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Ageing restructures the basal transcriptome of the core hypothalamic osmoregulatory control centre and alters the response to dehydration

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Abstract

Ageing is associated with altered neuroendocrine function. In the context of the hypothalamic supraoptic nucleus, which makes the antidiuretic hormone vasopressin, ageing alters acute responses to hyperosmotic cues, rendering the elderly more susceptible to dehydration. Chronically, vasopressin has been associated with numerous diseases of old age, including type 2 diabetes and metabolic syndrome. Bulk RNAseq transcriptome analysis has been used to catalogue the polyadenylated supraoptic nucleus transcriptomes of adult (3 months) and aged (18 months) rats in basal euhydrated and stimulated dehydrated conditions. Gene ontology and Weighted Correlation Network Analysis revealed that ageing is associated with alterations in the expression of extracellular matrix genes. Interestingly, whilst the transcriptomic response to dehydration is overall blunted in aged animals compared to adults, there is a specific enrichment of differentially expressed genes related to neurodegenerative processes in the aged cohort, suggesting that dehydration itself may provoke degenerative consequences in aged rats.

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Introduction

As we age, disorders of body salt and water composition become more commonplace. The UK Dehydration Recognition In our Elders (DRIE) study found that 20% of care home residents are dehydrated (1). Osmotic balance disorders are evident in many elderly patients admitted to hospital, and dehydration (DH) is a frequent cause of morbidity and mortality in old people (2,3). 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 (4).To promote healthy living well into old age, it is thus necessary to understand why such imbalances occur. Age-associated changes to both peripheral and central mechanisms that control salt and water homeostasis have been implicated. It is known that ageing is associated with a progressive decline in renal function, with less urine concentrating capacities in the elderly compared to younger subjects (5). This impaired ability to conserve body water, together with reduced thirst and inadequate fluid intake after periods of fluid deprivation, make the elderly more susceptible to DH (6,7).

The neuroendocrine reflexes regulating hydromineral balance are centred on the large peptidergic magnocellular neurones (MCNs) mainly located in the supraoptic nucleus (SON), the core hypothalamic osmoregulatory centre (8,9). Distinct groups of SON MCNs produce two major neuropeptide hormones, arginine vasopressin (AVP; encoded by the *Avp* gene) and oxytocin (OXT; encoded by the *Oxt* gene) (10), which are stored in its synaptic terminals located at the neurohypophysis. The rise in plasma osmolality that follows DH is detected by intrinsic MCN mechanisms (11,12) and by specialised osmoreceptive neurons in the circumventricular organs such as the subfornical organ, which provide excitatory inputs to shape the firing activity of MCNs for hormone secretion (13). Upon release, AVP and OXT travels through the blood to modulate water and sodium reabsorption at the kidney level (14-16), contributing to body water retention and sodium excretion (8.9). As a consequence of the depletion of pituitary stores that accompanies chronic osmotic stimulation, there is a need to synthesize more AVP, starting with an increase in *Avp* gene transcription (17), which results in an increase in the abundance of the precursor hnRNA (18), and the mature mRNA (19).

We have shown that aged rats drink less than their younger counterparts and exhibit higher plasma osmolality under both euhydrated (EH) and DH conditions (20). Following DH, aged animals consume less water and less salt (21). Pituitary vasopressin content is high in older animals under DH conditions, implying reduced secretion (20). AVP gene transcription (indirectly measured by quantifying AVP hnRNA by quantitative reverse transcriptase PCR, qRT-PCR) is increased in old age in the basal state (20). These physiological and molecular

data suggest that excitation-synthesis-secretion coupling in the SON (22) is altered in old age.

Interestingly, it is becoming clear that water intake or hydration status are linked to metabolic health. It appears that people who drink less water have a greater chance of developing type 2 diabetes and this is related to higher AVP levels (23). Furthermore, many studies have found that increased AVP release (usually assessed by measuring the surrogate precursor product copeptin) predicts insulin resistance and the onset of type 2 diabetes and major cardiovascular events in metabolic syndrome patients (24-27). There is also increasing experimental, pharmacological, and epidemiological evidence that supports a causative role for sustained increases in circulating AVP in the development of many diseases of old age (28-34).

