methylation status of individual CpG residues with expression of *hnAvp* in the same control sample (Fig. 4E). Of the 6 CpGs that showed significant changes in methylation status in dehydration, only 2 CpGs (CpG3 and CpG4) had methylation patterns that were strongly correlated with the *hnAvp* expression in control animals. The strong negative correlation between *Avp* promoter methylation level and *Avp* hnRNA abundance is consistent with a role for methylation in transcriptional inhibition. In dehydrated

and salt loaded animals, we observed no correlation between methylation of individual CpGs and *hnAvp* expression. A summary of the overall methylation changes is depicted in Fig. 4F.

***In vitro* Methylation of CpG sites in the *Avp* promoter alter promoter activity**

We then used *Avp* promoter-luciferase reporter assays to further explore the effect of methylation on *Avp* transcription (Fig. 5). Using the technique of overlap extension PCR, we replaced the nucleotide cytosine with adenine (C-A) for CpG sites (1-4, 6, 7) to prevent *in vitro* methylation of these residues by CpG methyltransferase (Fig. 5A). We did not examine CpG5 as the methylation status of this site was not altered in the SON by dehydration or salt loading. The methylation status of our promoter constructs was confirmed by failure to digest the plasmid DNA with PmlI (Fig. 5B). We performed luciferase assays to test if manipulation of these single nucleotides altered *Avp* promoter activity (Fig. 5C). The C-A substitution at CpG3, which resides in the CRE site increased *Avp* promoter activity, while at the more proximal CpG7 site *Avp* promoter activity decreased. Next, we transfected HEK293T cells with transcription factor *Creb3l1* overexpression construct to test if induced *Avp* transcription was altered by any of these manipulations. For CpG4, CpG6 and CpG7 C-A substitution reduced *Creb3l1* mediated transcriptional activation of the *Avp* promoter, suggesting that those nucleotides are important for transcriptional activation by *Creb3l1*. We then investigated the effect of *in vitro* methylation by CpG methyltransferase on *Avp* promoter activity of these constructs (Fig. 5D). After methylation, CpG1, CpG4 and CpG7 constructs displayed lower promoter activity, suggesting that methylation status of these sites was intrinsically important for *Avp* promoter activity. In

response to *Creb3l1*, methylation of CpG1, 4, 6 and 7 constructs decreased, whereas CpG2 and 3 increased, promoter activity.

Discussion

It has been suggested that epigenetic mechanisms underlie brain plasticity (19, 20, 39), a process in which stable modulation of gene expression occurs. In this regard the SON represents a perfect model as it undergoes huge plastic changes in response to dehydration and salt loading in order to cope with the increased demand for *Avp* synthesis (14). Thus, we proposed that the *Avp* gene maybe a target for epigenetic regulation in osmotic stress. The clustering of CpGs upstream of the transcriptional start site of *Avp*, a region that contains transcription factor binding motifs, implied that epigenetic modification of this segment of DNA maybe important for modulation of *Avp* transcription. We show that demethylation of the *Avp* promoter by 5-Aza treatment of hypothalamic 4B cells greatly increases basal and stimulated *Avp* synthesis, suggesting that methylation acts to inhibit *Avp* synthesis. We also show here DNA demethylation and *de novo* methylation of CpG residues in the proximal *Avp* promoter in the dehydrated, but not the salt loaded, rat SON.

We used hypothalamic 4B cells to investigate *Avp* promoter methylation *in vitro*. These cells have widely been used to study both corticotropin releasing hormone and *Avp* transcription (38, 40, 41). Hypothalamic 4B cells were found to express *Avp* as previously reported and treatment with DNA methyltransferase inhibitor 5-Aza dramatically increased *Avp* synthesis by forskolin treatment. Demethylation of gene promoter regions has been shown to increase the accessibility of DNA for activation by transcription factors (34). Notably, expression of house keeping gene

Rpl19 was not affected by treatment with 5-Aza. Therefore, this cell line represents a useful model for future investigation of transcriptional regulation of the *Avp* gene by methylation.

