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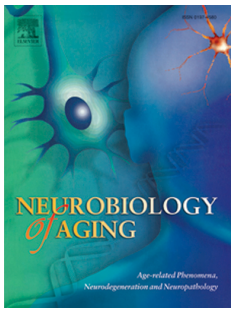


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# The effects of ageing on biosynthetic processes in the rat hypothalamic osmoregulatory neuroendocrine system

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**Abstract**

Elderly people exhibit a diminished capacity to cope with osmotic challenges such as dehydration. We have undertaken a detailed molecular analysis of AVP biosynthetic processes in the supraoptic nucleus (SON) of the hypothalamus, and secretory activity in the posterior pituitary of adult (3-months) and aged (18-months) rats, to provide a comprehensive analysis of age-associated changes to the AVP system. By MALDI-TOF MS analysis, we identified differences in pituitary peptides, including AVP, in adult and aged rats under both basal and dehydrated states. In the SON, increased Avp gene transcription, coincided with reduced Avp promoter methylation in aged rats. Based on transcriptome data, we have previously characterised a number of novel dehydration-induced regulatory factors involved in the response of the SON to osmotic cues. We found that some of these increase in expression with age, whilst dehydration-induced expression of these genes in the SON was attenuated in aged rats. In summary, we show that ageing alters the rat AVP system at the genome, transcriptome and peptidome levels. These alterations however did not affect circulating levels of AVP in basal or dehydrated states.

Keywords: Ageing, vasopressin, supraoptic nucleus, methylation, gene expression, peptidomics

## 1. Introduction

As we age, disorders of body salt and water composition become more commonplace. Cases of hypo/hypernatremia are much more prevalent in the elderly, where they have been linked to increased incidences of falls, fractures and osteoporosis, thus contributing to increased hospital admissions and morbidity and mortality (Cowen et al., 2013). To promote healthy living well into old age, it is thus necessary to determine why such imbalances occur. Age-associated changes to both peripheral and central mechanisms that control salt and water homeostasis are deemed responsible. There is a progressive age-related decline in renal function, with less urine concentrating capacities in the elderly compared to younger subjects (Ishunina and Swaab, 2002). Such impaired capacity to conserve body water, together with reports of reduced thirst and inadequate fluid intake after periods of fluid deprivation, make the elderly more susceptible to dehydration (Mack et al., 1994, Phillips et al., 1993). Inappropriate release of the antidiuretic hormone arginine vasopressin (AVP) in to the systemic circulation has been highlighted as one of the causes of irregular water homeostasis in ageing (Swaab and Bao, 2011).

AVP is synthesised in magnocellular neurones of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus. A change in plasma osmolality is detected by osmosensitive neurones in circumventricular organs of the brain that provide direct inputs to shape the firing of AVP magnocellular neurones which are osmosensitive themselves, and to co-ordinate AVP synthesis and secretion from the posterior (neural) lobe of the pituitary gland (Mecawi Ade et al., 2015, Nissen et al., 1994, Zhang and Bourque, 2003). Once released, following incidences such as rise in plasma osmolality or decrease in blood volume (Kondo et al., 2004), AVP promotes sodium and water reabsorption by the kidney (Ares et al., 2011, Breyer and Ando, 1994). When placed under stress, the capabilities of the AVP system have been shown to decrease with age (Frolkis et al., 1999, Keck et al., 2000, Sladek et al., 1981).

The AVP system has been interrogated on multiple levels, from synthesis to secretion, in aged subjects with differing results. For example, basal circulating AVP levels have been found to decrease, remain

unchanged and to increase with age in humans, as well as rodents (Frolkis et al., 1999). These discrepancies have been attributed to genetic, age and strain differences. Whilst there are many disputations, one area of agreement is that, whilst in many brain areas neuronal activity decreases with age (Burke and Barnes, 2006), paradoxically AVP neurones become more active (Palin et al., 2009, Terwel et al., 1992). This hyperactivity is thought to be a compensatory mechanism for decreased responsiveness to AVP in the kidney due to decreased receptor abundance in aged subjects (reviewed by (Ishunina and Swaab, 2002)), although this theory has been questioned (Preisser et al., 2004), and is not intuitive with the profound difference in circulating AVP described in ageing models. The AVP magnocellular neurones undergo numerous morphological changes as they age including increased size of perikarya, nucleoli and Golgi apparatus in humans as well as rodents (Ishunina and Swaab, 2002), analogous to morphological changes in these neurones with dehydration (Hatton and Walters, 1973). Increased AVP neurone size in states of dehydration is recognised as a necessary measure to meet cellular demands for increased transcription and protein synthesis under hypertonic stimulation where circulating levels of AVP are robustly increased (Zhang et al., 2001). It has been suggested that such hyperactivity of AVP neurones may in itself lead to electrolyte disorders in the elderly (Swaab and Bao, 2011), but the relationship between the activity of AVP neurones and circulating levels of AVP is poorly understood

Transcriptional changes have been identified in AVP neurones with ageing. A study by Palin *et al.* (Palin et al., 2009) showed increased expression of immediate early gene c-Fos, a commonly used marker of neuronal activity, in the rat SON, consistent with hyperactivation of the AVP neurones. In contrast to increased activity under basal conditions, reports have described an attenuation of the evoked AVP secretion in response to osmotic stress with ageing in rodents (Sladek and Olschowka, 1994, Swenson et al., 1997). This has led some to suggest that deficits in mechanisms controlling transcription, mRNA stability or translation in the ageing SON magnocellular neurones may be responsible (Lucassen et al., 1997). Moreover, we recently showed that dehydration initiates the formation of new methylation marks

on the rat Avp promoter (Greenwood et al., 2016a), suggesting that altered methylation patterns could lie beneath these transcriptional changes in ageing AVP neurones.

Few studies have sought to combine information on the physiological aspects of ageing with analyses of the molecular changes occurring in the hypothalamus. The reasons why old AVP neurones have elevated basal activity, or why they can fail to adequately respond under stress, is not well understood. In particular, the molecular basis for these changes in relation to circulating levels of AVP has received little attention. We reasoned that ageing-associated deficits in the AVP system may be due to a combination of changes at the genome, transcriptome and peptidome levels, and that these changes might be responsible for disturbances in osmotic stability. In this study we have interrogated the physiological aspects of ageing, performed metabolic measurements, and analysed peptide levels in plasma and pituitary, correlating the results with molecular events occurring within the hypothalamus of aged rats. Further, we have utilised our extensive knowledge of the transcriptome of the adult rat SON in euhydrated and dehydrated states to uncover novel changes surrounding altered Avp transcription in ageing.

## 2. Materials and Methods

### 2.1. Animals

All experiments were performed under a Home Office UK licence held under, and in strict accordance with, the provisions of the UK Animals (Scientific Procedures) Act (1986); they had also been approved by the University of Bristol Animal Welfare and Ethical Review Board. We choose to use male Wistar Han rats from the international standard program (IGS) in our ageing study (Charles River, France). The carefully managed breeding program for these animals helps to manage genetic drift so colonies bred in different locations around the world are not significantly divergent from each other giving a level continuity in ageing studies performed in laboratories worldwide. The Charles River Han Wistar rats have been extensively studied at 2 years of age when these rats are reaching the end of their natural lifespans.

Incidences of neoplastic and non-neoplastic lesions were high in tissues including the kidney and pituitary gland at this age. Furthermore, rats surviving to this age varied from 30-80% across 20 control studies (www.criver.com). Therefore, in this ageing study we opted for rats of 18 months of age to minimise pathophysiological effects and thus allow investigation of the ageing process in healthy animals. All adult rats used in this study were free of pituitary tumours, however 6/50 aged animals were removed from this study because of tumours on the pituitary gland. On arrival, rats were 2 weeks younger than the desired ages, 3 months (adult) and 18 months (aged), to enable sufficient time for acclimatisation before experimentation. Rats were housed at a constant temperature of 22°C and a relative humidity of 50–60% (v/v) under a 14:10 hour light/dark cycle (lights on at 0500) with food and water *ad libitum* for 2 weeks. To induce hyperosmotic stress, both adult and aged rats were randomly assigned to two groups: control (free access to drinking water) and dehydrated (removal of drinking water for 3 days). All rats were humanely killed by striking of the cranium (stunning), and then immediately decapitated with a small animal guillotine (Harvard Apparatus, Holliston, MA). Trunk blood was collected in heparin-coated tubes. Brains were rapidly removed from the cranium and immediately frozen by covering with powdered dry ice (within 3 minutes of stunning). The pituitary gland was removed from the base of the skull within 2 minutes after decapitation. The neurointermediate lobe (NIL) was carefully separated from the anterior pituitary using a scalpel blade, and then, either placed into 1.5 ml tubes containing 500 µl of 0.1M HCl or 0.2 ml tubes containing 150 µl of 15 mg/ml of 2, 5-dihydroxybenzoic acid (DHB) solution. Frozen brains and NIL in HCl solution were stored at -80°C, whilst NILs in DHB solution were stored at 4$^{o}$C. Animal experiments were performed between 9 am -2 pm.