In contrast to the physiological alterations evident during ageing, the anatomical structure of the SON is remarkably stable with age and resistant to deterioration consequential to Alzheimer's disease (AD). Studies on the ageing human brain have found preservation of the SON cytoskeletal architecture, neuronal activity, and connectivity (35-40). The most common pathological findings in AD, amyloid ß (Aß)-positive plaques and tau-positive neurofibrillary tangles (NFTs), are rarely seen in the SON (41-45).

Chronic osmotic stimulation evokes a dramatic functional remodelling of the HNS (46,47). A plethora of activity-dependent changes in the morphology, electrical properties and biosynthetic and secretory activity of the SON have been described (48), which contribute to the facilitation of hormone production and secretion, and hence survival. We wish to understand this function-related plasticity at the level of the transcriptome. Thus, we have used Affymetrix GeneChip (49-51), and latterly RNASeq (52,53), to comprehensively document the transcriptomes of the young adult male and female rat SON, and to describe how this change following the osmotic challenges of DH or salt loading. Some of the novel differentially expressed genes identified have been subjected to detailed functional investigation, for example *Creb3l1* (54). We have thus demonstrated that the SON is an excellent model that enables us to progress from transcriptome datasets to novel physiological understanding (55).

In this report, we describe the use of Illumina RNAseq to comprehensively describe the polyadenylated transcriptomes of the Wistar Han rat SON from 18-month old euhydrated and dehydrated rats. Comparison of these catalogues with our previously published datasets from 3-month old EH and DH rats (53) has enabled us to describe changes in gene expression that accompany ageing, as well as common and age-specific responses to DH. These datasets are a valuable resource for continued studies on the ageing SON, which may we have implications for the health and wellbeing of the elderly.

Results

We have asked about the process of ageing in the Wistar Han supraoptic nucleus (SON). We compared adult (3-month old) and aged (18-month old) animals. Physiological assessment of the ageing process in these rats was performed and revealed that aged rats had a significantly higher weight than adult rats, which, in both adult and aged animals, was significantly decreased by DH (Supplementary Figure S1A). Whilst rats in both age groups showed a significant increase in plasma osmolality and plasma AVP following DH (Figure S1A), both basal and dehydrated plasma osmolaity were eleveted in aged animals compared to adults (Supplementary Figure S1A).

We have previously sequenced the SON polyadenylated transcriptome from EH (n=5) and DH (n=5) adult rats. Applying a padj value cut-off of 0.05 (with Benjamini-Hochberg correction) with no log2 fold change (LFC) filter, a total of 2246 differentially expressed (DEGs) were identified (53). Here we report the sequencing of the SON polyadenylated transcriptome from EH (n=5) and DH (n=5) aged rats. RNASeq samples from the 4 groups (adult EH, adult DH, aged EH, aged DH) clustered separately illustrating transcriptome differences between the experimental conditions (Supplementary Figure S1B). Using the same statistical and filtering parameters as for the adult samples, we have identified genes that change in expression as a consequence of ageing (1283 genes) and genes that alter their expression following DH in aged rats (1536 genes). Our new, ageing related transcriptomic datasets are presented in Supplementary Table S1 and are publically accessible (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214353; token ctediycahjodtmx). Several identified DEGs were validated using quantitative reverse transcription PCR (qRT-PCR) in SON samples from an independent cohort of adult (EH and DH) (Supplementary Figure S1C) and aged (EH and DH) animals (Supplementary Figure S1D), indicating a low false discovery rate in our findings. Note that LogFC of the RNAseq and qRT-PCR expression are closely correlated (Supplementary Figures S1E and S1F).

Weighted Correlation Network Analysis of SON transcriptomes and physiological parameters

We used Weighted Correlation Network Analysis (WGCNA) (56) to tease physiological meaning from our transcriptome datasets. WGCNA uses correlations of gene expression patterns to define modules of closely related genes, with modules assigned arbitrary colour names. Genes within modules are assumed to have some functional overlap. Here, we used WGCNA to define groups of genes that may share functional pathways in response to dehydration that are also influenced by aging and identify influential “hub” genes within these modules that may be promising candidates for further investigation. Modules of genes identified by expression can then be correlated with physiological trait data. We used weight

as a proxy for the age of the animals, and plasma osmolality and plasma AVP as markers for the dehydration response. WGCNA is an especially useful method when there are more than two groups to compare. Therefore, all four groups (EH adult, EH aged, DH adult, and DH aged) were used in this WGCNA analysis. Eigengenes (an abstract representation of the genes in a module) for each module identified by WGCNA were correlated (bicor) to trait data and the categorical groups of adult/aged and EH/DH (Supplementary Figure S1G).