Increased neuronal activity has been linked with global methylation changes in the brain. In mouse dentate gyrus neurons, activation was shown to be associated with active demethylation or *de novo* methylation of specific genes related to neuronal plasticity (20). It well known that *c-Fos*, a marker of neuronal activity, is dramatically increased in abundance in magnocellular neurons of the SON by hyperosmotic stress (42, 43), as observed here by increased mRNA expression. With this in mind, we investigated mRNA expression of genes regulating DNA methylation processes in the SON. The expression of DNA methyltransferases, *Dnmt1* and *Dnmt3a*, have been described in the adult brain (21), and we found expression of these genes in the SON, but levels were not altered by dehydration or salt loading. Nevertheless, one member of *Tet* family, *Tet2*, known to be involved in active and passive demethylation of DNA (23), was altered by both stimuli in the SON. Interestingly, the knockdown of *Tet2* expression *in vitro* has been associated with hypermethylation of DNA (44, 45). Therefore, we reasoned that decreased *Tet2* may result in hypermethylation of CpG sites in the SON *Avp* promoter.

The only report of methylation status regarding the proximal *Avp* promoter region was in mouse PVN, where sparse methylation was reported (27). Indeed, previous studies on methylation of the *Avp* gene have examined regions outside of the proximal promoter, focusing on more distal extremities of the promoter or on an enhancer within the intergenic region linking the *Avp* and oxytocin genes (25-27). In fact, no study has sought to elucidate the steady-state methylation status of the *Avp* gene in magnocellular neurons of the PVN or in the SON. We have addressed

this issue here. To our surprise, methylation increased at CpG2, 3, 4, 6 and 7 only in dehydrated animals, highlighting that methylation of these CpGs is not stable. The fact that decreased Tet2 expression was observed in SONs of both dehydrated and salt loaded animals suggests that Tet2 may not be responsible for increased *Avp* promoter methylation in dehydration.

Notably, methylation levels were higher at CpG3, which resides in the middle of the cAMP responsive element (CRE), and CpG2 and CpG4, which flank this motif. Methylation at CRE sites has been shown to inhibit CREB mediated transcription (46, 47). Furthermore, promoter deletion studies in cell line JEG3 showed that deletion of both CRE sites significantly reduced forskolin stimulation of the *Avp* promoter (33). We have previously reported *Creb3l1* as a transcription factor of the *Avp* gene in the rat hypothalamus (30). A G-box element (GCCACGTGTGT) that flanks the previously identified E-box enhancer element (CACGTG) was identified as a putative binding site for CREB3L1. When we deleted the core nucleotides (ACGT) from this motif, which corresponds to CpG5, *Avp* promoter activity dropped significantly, confirming the importance of this site in *Avp* promoter activity. It is interesting that the methylation status of CpG5 was the only CpG not affected by dehydration in the SON, perhaps suggesting stringent regulation of this site for appropriate *Avp* transcription. The increased methylation of individual CpGs may alter promoter activity by interfering with the DNA binding sites of proteins that affect *Avp* transcription. Increased promoter methylation is commonly associated with transcriptional silencing though there is evidence that methylation can also stimulate gene expression (24, 34). What we do know is that demethylation of the *Avp* promoter *in vitro* increases *Avp* expression, suggesting that methylation silences the *Avp* gene. In agreement, CpG methylation at sites 3 and 4 inversely correlated with *Avp* expression in

control SON samples suggesting that DNA methylation acts to confer *Avp* gene silencing *in vivo*. It is important to note that in dehydration *Avp* promoter methylation and *Avp* expression both increased in the SON. Therefore, we can not rule out the possibility of methylation having a positive influence on *Avp* transcription *in vivo*.