**2.2. Metabolic measures in adult and aged rats**

For metabolic measurements, animals were individually housed in metabolic cages (Techniplast, Italy) to allow precise daily measures of fluid and food intake, and urine output. A plastic gnawing disc was suspended from the lid of the cage to provide environmental enrichment throughout the study. Animals were firstly acclimatised to metabolic cages for 48 hours. Measures of food hoppers, water bottles and

urine collection tubes were performed for 3 consecutive days, by weight. Extra-renal secretion of fluid was calculated by subtracting water intake from urine output. Plasma and urine osmolalities were measured by freezing point depression using a Roebling micro-osmometer (Camlab).

### 2.3. Vasopressin measures

The NIL of the pituitary was sonicated for 15 seconds in 0.1M HCl and incubated at 85°C for 20 minutes. Cellular debris was removed by centrifugation at 3,000×g for 30 minutes at 4°C. Pituitary AVP content was determined using an AVP[8]-Vasopressin ELISA (Enzo; ADI-900-017A) kit. The supernatant was diluted (1:10,000) with assay buffer and ELISA was performed following the manufacturer's protocol. The signal was detected on an iMark Microplate absorbance reader (Biorad). For radioimmunoassay, trunk blood was centrifuged at 1,600×g for 15 minutes at 4°C. Extractions were performed from 1 ml of plasma. Two sample volumes of ice-cold acetone were added and samples were vortexed for 1 minute. Protein precipitates were removed by centrifugation at 2,500×g, 4°C, for 25 minutes. The supernatant was transferred to a new tube and mixed with 2 ml of cold petroleum ether by vortexing for 1 minute. The tubes were left to stand for 1 minute at room temperature before discarding the upper phase. The lower phase solution was lyophilised using a freeze dryer (Benchtop Pro, Biopharma). AVP concentration was determined by specific radioimmunoassay (Husain et al., 1973).

### 2.4. Peptide analysis of the NIL

Peptides were measured directly in individual NIL extracts by mass spectrometry (MS) (Romanova et al., 2014, Romanova and Sweedler, 2015).

*2.4.1. Extraction of peptides.* NIL samples were incubated in 15 mg/mL DHB solution for 48 hour as described by Romanova et al. (Romanova et al., 2008). The samples were grouped as follows: adult control (n=14), adult 3-day dehydrated (n=16), aged control (n=11), aged 3-day dehydrated (n=13).

*2.4.2. Measurement of the NIL peptide profiles by MALDI-TOF MS.* For MALDI-TOF MS measurements, 0.7 µl of the NIL extraction solution was spotted on a stainless steel MALDI target in triplicates and co-crystallized with 0.7 µl of freshly prepared concentrated DHB matrix (50 mg/mL, 50% (v/v) acetone). Positive ion mass spectra of each spotted sample were acquired automatically at 1 KHz laser frequency and constant power optimized for the sample type in the 600–6000 *m/z* region using an ultrafleXtreme mass spectrometer (Bruker Daltonics) operated in reflectron mode via the AutoXecute protocol. Acquisition parameters included laser Fuzzy control logic, random laser walk over the entire sample area, 250 laser shots per raster step, maximum of 5000 shots per sample in 250-shot increments, and dynamic termination of spectrum acquisition when the signal intensity reached 30000 counts for 3 peaks, regardless of the number of fired laser shots. Peak evaluation was set to a signal intensity per shot of 20 or above, minimal resolution of 10000, S/N=3, maximum 300 peaks per spectrum, and centroid peak detection algorithm; 50 failed spectrum judgments were required before acquisition moving to the next sample. External quadratic calibration was adjusted automatically for every 5 x 5 sample spot square.

*2.4.3. Principal component analysis of the peptide profiles.* Statistical analysis of raw MALDI MS data was performed using ClinProTools 2.2 software (Bruker Daltonics). All spectra were normalized to total ion count upon loading into ClinProTools, and level scaled. Spectra were processed for convex hull baseline correction within 800–5500 *m/z*, smoothed with 0.1 Da x 2 cycles of the Savitzky-Golay method and a data reduction factor of 2, null spectra exclusion was enabled, and spectra grouping applied. Other criteria included automatic peaks selection on the total average group spectrum by intensity, S/N=5 cut off, 1% relative threshold base peak on average group spectrum, unlimited picking. Manual peak editing for the integration area after automatic peak picking was done on the mean spectrum representative of each sample group in order to include entire isotopic clusters of highly resolved peaks. Peptide profiles of the mean spectra were compared by principal component analysis (PCA) followed by the Anderson-Darling (AD) normality test and *Student's unpaired t*-test for normal distributed data. Data not showing normal distributions ($p_{AD} \leq 0.05$) were evaluated by Kruskal-Wallis tests, respectively (Kruskal and

Wallis, 1952, Stephens, 1974, Wilcoxon, 1945). To decrease the number of false positives while computing individual peak statistics on the complex spectra, the Benjamini-Hochberg procedure incorporated into ClinProTools was automatically applied for p-value adjustment during analysis (Dudoit and shaffer, 2003). Unsupervised clustering of spectra was performed on PCA-modified data using Euclidean distance, average distance methods and a Minkowski exponent of 1.5. The following peptide profile differences were investigated: 1) between control aged and adult rats, 2) between dehydrated aged and adult rats, and 3) between control and dehydrated rats of either age.

**2.5. Dual DNA and RNA extraction from SON punch samples**

SON samples (12 unilateral punches) were collected from 12 coronal slices using a 0.35 mm sample corer (Fine Scientific Tools) using the optic chiasm as a reference. Total RNA and genomic DNA were extracted from each sample as previously described (Greenwood et al., 2016a).

**2.6. cDNA synthesis and quantitative PCR**

For cDNA synthesis, 40 ng of total RNA was reverse transcribed using the Quantitect reverse transcription kit (Qiagen). Primers for rat genes used in this study: Avp (5'-TGCCTGCTACTTCCAGAACTGC-3' and 5'-AGGGGAGACACTGTCTCAGCTC-3'), heteronuclear Avp (hnAvp) (5'-GAGGCAAGAGGGCCACATC-3' and 5'-CTCTCCTAGCCCATGACCCTT-3'), ras related dexamethasone induced 1 (Rasd1) (5'-CCCTCAGCGTTGTGCCTACT-3' and 5'-AAAGAGCGCACGGAACATCT-3'), caprin famiy member 2 (Caprin2) (5'-CAGGGTTAAGTGCAAGCGAT-3' and 5'-CTGGTGGTTGACTGGTTGAG-3'), c-Fos (5'-AGCATGGGCTCCCCTGTCA-3' and 5'-GAGACCAGAGTGGGCTGCA-3'), cAMP responsive element binding protein 3 like 1 (Creb3l1) (5'-GCCAACAGGACCCTGCTCCA-3' and 5'-AGTGCCAGTCTGTGTGGCCG-3'), gonadotropin inducible ovarian transcription factor 1 (Giot1) (5'-GACACTTCCGGTCCGTCATAG-3' and 5'-GCCTCACTCAAGCACCCAGT-3'), DNA

methyltransferase 1 (Dnmt1) (5'-AACCACTCAGCATTCCCGTA-3' and 5'-TGCTGGTACTTCAGGTCAGG-3'), Dnmt3a (5'-AAGACCCCTGGAACTGCTAC-3' and 5'-TGGCGAAGAACATCTGGAGT-3'), mature oxytocin (Ot) (5′-TGCCCCAGTCTTGCTTGCT-3′ and 5′-TCCAGGTCTAGCGCAGCCC-3′), heteronuclear Ot (hnOt) (5′-TGAGCAGGAGGGGGCCTAGC-3′ and 5′-TGCAAGAGAAATGGGTCAGTGGC-3′), proprotein convertase subtilisin/kexin type 1 inhibitor (proSAAS) (5'-GAGCTGCTGAGGTACTTGCT-3' and 5'-ACCCAAATCCTGGTCCACAG-3'), heteronuclear proSAAS (hnproSAAS) (5'-GAAGTGACGACCGAGGTGTA-3' and 5'-GCAGTATTGTAGGGCGTTCG-3'), tet methylcytosine dioxygenase 1 (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 ribosomal protein L19 (Rpl19) (5'-GCGTCTGCAGCCATGAGTA-3' and 5'-TGGCATTGGCGATTTCGTTG-3') were synthesised by Eurofins MWG Operon. Quantitect Primer Assays for solute carrier family 12, member 1 (Slc12a1) were purchased from Qiagen. The optimisation and validation of primers was performed using standard Applied Biosystems protocols. The cDNA from reverse transcription reaction was diluted 1:4 with $H_2O$ and used as a template for subsequent PCRs, which were carried out in duplicate using SYBR green (Roche) on an Applied Biosystems StepOnePlus Real-Time PCR system. For relative quantification of gene expression the $2^{-\Delta\Delta CT}$ method was employed (Livak and Schmittgen, 2001). The internal control gene used for these analyses was the housekeeping gene Rpl19.