In salt loaded animals, there were no changes in *Avp* promoter methylation in the SON. One explanation could be the physiological differences between these two osmotic stimuli. We recently compared the physiological and transcriptome responses to dehydration and salt loading in the rat SON (16). In this study we identified several fundamental differences between dehydration and salt loading in terms of natriuresis, drinking behaviour and circulating hormone levels. Despite these differences we found that the transcriptional response by the SON was very stable leading to the hypothesis that the SON may not be able to discriminate between these two osmotic cues. Indeed, both dehydration and salt loading result in significant increases in plasma osmolality compared to control, but we found no difference when comparing dehydration with salt loading (16).

In the absence of drinking fluid, both extracellular and intracellular fluid volumes decrease leading to increased plasma osmolality and, unlike salt loading, hypovolemia (48, 49). Acute hypovolemia, caused by haemorrhaging or, pharmacologically, by polyethylene glycol injection into the rat, increases AVP secretion from the posterior pituitary, which is accompanied by increased synthesis of *Avp* in the PVN and SON (3, 5, 9), in the absence of osmotic stimulation (3). A decrease in plasma volume of around 15-20% has been shown to increase *Avp* synthesis approximately 2-fold in the SON and PVN in acute experiments. Therefore, one may perhaps

question why the two stimuli of dehydration and salt loading similarly increase *Avp* mRNA levels by approximately 2-fold. Indeed, one would predict a greater increase in *Avp* synthesis in dehydrated compared to salt loaded animals, in response to both increased plasma osmolality as well as hypovolemia provoked by this stimulus. Therefore, one can speculate that the formation of new epigenetic marks on the *Avp* promoter in dehydration may act to control synthesis of *Avp*, which is being driven by a combination of increased plasma osmolality and hypovolemia. In support of this concept, a study of sustained hypovolemia produced by injection of the diuretic furosemide showed that plasma AVP was increased by 8 h but decreased by 32 h, despite further decreases in blood volume (10). The authors proposed that this decrease in plasma AVP may be due to adaptive resetting of volume control mechanisms. Unfortunately, *Avp* mRNA expression was not reported in this study. However, Hayashi et al. (2006) (9) showed that acute hypovolemia can still induce *Avp* synthesis in the PVN and SON of rats dehydrated for 3 days. This ability to increase *Avp* expression may underlie differing mechanisms governing chronic versus acute hypovolemia (10).

The differences in plasma AVP, oxytocin and angiotensin II levels between dehydration and salt loading (16) may also influence DNA methylation. It has been suggested that the methylation status of the *Avp* gene is maintained by the hormonal environment (25). Castration of rats was shown to increase methylation of 2 CpGs in the bed nucleus of the stria terminalis *Avp* promoter. This methylation was successfully reversed by administration of testosterone. We have previously described differences in circulating angiotensin II in dehydrated and salt loaded animals (16), and there is evidence that angiotensin II influences methylation (50), though effects on methylation patterns in the brain are not known. The circumventricular organs, such as the

subfornical organ (SFO) and organum vasculosum lamina terminalis (OVLT), lack a functional blood-brain barrier so are sensitive to changes in circulating hormones (49). The SFO and OVLT expresses angiotensin II type 1 receptor (51) and the expression of this receptor is increased in the SFO by dehydration (52). The SFO directly projects to AVP expressing magnocellular neurons in the SON and electrophysical studies have shown that stimulation of the SFO effects AVP neurons (53). Therefore, one could speculate that the increased circulating levels of angiotensin II in dehydration, via activation of angiotensin II type 1 receptor in the SFO, may influence *Avp* methylation events in the SON.

One of the limitations of investigating DNA methylation patterns in complex tissues such as the brain is the number of different cellular phenotypes in punch samples. In the SON there are primarily two neuronal phenotypes (either *Avp* or oxytocin), but this nucleus also contains glial and vascular cellular phenotypes. Consequently, DNA will be extracted from all these cell types and thus will contribute to the *Avp* promoter methylation profiles in our study. While it is not possible to say in which cell types these promoter changes are occurring, we observed a strong correlation at specific methylation landmarks with heteronuclear *Avp* expression in control animals, which implied that the methylation profiles may be important in maintaining steady-state *Avp* expression in the SON. We also showed by mutation of individual CpG sites that these residues are important for regulating *Avp* promoter activity in cell lines.

It has been shown that children and adolescents fail to completely replete their hydrational needs by drinking water or other fluids. This has led some to speculate that over the long-term such deficiencies may result in low level of dehydration and perhaps even hypovolemia with potential

consequences later in life (54). Indeed, in the elderly disturbance in serum sodium and blood volume are major causes of hospital admissions (55). Our data suggest that epigenetic regulation of the proximal *Avp* promoter may be an important mechanism in controlling the *Avp* synthesis in response to dehydration. We speculate that this extra level of regulation may be necessary to co-ordinate the dual inputs received in this nucleus derived from increased plasma osmolality and hypovolemia provoked by decreased blood volume in chronic dehydration. The stability or indeed the longevity of these newly established epigenetic marks has yet to be determined. The striking differences in methylation patterns that exist in the *Avp* promoter of dehydrated and salt loaded rat are likely to be important for furthering our understanding of *Avp* transcriptional control.

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Figure legends

Figure 1: Schematic diagram of the *Avp* gene and presence CpG sites in the proximal promoter region.

(A) *Avp* gene contains 3 exons indicated by open boxes. The primers used to amplify 302 bp *Avp* promoter are indicated. (B) The diagram shows transcription factor binding sites (highlighted) and CpG sites (Red) investigated within the 350 bp of *Avp* promoter. The location of forward and reverse primers for amplification of bisulfite converted DNA are underlined. Lower panel indicates the location of CpG sites in the *Avp* promoter. TSS, transcription start site; CAAT box; CRE, cAMP response element; AP, activator protein.

Figure 2: Demethylation of the *Avp* promoter dramatically increases *Avp* transcription in hypothalamic 4B cells. (A) Tile diagram showing the methylation status of CpG sites for individual clones of the *Avp* promoter from the hypothalamic 4B cells. (B) Treatment of hypothalamic 4B cells with DNA methyltransferase inhibitor 5-Aza-dc (1-10 μ M) increases *Avp* synthesis. (C) Forskolin (10 μ M) induced *Avp* synthesis was further enhanced by 5-Aza

treatment. Error bars, mean+SEM of n=4 per group; ***, p<0.001 (B, One-way ANOVA with Tukey's post hoc test; C, Two-way ANOVA with Bonferonni post hoc test).

Figure 3: mRNA expression of genes involved in hyperosmotic stress and methylation in the SON and cortex of dehydrated and salt loaded rats

(A) Brain section stained with 1% (w/v) toluidine blue/70% (v/v) ethanol shows the punched area of the SON. The high magnification image shows that the punch samples were confined within the area of magnocellular neurons of the SON. cDNA synthesis and subsequent qPCR analysis was performed using RNA extracted from these punch samples. The mRNA expression of *hnAvp*, *c-Fos*, *Creb3l1*, *Dnmt1*, *Dnmt3a*, *Tet1*, *Tet2* and *Tet3* were examined in SON (A) and cortex (B). Error bars, mean+SEM of n=4-5 per group; *, p<0.05, **, p<0.01, ***, p<0.001 (One-way ANOVA with Tukey's post hoc test). SON, supraoptic nucleus; OC, optic chiasm; MCN, magnocellular neuron; DH, dehydration; SL, salt loading; *hnAvp*, heteronuclear RNA of *Avp*. Scale bar = 200 μ m.