**2.7. poly(A) tail-length assay**

The poly(A) tail-length of the Avp mRNA was examined using the USB poly(A) Tail-Length Assay Kit (Affymetrix). RNA extracted from SON (50 ng) was used as the starting material. Guanosine and inosine residues were added to the 3' ends of poly(A)-containing RNAs using poly(A) polymerase enzyme. After incubation at 37°C for 1 hour, stop solution was added and the tailed-RNAs were converted to cDNA by reverse transcription (RT) using the newly added G/I tails as priming sites. PCR

amplification products were generated by using two primer sets: Set 1, gene-specific forward and reverse primer set for Avp (forward 5'-CGAGTGTCGAGAGGGTTTTT-3', reverse 5'-TTTATTTTCCATGCTGTAGG-3') and Set 2, Avp gene-specific forward primer and a universal reverse primer provided in the kit. PCR reactions were performed using 2 µl of undiluted RT sample. PCRs were performed using the following cycling conditions; 94°C for 2 minutes followed by 40 cycles of 94°C for 10 seconds, 60°C for 45 seconds and 72°C for 5 minutes. The PCR products were separated on 2.5% (w/v) agarose/TAE gel. The PCR products were visualised on ethidium bromide-stained gels using a Syngene G:BOX imaging system.

### 2.8. Bisulfite conversion and sequencing

Genomic DNA from SON punches (25 ng) was bisulfite converted using an EZ DNA Methylation-Gold kit (Zymo Research). The amplification and sequencing steps were performed as previously described (Greenwood et al., 2016a).

## 3. Results

### 3.1. Physiological assessment of adult and aged rats

We singly housed adult and aged rats in metabolic cages to assess their ingestive behaviours. As expected, the average weight of aged rats was significantly higher than that of adult rats (Fig. 1A). Despite their larger size, aged rats consumed significantly less food (Fig. 1B) and water (Fig. 1C) over consecutive 24 hour periods compared to adult rats. The lower water intake in aged rats was not accompanied by a significant decrease in urine output compared to adult rats (Fig. 1D), but reflected a decrease in extra-renal water loss compared to their younger counterparts (Fig. 1E). Urine osmolality was not affected by age (Fig. 1F).

### 3.2. The AVP system in adult and aged rats

To test how fluid homeostatic systems respond to osmotic stress, rats were deprived of water for 3 days. A decrease in weight from the starting body weight was observed for both adult and aged rats (Fig. 2A). This period of dehydration increased plasma osmolality by similar degrees in both aged and adult rats (Fig. 2B). However, a higher basal plasma osmolality was observed in aged rats, a difference that was preserved in response to 3 days of dehydration, suggestive of different osmolality set points in adult and aged rats. We investigated the expression of Avp mRNA and hnAvp RNA, a surrogate measure of Avp transcription (Herman et al., 1991), in the SON of control and dehydrated adult and aged rats using qRT-PCR (Fig. 2C-D). The abundance of Avp mRNA under basal conditions was not influenced by age (Fig. 2C), whilst increased hnAvp expression in aged animals indicated increased transcription of the Avp gene compared to adult rats (Fig. 2D). In contrast, the osmotic stimulus of dehydration, increased hnAvp levels above adult basal measures for both adult and aged rats, though this response was only significant in adult rats. The AVP content in the pituitary was investigated by AVP ELISA (Fig. 2E). There was a decrease in NIL AVP content in rats subjected to dehydration for both age groups. AVP NIL content was unchanged by age. Interestingly, the expected decline of AVP content with dehydration was marginally attenuated in aged rats, with higher AVP levels detected in aged dehydrated compared to adult dehydrated rats. However, there was no significant effect of age on basal or osmotically induced plasma AVP levels when comparing adult and aged rats (Fig. 2F).

### 3.3. Peptide analysis of the NIL in control and dehydrated states

*3.3.1. Effect of dehydration on NIL peptide profiles in adult rats*

The abundance of peptides in the NIL provides a good measure for assessing changes in peptide synthesis/secretion. To study effects of ageing on peptide profiles in the NIL, we used MS-based peptide measurements (Romanova et al., 2013, Romanova and Sweedler, 2015) to characterize and quantify the neuropeptide changes of the NIL. In adult rats, control and dehydrated profiles were easily classified by PCA according to PC1 (~60% of variance) (Fig. 3A). Loading plots indicated that peptides contributing to this difference matched the masses of AVP and its sodiated ion, the sodiated ion of OT, acetylated

alpha-MSH, di-acetylated alpha-MSH, a portion of the ACTH domain, and other POMC-derived peptides (Fig. 3B). The level of AVP and OT decreased with dehydration, whilst alpha-MSH and proSAAS levels increased (Fig. 3C).

*3.3.2. Comparison of the NIL peptide profiles between aged and adult rats*

A comparison was performed for adult and aged rats. In PCA, 10 PCs were required to explain 93% of variance in the dataset, with most sample segregation achieved along PC1 accounting for about 30% of variance. Spectra from aged animals showed more broad distribution within the 3D space constructed of the first 3 PCs. In this dataset, a total of 78 peaks passed the criteria for statistics selection, of which 22 (~30%) were detected at statistically different intensities ($p \leq 0.05$) between compared age groups (see Tables 1S and 2S). Some of the peaks can be matched to proopiomelanocortin (POMC) by peptide mass fingerprinting, or other previously reported neuropeptides expressed in pituitary including OT (Fig. 3D). Relative to adult rats, the aged rats exhibited significant decrease in the sodiated ion and potassiumated ion of OT and increases in the intensity of peptides matching the masses of alpha-MSH, acetylated alpha-MSH, di-acetylated alpha-MSH, and five other POMC-derived peptides..

*3.3.3. Effect of ageing on NIL peptide profile in dehydrated rats*

A comparison was performed for NIL of adult and aged dehydrated rats. In PCA, 13 PCs were required to explain 95% of variance in the dataset. Both adult and aged rats showed a range of profiles that could not be reliably classified by PCA and unsupervised clustering. Four of 13 adult rats and 2 out of 16 aged rats were particularly different. A total of 121 peaks were selected for statistics, of which 20 (~16%) were detected at statistically different intensities ($p \leq 0.05$) between compared age groups (see Tables 3S and 4S). Similar to the control groups, aged animals had higher levels of peptides matching by mass to the POMC prohormone as well as proSAAS and AVP-copeptin (Fig. 3E).

*3.3.4. Effect of dehydration on NIL peptide profiles in aged rats*

With the set of 24 aged rats (11 control and 13 dehydrated), no significant changes in NIL profiles were seen with dehydration, and no clear segregation was observed on a PCA plot.