Figure 4: Methylation status of the *Avp* promoter in response to dehydration and salt loading in the SON

Genomic DNA was extracted from the SON and cortex of control, 3 days dehydrated and 7 days salt loaded rats (n=4-5). The DNA was bisulfite converted, *Avp* promoter region amplified and cloned into TA vector for sequencing (n=20 for each sample). (A) Tile diagrams show the methylation status of seven CpG sites for individual clones of the *Avp* promoter extracted from the SON. (B) Percentage of global methylation on *Avp* promoter in the SON and cortex of

dehydrated and salt loaded rats is shown. (C-D) Change in methylation status of CpGs in (C) SON and (D) cortex *Avp* promoters in response to dehydrated and salt loaded. (E) Correlation analysis of methylation level with expression of *hnAvp* in control sample. (F) The diagram presents the methylation level of CpG 1-7 on *Avp* promoter of dehydrated and salt loaded rats compared to control. Black indicates hypermethylation. White indicates hypomethylation and grey indicates no change in methylation level compared to control. Error bars, mean+SEM of n=4-5 per group; *, p<0.05, **, p<0.01 (one-way ANOVA with Tukey's post hoc test); #, p<0.05 (unpaired t-test); SON, supraoptic nucleus; DH, dehydration; SL, salt loading.

Figure 5: Methylation of CpG sites on *Avp* promoter *in vitro*

The substitution (C-A) at CpG sites in the *Avp* promoter by overlap extension PCR was used to prevent methylation at specific CpG sites. The mutation sites are shown in (A). The mutated plasmids were subsequently methylated by methyltransferase enzyme. (B) Successful methylation was determined using methylation sensitive restriction enzyme PmlI. (C-D) Luciferase assays were performed by co-transfection of plasmid expressing *Creb3l1* and 350bp *Avp* promoter constructs with (C) unmethylated and (D) methylated plasmid. Error bars, mean+SEM of n=4 per group; *, p<0.05; **, p<0.01; ***, p<0.001 (One-way ANOVA with Tukey's post hoc test).

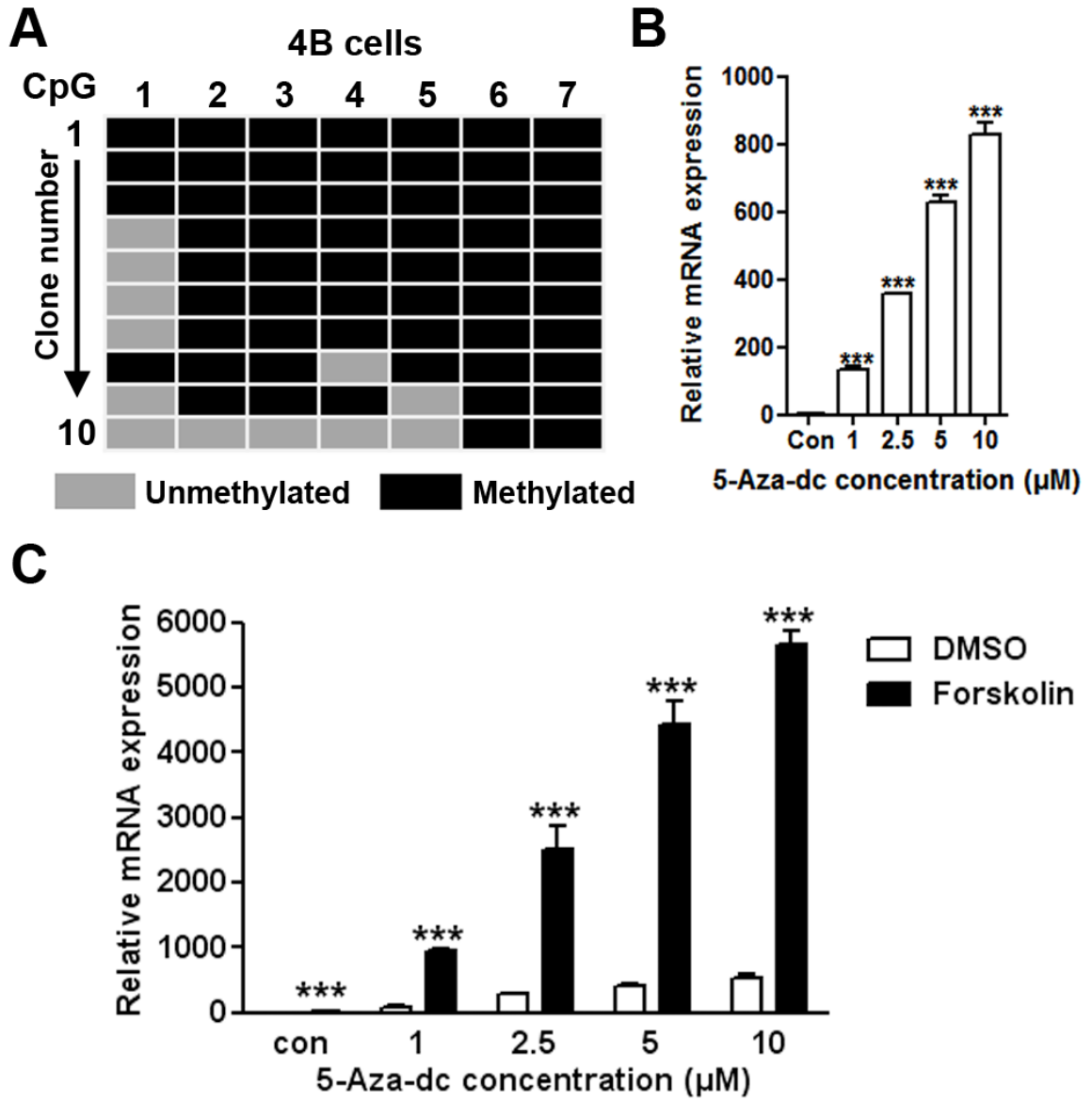


Fig. 2