*3.3.5. Peptide changes specific to age or dehydration*

A number of peptides profiles in the NIL were altered only as a function of ageing (Fig. 3F). In addition a separate cohort of peptides were found to only differ between adult and aged rats in dehydration. (Fig. 3F). These included OT (ageing) and proSAAS (dehydration) whose precursor proteins are known to be synthesised in magnocellular neurons in the supraoptic nucleus (Murphy et al., 2012). Using qRT-PCR we show that expression of these genes in the SON is not altered by age or dehydration (Fig. 3G)

**3.4. Changes in Avp promoter methylation as a consequence of ageing**

To see if changes in methylation could account for Avp gene transcriptional differences in the SON with age, we looked at the expression of genes known to regulate methylation status of DNA, namely the Dnmt and Tet families, in the SON (Fig. 4A). We found decreased Dnmt1 and Tet1 in the SON of aged compared to adult rats, whilst expression of the closely related genes Dnmt3a and Tet2/3 remained unchanged with age. In the dehydrated state, Dnmt1 increased and Tet1 decreased in adult rat SON samples, whilst no changes in these genes were observed with dehydration and ageing. To analyse gene-specific methylation changes we chose to examine the methylation profile of the Avp promoter within the SON by sequence analysis of bisulfite-converted DNA. Using primers spanning the proximal Avp promoter (-325 to -24bp) we investigated the methylation status of a cluster of 7 CpG sites (Fig. 4B). Analysis of the methylation pattern of CpGs in single clones from individual control and dehydrated animals with ageing are depicted in Fig. 4C. Analysis of the overall methylation of the Avp promoter for the SON revealed decreased methylation in aged compared to adult animals by two-way ANOVA ($p<0.002$), whereas methylation levels increased in response to dehydration in aged rats (Fig. 4D). In comparison, overall methylation was not significantly altered by dehydration in the SON of adults.

We next compared the methylation profiles of individual CpGs (Fig. 4E). Of the 7 CpGs analysed, only CpG2 was significantly influenced by age, with lower methylation compared to adult controls. In aged rats dehydration increased methylation of CpGs 1, 3-5, and 7 compared to aged controls. By contrast, only CpG4 showed increased methylation in dehydrated adult rats compared to adult controls. Of note, the methylation of individual CpGs was found to be similar in dehydrated adult and aged rats.

**3.5. Ageing changes gene expression in the SON and alters the effect of dehydration**

We have used transcriptomics to catalogue all of the genes expressed in the adult male SON, and to identify genes that are differentially regulated by dehydration (Hindmarch et al., 2006). The challenge now is to place these genes into physiologically relevant pathways; thus, in pursuit of this aim, our functional investigations have revealed novel genes involved in AVP elaboration (Creb3l1, (Greenwood et al., 2014, Greenwood et al., 2015a, Greenwood et al., 2015b); Slc12a1, (Konopacka et al., 2015b); Caprin2, (Konopacka et al., 2015a); Giot1, (Qiu et al., 2007); and Rasd1, (Greenwood et al., 2016b)). We have now asked if the expression of these genes is altered with ageing, under both euhydrated and dehydrated conditions (Fig. 5). We used qRT-PCR to reveal age-related increases in mRNA expression of transcription factors c-Fos (a general marker of neuronal activation), Creb3l1, Giot1 and RNA binding protein Caprin2 under basal conditions, whilst levels of the small G-protein Rasd1 and the Na-K-2Cl cotransporter Slc12a1 were unchanged. The expression of all of these genes was increased by dehydration in both adult and aged animals. In aged rats, dehydration induced smaller rises in the expression of all genes analysed, reaching statistical significance compared to adult dehydrated rats, with one notable exception, Caprin2.

**3.6. Post-transcriptional modification to Avp mRNA in ageing**

A known feature of the Avp mRNA is that the unusually long 3' poly(A) tail further increases in length in response to osmotic stress (Carter and Murphy, 1991). Here we have used poly(A) tail assays to determine the length of Avp mRNA poly(A) tail in adult and aged rats in the basal condition and in

response to dehydration (Fig. 6A). The length of Avp poly(A) tail was found to be susceptible to change with ageing. In aged rats the poly(A) tail was longer than adult control rats (Fig. 6B), perhaps suggesting altered transcript stability with age. The Avp poly(A) tail-length increased more in adult rats in dehydration, but overall poly(A) tail lengths ended up being the same size in both dehydrated groups reflecting the smaller starting point in adult rats.

**4. Discussion**

With increased life expectancy, maintaining a health and well-being into old age is becoming a priority, making the push toward understanding our ageing homeostatic systems ever more pertinent. A decline of appetite accompanied by a reduction in daily fluid intake, as we observed in the rat, are common behavioural characteristics observed in the elderly (Kmiec, 2006, Phillips et al., 1993), suggesting that our rat model is of particular value to study metabolic changes related to ageing. Comparisons of basal and dehydrated urine osmolalities in both age groups suggested that urine concentrating capacity and thus renal function is not impaired in our model at this age. Furthermore, AVP circulating levels were comparable in adult and aged rats in the basal state and in response to dehydration suggesting no changes in hypothalamo-neurohypophysial system (HNS) responsiveness to osmotic and volume stimuli. Hence, the circulating levels of AVP and the renal response to it are adequate to concentrate urine in these ageing rats.

Chronic dehydration depletes AVP stores in the posterior pituitary to meet necessary circulatory demands for AVP to facilitate increased water uptake by the kidney (Antunes-Rodrigues et al., 2014). The large stores of AVP in the posterior pituitary were depleted by comparable amounts in both age groups by dehydration. However, when compared to adult rats, aged rats had higher levels of pituitary AVP as a consequence of dehydration. We propose that increased basal AVP pituitary is responsible for this difference as opposed to ineffective stimulation of AVP secretion. This concept is consistent with the comparable increases in circulating levels of AVP after dehydration. Therefore, the ability to store and

secrete adequate quantities of AVP in response to 3 days of dehydration was not compromised in these Han Wistar rats at this age. Taken together, these data show that changes to AVP secretion cannot account for the observed metabolic changes in aged compared to adult rats. The altered fluid intake and plasma electrolytes may represent changes to other systems co-ordinating salt and water balance. For example, the renin angiotensin aldosterone and atrial natriuretic peptide systems that are known to be altered in rats and humans as a function of ageing (El-Sharkawy et al., 2014, Pollack et al., 1997, Silver et al., 1993). However, any involvement of these systems in this particular ageing model remains to be investigated.

A higher set-point for basal plasma osmolality in our aged model, one of the reported characteristics of ageing in humans and rodents (McLean et al., 1992, Terwel et al., 1992), provides one possible explanation for AVP neurone hyperactivation in the basal state. A small rise in plasma osmolality of approximately 1% is normally sufficient to activate Avp transcription in magnocellular neurons of the SON and PVN and these transcriptional events are well known to occur together with increased AVP secretion from the posterior pituitary in adult rats (Arima et al., 1999). The higher plasma osmolality in aged rats, being approximately 1% above adult rats, was indeed associated with increased transcription, but not with increased secretion of AVP. In contrast, 3 days of dehydration, a well-characterised model for activating Avp transcription in the SON (Greenwood et al., 2014), increased AVP secretion, but not Avp transcription in the aged group. This is despite a rise in plasma osmolality of greater than 2% by this osmotic stimulus.

The synthesis and secretion of AVP are normally twinned to maintain neurohypophysial homeostasis as AVP stores in the pituitary become depleted and need to be replenished with newly synthesised AVP (Murphy and Carter, 1990). Any delay in replenishing pituitary AVP stores to pre-stimulus levels might leave the system at greater risk from further hyperosmotic insults. The elderly living in care homes have been shown to have lower daily intakes of fluid than those that live at home. Furthermore, elderly people

with cognitive impairments such as dementia often forget to drink. These behavioural characteristics, coupled with reduced thirst perception in elderly people, greatly increase their risk of dehydration (El-Sharkawy et al., 2014). In elderly patients admitted to hospital, hypernatremia has been associated with an increased mortality rate (Snyder et al., 1987). In addition, clinical studies of care home patients who develop acute illness and require hospital treatment, reported that approximately 34% became markedly hypernatraemic in hospital (Millet et al., 1991). The uncoupling of plasma osmolality and Avp transcription did not alter AVP secretion here in healthy ageing rats but may become important in pathophysiological conditions if the rate of Avp transcription ever fails to meet secretory demands.

We next investigated if the observed uncoupling of synthesis and secretion was unique to AVP in the ageing HNS. We revealed a cohort of peptides in particular POMC-derived peptides, N-terminal truncated form of copeptin, proSAAS and OT in addition to AVP, which were susceptible to changes with age and also dehydration. POMC has been shown to be expressed in the pituitary intermediate lobe, with its expression altered by osmotic stimulation and changes in blood pressure (Felder and Garland, 1989, Pardy et al., 1990). In rats supplied with a drinking diet of 2% NaCl, POMC mRNA expression was shown to decrease in the intermediate lobe of the pituitary (Pardy et al., 1990). In relation to blood pressure, SHR rats have lower POMC expression in the intermediate lobe compared to WKY rats. Lowering blood pressure in SHRs with antihypertensive agents normalises POMC expression in the intermediate lobe to those of the WKY rat (Felder and Garland, 1989). We show here age-related increases in an array of POMC-derived peptides in the NIL under basal and dehydrated conditions. How, or if, these POMC peptides contribute to age-related changes to physiology is currently not known.