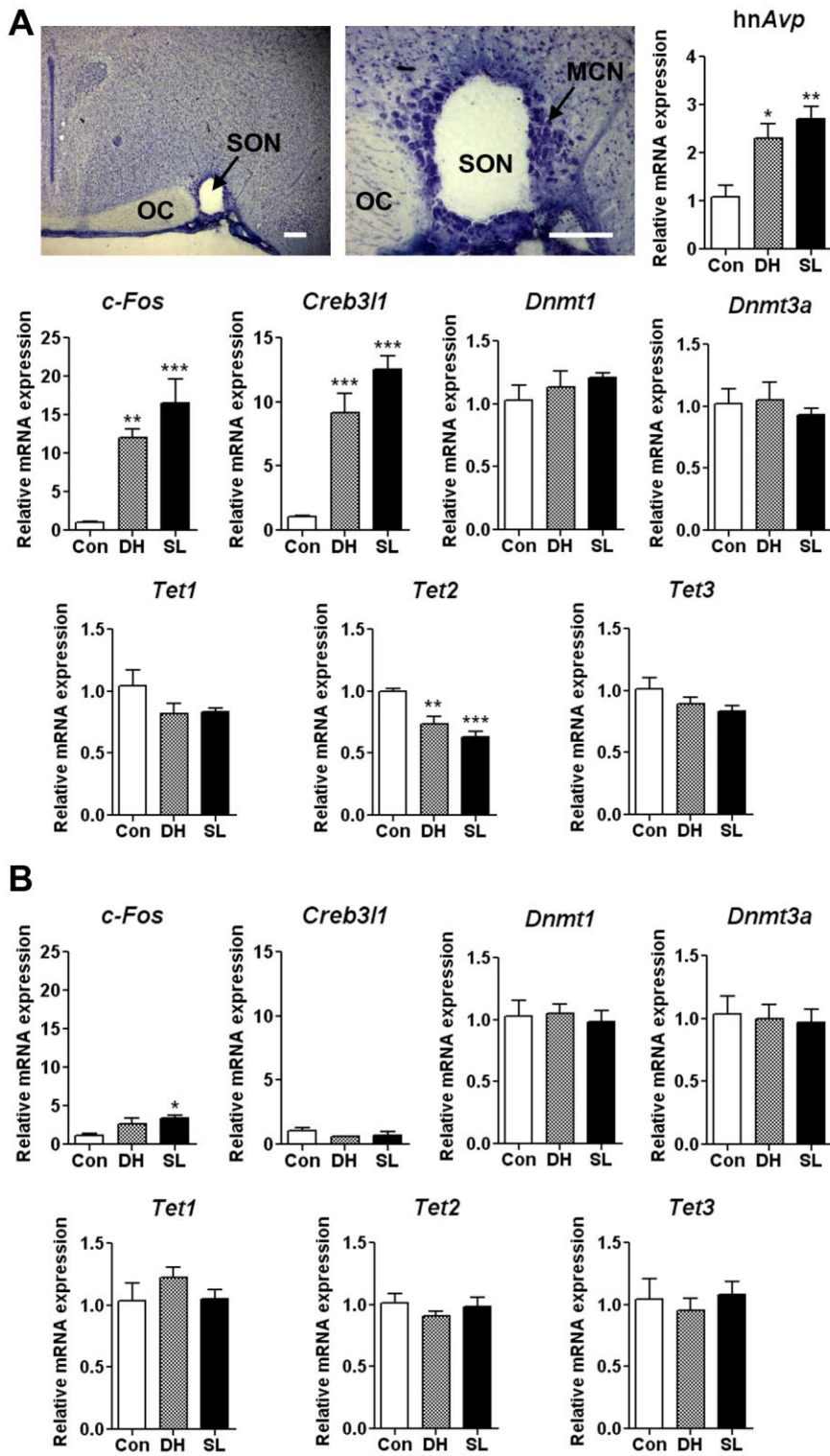


Fig. 3

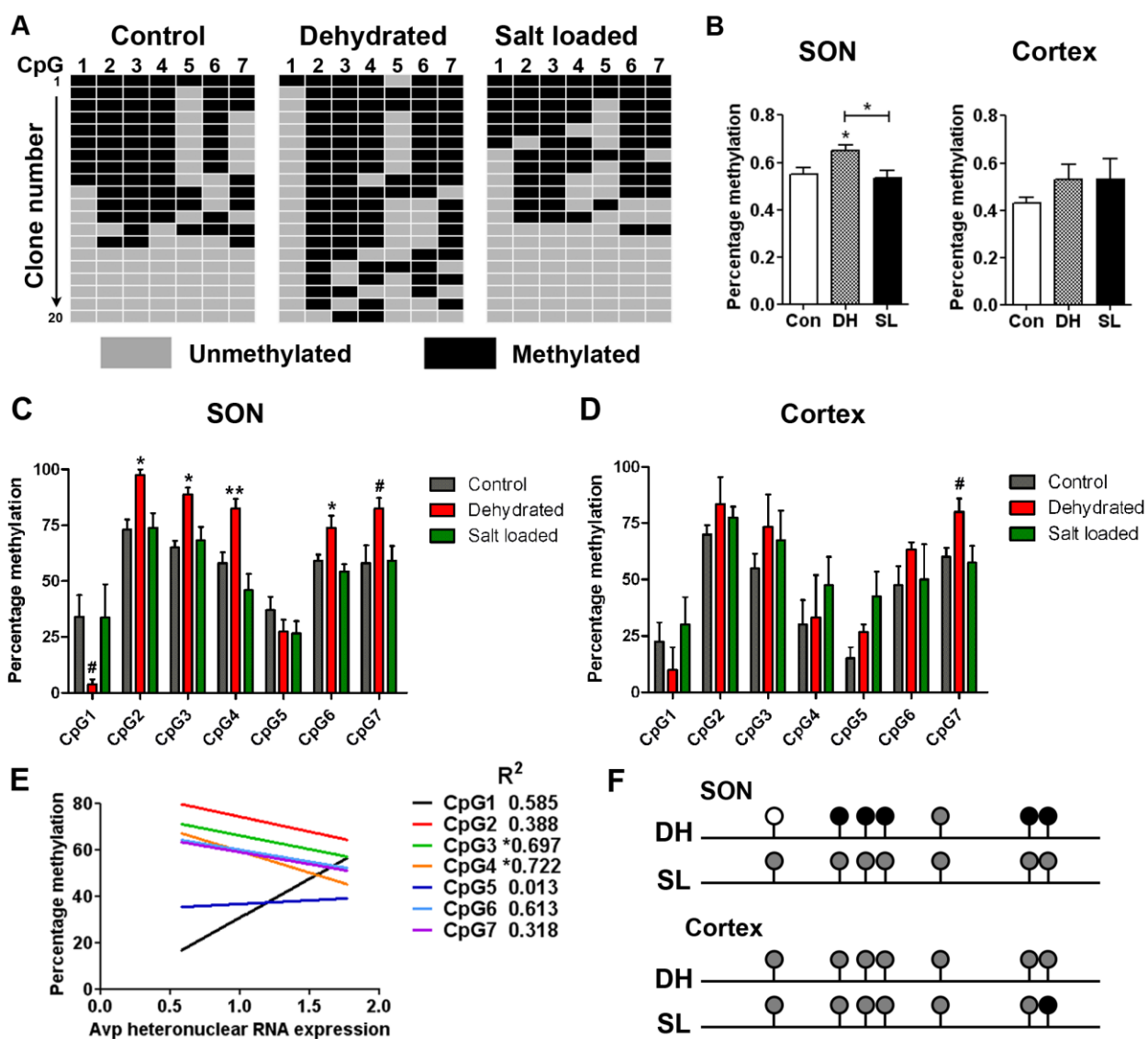


Fig. 4

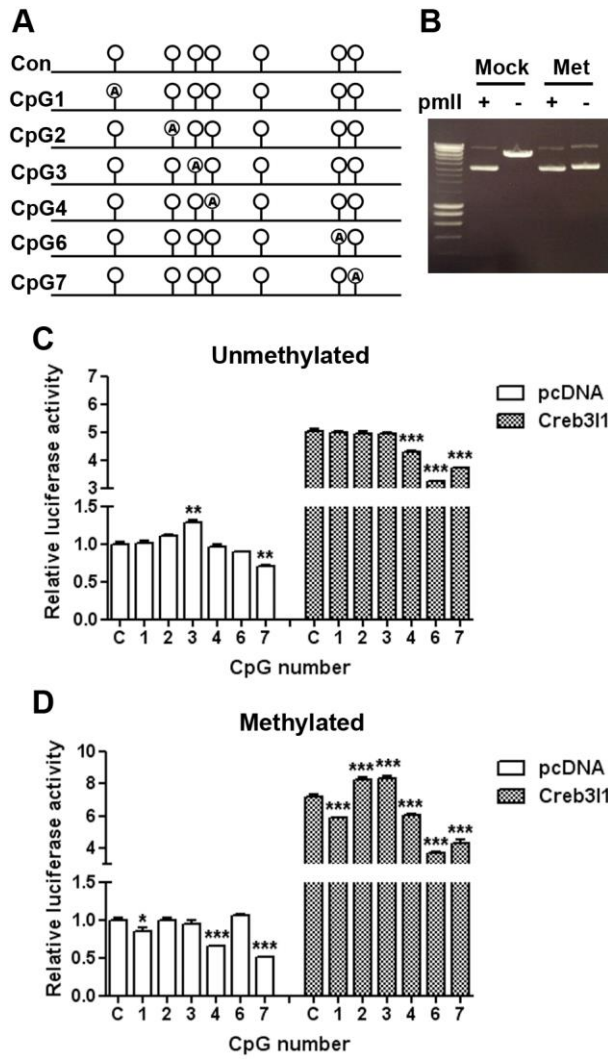


Fig. 5