Copeptin and AVP are derived from the same common precursor molecule. Copeptin is the C-terminal part of pro-AVP that is cleaved during processing and released with AVP into the circulation. The functions of copeptin are not known, but due to its higher stability in plasma it is commonly used as surrogate measure for circulating levels of AVP (Christ-Crain and Fenske, 2016). Here, we have

identified an N-terminal truncated form of copeptin in the NIL, and further show that the abundance of this peptide increases in the dehydrated rat as a function of ageing. This peptide has previously been identified in a peptidomic study of the rat SON (Bora et al., 2008). Therefore, increased abundance of this peptide in the NIL might suggest increased processing of pro-AVP in ageing magnocellular neurones in response to dehydration. In support of this concept, a study using microdialysis probes to measure release patterns of AVP in the PVN and SON in the ageing male Wistar rat showed an age-associated increase in AVP release in the PVN, though not in the SON (Keck et al., 2000).

ProSAAS and OT are known to be expressed in magnocellular neurons of the hypothalamus and the posterior lobe of the pituitary gland (Bora et al., 2008, Gouraud et al., 2007). The propeptide precursor ProSAAS is processed into a number of smaller peptides in the brain and pituitary including big SAAS, little SAAS, PEN, big LEN and little LEN (Mzhavia et al., 2001). Here we identify age-associated alterations in truncated forms of little SAAS (ProSAAS 42-57) and PEN (ProSAAS 221-237) in the dehydrated NIL. Interestingly, dehydration for 3 days also increases ProSAAS expression in the SON whilst decreasing ProSAAS expression in the NIL, suggesting that ProSAAS peptide might be secreted (Gouraud et al., 2007), although this remains to be determined. The previous peptidomic study performed on rat SON samples also identified this cleavage of ProSAAS (42-57) as well a multiple PEN peptides in magnocellular neurons. One possibility for the involvement of ProSAAS in the regulation AVP is through its interactions with proprotein convertase 1. Pro-AVP is processed by proprotein convertase1, and ProSAAS inhibits the activity of this convertase (Murphy et al., 2012). Therefore, changes in ProSAAS expression in ageing might alter the processing and thus the availability of AVP.

OT is probably best known for its role in female reproduction but also functions as a natriuretic hormone that reduces sodium appetite and increases sodium excretion at the kidney (Verbalis et al., 1991). Circulating levels of OT increase in response to stimulation by osmotic stress and this depletes pituitary stores of this peptide consistent with this study (Silverman et al., 1990). Our data suggests that OT stores

are depleted in the NIL as a consequence of ageing, in agreement with earlier studies of the ageing HNS (Silverman et al., 1990, Zbuzek et al., 1988). Because OT and ProSAAS are synthesised in magnocellular neurons of the hypothalamus, like AVP, we have also investigated their transcript abundance in the SON to assess this phenomena of uncoupling observed for AVP. We report no age-related effects on Ot or ProSAAS transcription in the SON even though our data indicates altered peptide levels in the pituitary.

We have made significant steps to understanding the mechanism regulating AVP in the adult rat and have applied our understanding to the Avp transcriptional changes in this ageing model. Based on our recent study where we described altered methylation patterns of the Avp promoter with dehydration, we hypothesised that altered methylation marks in the Avp promoter could be responsible. Cellular ageing is closely associated with a decrease in expression of Dnmt1, an enzyme that stabilises methylation marks on DNA (Casillas et al., 2003), as we observed here in the SON. This is thought to be one reason for hypomethylation of DNA sequences in rodents as well as humans, and is consistent with hypomethylation of the Avp promoter in the aged rat SON. We also found lower levels of Tet1 expression in the aged rat and adult SON following dehydration. Tet1, by hydroxylation of 5hmc, has been shown to promote active demethylation of DNA in the rodent brain (Guo et al., 2011).

The decrease in Avp promoter methylation in aged rats may perhaps explain the increased Avp transcription in the ageing SON. Many studies have shown that lower levels of promoter methylation correlate with increased gene transcription. We previously reported increased Avp transcription in hypothalamic 4B cells following demethylation by 5-aza-2'-deoxycytidine treatment, consistent with this hypothesis (Greenwood et al., 2016a). Individual CpG sites were largely unaffected by age, apart from CpG2, which resides close to a cAMP responsive element which underwent hypomethylation with age. Methylation at cAMP response element sites has been shown to inhibit cAMP response element binding protein (CREB) mediated transcription (Elliott et al., 2010, Zhang et al., 2005), so hypomethylation of this site may serve to enhance Avp promoter activation by CREB (Iwasaki et al., 1997). Interestingly,

methylation signatures on this segment of the Avp promoter remained largely unchanged by dehydration in adult rats, differing from our findings in the Sprague Dawley rat (Greenwood et al., 2016a). We suggest this is due to strain differences. Nonetheless, dehydration induced the hypermethylation of CpG3, 4, and 7 in aged rats, as observed in adult Sprague Dawley rats, thus adding strength to the argument for a relationship between the methylation status of specific Avp promoter CpGs and Avp gene transcription.

To further aid our understanding of AVP neurone activity in ageing, we looked at the expression of genes known to be robustly induced by osmotic stimuli, and whose functions have been the subject of interrogation by us in relation to AVP biosynthesis in the SON, and the overall regulation of fluid balance in the rat (Greenwood et al., 2014, Greenwood et al., 2016b, Konopacka et al., 2015a, Konopacka et al., 2015b, Qiu et al., 2007). We recently identified Creb3l1 as a putative transcription factor regulating the expression of the Avp gene (Greenwood et al., 2014). Therefore, in the basal condition, increased Avp transcription can perhaps be explained by the upregulation of Creb3l1 expression and, conversely, the attenuated Creb3l1 induction in aged dehydrated rats following osmotic stimulation may explain the reduced capacity to elevate Avp. The altered expression of genes regulating transcriptional events (c-Fos, Creb3l1, and Giot1) in basal and dehydrated states, implies dramatic changes in the SON transcriptome with ageing. These genes are all activated by cAMP pathways (Greenwood et al., 2015a, Qiu et al., 2007), suggesting that altered cAMP signalling may determine altered transcriptional responses in the aged SON. The source of these altered signalling responses is not known, but may be a consequence of either altered inputs from the circumventricular organs due to changes in plasma osmolality (McKinley et al., 2004), or changes within the magnocellular neurones themselves.

It is interesting to note that basal Rasd1 and Slc12a1 expression levels were not influenced by age implying activation of these genes by separate signalling pathways not affected by age. Rasd1 is a member of the Ras family of small G-proteins that is expressed in AVP magnocellular neurones of the PVN and SON, where increased circulating glucocorticoid and/or raised plasma osmolality induce its

expression (Greenwood et al., 2016b). Interestingly, plasma corticosterone levels increase in ageing rodents as a result of hyperactivity of the hypothalamo-pituitary-adrenal axis (Goncharova, 2013). At the same time there is an age-associated decrease in the sensitivity of the hypothalamus, along with other brain nuclei, to glucocorticoids (Goncharova, 2013), which may account for the blunted increase in Rasd1 expression in dehydrated aged animals, despite changes to corticosterone levels. By lentiviral vector mediated overexpression of Rasd1 in the SON, we recently showed that Rasd1 inhibits osmotically induced Avp transcription in this nucleus (Greenwood et al., 2016b). The aged dehydrated rats appear to have lost this dehydration-induced inhibitory input on Avp transcription in the ageing SON.

The expression of Slc12a1 in magnocellular neurones of the SON and PVN is also known to be induced by chronic and acute osmotic stimulation (Konopacka et al., 2015b). We recently showed that lentiviral-mediated knockdown of Slc12a1 in these hypothalamic nuclei altered fluid homeostasis by increasing fluid intake and urine output during salt loading. Furthermore, the loop diuretics bumetanide and furosemide were found to inhibit gamma-aminobutyric acid-mediated excitation of AVP neurones and AVP release, respectively. Therefore, an altered abundance of Slc12a1 might be expected to alter neuronal activity in ageing AVP neurones.

The Avp mRNA is subject to post-transcriptional modification in the form of an increase in the length of the poly(A) tail, as seen in the SON in response to osmotic challenges (Carter and Murphy, 1991), and as we show here by age. An increased poly(A) tail length is thought to reduce the degradation and increase the stability of many transcripts (Zeevi et al., 1982), and maybe involved in the control of translation (Palatnik et al., 1984). We recently showed that the RNA binding protein Caprin2 binds to the Avp mRNA, and in doing so, mediate an increase in the length of the poly(A) tail (Konopacka et al., 2015a). It is interesting to note that the expression of Caprin2 in the ageing SON increases in parallel with the Avp mRNA poly(A) tail length. The increase in Avp mRNA poly(A) tail length in response to dehydration was not affected by age, as previously reported (Sladek and Olschowka, 1994), which was

further corroborated by there being no difference in Caprin2 expression in dehydrated adult and aged rats. A previous Northern blot study on Fisher344 rat SON samples reported no change in Avp poly(A) tail length with age (Sladek and Olschowka, 1994). We suggest that this discrepancy could be due either to strain differences, or the different methodological approaches employed.

### 4.1. Conclusions

In summary, we have performed a comprehensive analysis of the AVP system in ageing Han Wistar rats. There were no age-related changes to AVP circulating levels in basal or dehydrated states suggesting that the functioning of the HNS in body water homeostasis is intact in healthy rats at this age. In stark contrast, we describe in the magnocellular neurons of the SON a plethora of molecular changes known to alter Avp expression. These include methylation changes to the Avp promoter and altered expression of genes involved in transcriptional and posttranscriptional regulation of the Avp gene (Fig. 7). We currently do not understand the origin of these changes or why they are a necessary part of normal ageing in the rat. The current rat model was perfectly capable of coping with 3 days of dehydration by secreting adequate quantities of AVP. However, this may not be true for pathophysiological conditions commonly encountered in the ageing process. The stimulus secretion uncoupling of Avp transcription as seen here in normal ageing could increase the likelihood of fluid and electrolyte disorders in critically ill elderly patients. Not being able to adequately correct or respond to fluid and electrolyte disturbances such as a rise plasma osmolality would certainly lead to hypernatremia, a condition which is regularly observed in elderly patients admitted to hospital (El-Sharkawy et al., 2014). Further studies are necessary to address whether Avp transcription can be a rate-limiting step in the ageing HNS. The identification by MS of additional peptides that are also influenced by age in the NIL gives further scope for exploring other peptidergic systems in the ageing hypothalamus, in the context of healthy ageing. Understanding how ageing alters these hormonal systems may help to improve treatment regimens and perhaps improve the clinical outcomes for elderly patients.

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**Figure Legends**

**Figure 1. Comparison of metabolic parameters in adult and aged rats**

Physiological parameters: (A) body weight, (B) food intake, (C) water intake, (D) urine output, (E) extra-renal water secretion and (F) urine osmolality were recorded in adult (3-months old) and aged (18-months old) male rats (n=6) housed in metabolic cages. **,$p<0.01$; ***, $p<0.001$ by two-way ANOVA with Bonferroni post-hoc test.

**Figure 2. Effect of ageing on the rat AVP system**

Adult and aged male rats were subjected to dehydration for 3 days and AVP measures were performed. (A) Body weights were recorded before and after dehydration (n=11-14). (B) Plasma osmolality was measured (n=19-23) by freezing point depression. The effect of ageing on Avp expression was examined at the transcriptional level in control and dehydrated adult and aged rats. (C-D) The RNA expression level of Avp (both heteronuclear and mature form) was examined by qRT-PCR (n=6). AVP measures were performed on NIL and plasma extracts. (E) AVP content in NIL was measured by ELISA (n=9-10). (F) Plasma AVP level was determined by radioimmunoassay (n=9). DH, dehydrated. *, $p<0.05$ ***, $p<0.001$ by two-way ANOVA with Bonferroni post-hoc test. $^{\#}$, $p<0.05$ by unpaired t-test.

**Figure 3. Peptide analysis in aged rats**

Adult and aged male rats were subjected to dehydration for 3 days and NIL peptide measures were performed by MALDI-TOF MS. (A-E) Signal intensity of peptides in NIL was measured by MALDI-TOF MS in individual tissue extracts from adult and aged rats in basal and dehydrated states (n=11-16). (A) Control and dehydrated peptide profiles are easily classified by principal component analysis (PCA) according to PC1 (~60% of variance) in adult rats; PCA plot is shown for the first 3 PCs. (B) Loading plot indicates that a small subset of peptides contributes to differences between control and dehydrated adult rats, among which are masses matching AVP, acetylated alpha-MSH, and di-acetylated alpha-MSH. (C) The AVP and OT signal decreases significantly, whilst the alpha-MSH signals and proSAAS (221-237) increase significantly with dehydration in adult rats. (D-E) The effect of age on peptide signals in the NIL. (D) The OT signals decreased, whilst the signals of POMC derived peptides increased in aged compared to adult rats. (E) Peptide signals in the NIL of adult and aged rats subjected to 3 days of dehydration. The AVP-copeptin signal increased whereas the proSAAS signal decreased with age. (F) Peptides uniquely altered as a function or ageing or by just dehydration. (G) Relative RNA expression of proSAAS and Ot in the SON of adult and aged rats. DH, dehydrated. *$p<0.05$ by unpaired t-test or peptide names beginning with * by Kruskal-Wallis tests.

**Figure 4. Epigenetic changes in Avp gene promoter in ageing**

Methylation status of the Avp promoter in the SON of control and dehydrated adult and aged male rats. (A) Relative mRNA expression of Dnmt1, Dnmt3a, Tet1, Tet2, and Tet3 was determined by qRT-PCR. (B) Diagram showing seven CpG sites on the Avp promoter that were examined by colony-based PCR. (C) Representative tile diagrams showing the methylation status of seven CpG sites for individual clones of the Avp promoter extracted from the SON. (D) Percentage of global methylation on this region of the Avp promoter in control and dehydrated adult and aged rats. (E) Percentage methylation of individual

CpG sites on the Avp promoter in control and dehydrated adult and aged rats. *, $p<0.05$; **, $p<0.01$;***, $p<0.001$ by two-way ANOVA with Tukey's post-hoc test. #, $p<0.05$ by unpaired t-test.

**Figure 5. Effect of ageing on the expression of osmotically induced genes**

Relative mRNA expression of genes involved in hyperosmotic stress in the SON of the hypothalamus in control and 3-day dehydrated adult and aged rats. DH, dehydrated. *, $p<0.05$; **, $p<0.01$;***, $p<0.001$ by two-way ANOVA with Bonferroni post-hoc test. #, $p<0.05$ by unpaired t-test.

**Figure 6. Effect of ageing on transcriptional and post-transcriptional Avp gene expression**

The effect of ageing on Avp poly(A) tail length in the SON in control and dehydrated adult and aged rats. (A) Diagram of poly(A) tail assay design for the rat Avp gene. (B) poly(A) tail length of the Avp mRNA was examined using a PCR-based poly(A) tail assay. DH, dehydrated; Ag, aged; Ad, adult; RT, reverse transcription.

**Figure 7. Modelling of molecular events in the SON in ageing and dehydrated rats**. (A) How ageing may alter Avp expression in the SON compared to younger counterparts. (B) Proposed differential regulation of Avp expression in the SON as a function of both ageing and dehydration.

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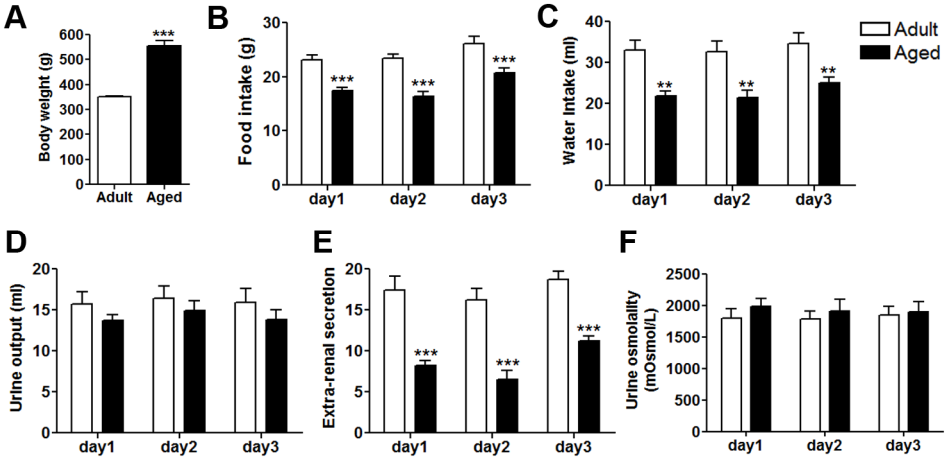
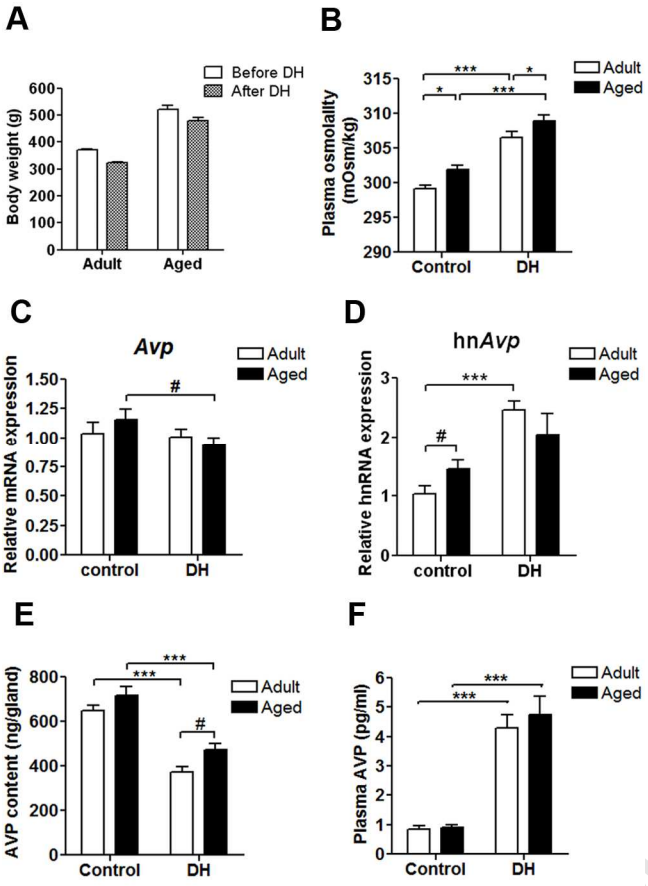
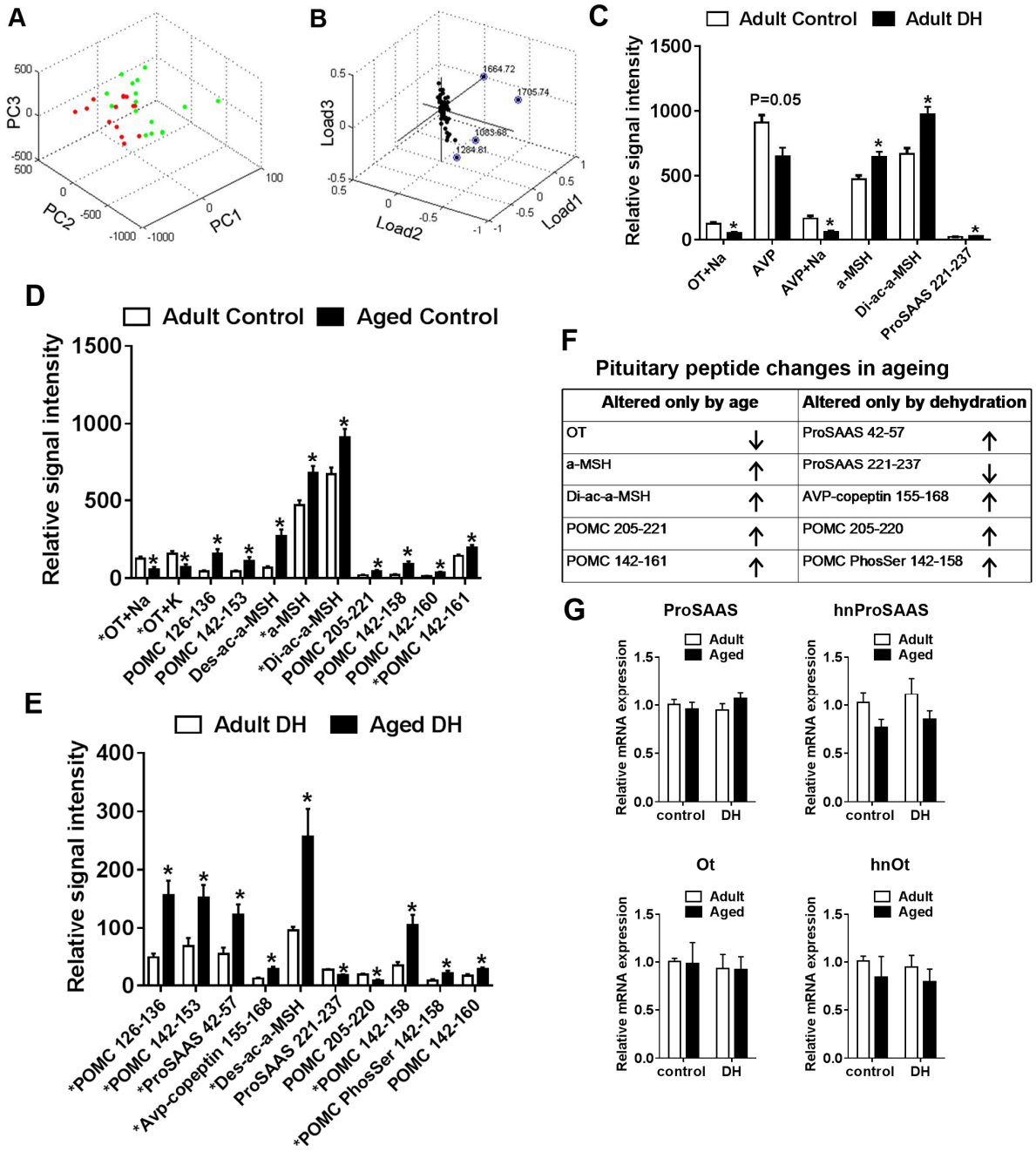
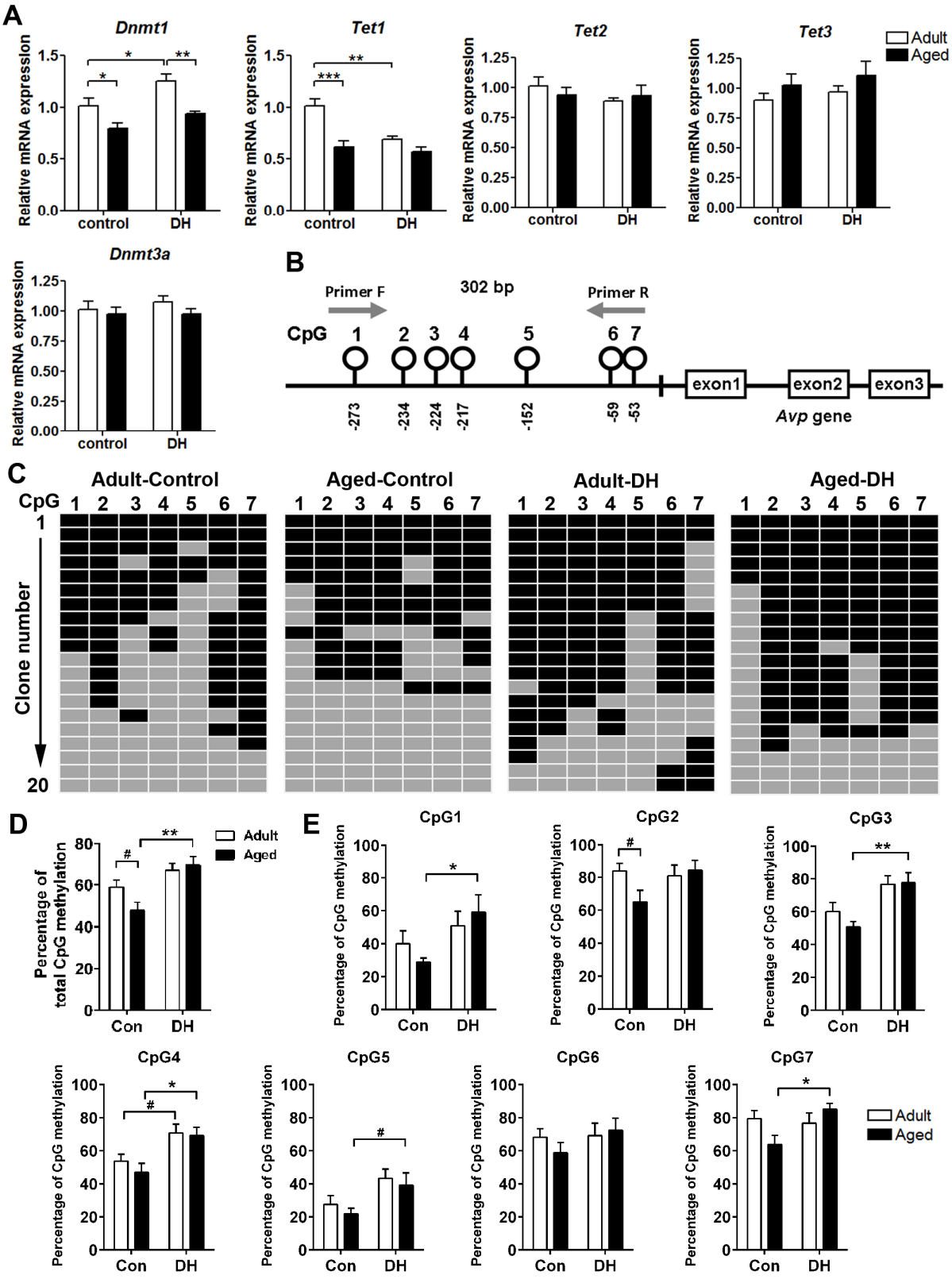







| Altered only by age | | Altered only by dehydration | |
|---|---|---|---|
| OT | ↓ | ProSAAS 42-57 | ↑ |
| a-MSH | ↑ | ProSAAS 221-237 | ↓ |
| Di-ac-a-MSH | ↑ | AVP-copeptin 155-168 | ↑ |
| POMC 205-221 | ↑ | POMC 205-220 | ↑ |
| POMC 142-161 | ↑ | POMC PhosSer 142-158 | ↑ |


A
Dnmt1
Tet1
Tet2
Tet3
Dnmt3a
Relative mRNA expression
control
DH
Adult
Aged
B
Primer F
302 bp
Primer R
CpG 1 2 3 4 5 6 7
exon1
exon2
exon3
-273
-234
-224
-217
-152
-59
-53
Avp gene
C
Adult-Control
Aged-Control
Adult-DH
Aged-DH
CpG 1 2 3 4 5 6 7
Clone number
1
20
D
Percentage of total CpG methylation
Con
DH
E
CpG1
CpG2
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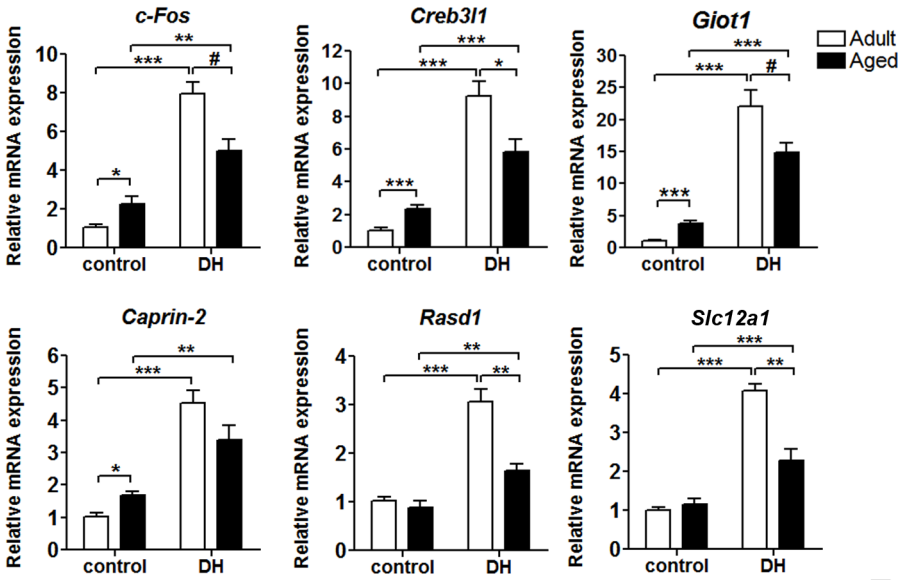
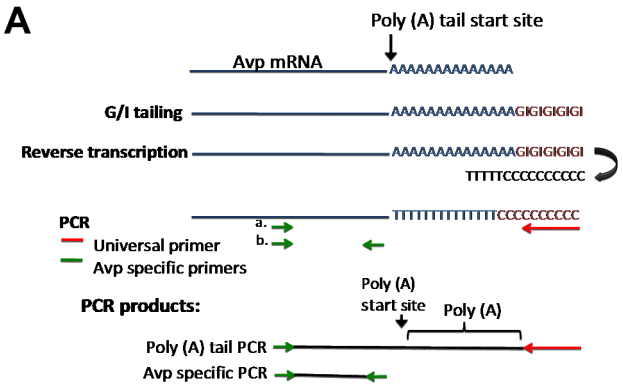
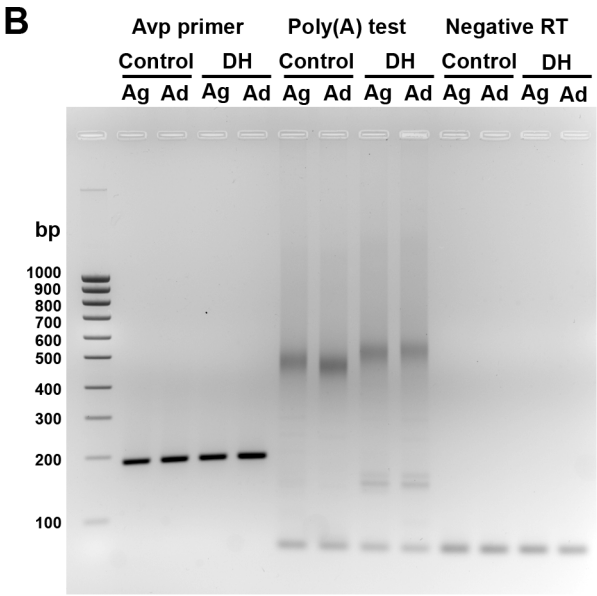
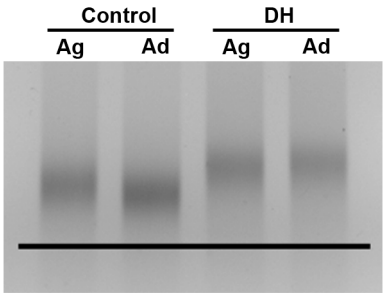
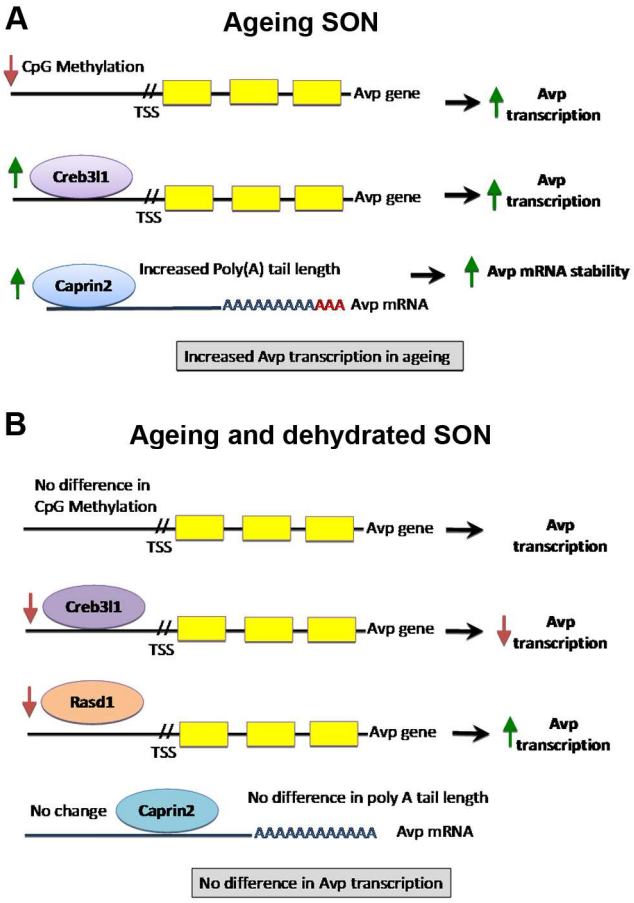
CpG5
CpG6
CpG7
Percentage of CpG methylation







**Highlights**

- Uncoupling of plasma osmolality and Avp transcription in the ageing supraoptic nucleus
- Age-associated change to methylation and gene transcription in the rat hypothalamus
- Ageing diminishes transcriptional responses to dehydration in supraoptic nuclei