Ageing induced transcriptome dynamics in the rat SON

In order to identify DEGs associated with ageing in the rat SON, we compared the adult EH and the aged EH datasets. These datasets clustered separately in a PCA plot (Figure 1A). Of the 1283 DEGs associated with ageing in the SON, 586 were upregulated and 697 were downregulated. To help visualise the data intuitively, a volcano plot was used (Figure 1B). Note that all gene symbols referred to in this manuscript are defined in Supplementary Table S2.

We categorized the global gene expression profile of the ageing SON, firstly by plotting the top 50 DEGs (P_{Adj}< 0.05) ordered by LFC in a lollipop graph that also plots significance (by padj value) and expression level represented by the size of the dot (Figure 1C). DEGs were then catalogued according to their identity as transcription factors (57) or their physiological or pharmacological classifications using the International Union of Basic and Clinical Pharmacology and British Pharmacological Society (IUPHAR- BPS; 58) (Figure 1D). The full classification list can be found in Supplementary Table S3.

To assist with biological understanding of the ageing data, Gene Ontology (GO; 59), Reactome (60) and KEGG (61) analyses were performed on the ageing DEG set. The DEGs list was used without any filtering being applied to the LFC nor the basemean counts. 18 Molecular Function (MF) GO enriched terms were identified to be significantly over-represented in adult verses aged rat SON. Cell adhesion (GO:0050839; P_{Adj}= 1.17E-7), extracellular matrix (ECM) structure (GO:0005201; P_{Adj}= 1.17E-7), and integrin binding (GO:0005178; P_{Adj}= 1.22E-6) were the main enriched molecular function terms (Supplementary Figure S1H, Supplementary Table S4).

Over-representation analysis (ORA) using the Reactome databases (Supplementary Figure S1I, Supplementary Table S5) identified extracellular matrix organization (R-RNO-1474244; P_{Adj}= 6.61E-9) as the most overrepresented Reactome pathway, which was also present in the MF GO analysis. Multiple pathways connected to the ECM were highly represented in this analysis, such as collagen formation (R-RNO-1474290; P_{Adj}= 3.6E-4), ECM proteoglycans (R-RNO-3000178; P_{Adj}= 2.8E-4), laminin interaction (R-RNO-3000157; P_{Adj}= 6.6E-4), and collagen degradation (R-RNO-1442490; P_{Adj}= 2.9E-3).

Ageing of the SON was defined by 20 over-represented KEGG pathways. The analysis confirmed the enrichment of the ECM pathways including lysosomes (rno04142; P_{Adj}= 1.96E-8) and ECM receptor interaction (rno04512; P_{Adj}= 3.14E-8) (Figure 1E, Supplementary Table S6).

Considering the WGCNA analysis, the “blue module” showed a strong negative correlation with weight (bicor -0.9, p=<0.001) but little correlation with plasma AVP or plasma osmolality (Supplementary Figure S1E) suggesting it represents genes influenced specifically by ageing. GO analysis using genes assigned to ‘blue module’ corroborated ORA results described above, with some structural terms such as extracellular matrix organisation (GO:0030198, P_{Adj}=1.9E-17) and collagen-containing extracellular matrix (GO:0062023, P_{Adj}= 1.8E-25). The "blue module” showed a very strong correlation between within-module connectivity and correlation to the weight trait (bicor 0.76, p<1e-200). The gene with the highest within module connectivity was *Igf2*, which is involved in cell proliferation and growth. Other high connectivity genes in the “blue module” include the gene encoding the extracellular proteoglycan *Bgn* and the collagen encoding genes *Col4a5* and *Col1a1* (Figure 1F).

DH induced transcriptome dynamics in the aged rat SON

In order to identify DEGs associated with DH in the aged rat SON, we compared the aged EH and the aged DH datasets. These datasets clustered separately in a PCA plot (Figure 2A). Of the 1536 DEGs associated with DH in the aged SON, 842 were upregulated and 694 were downregulated. To help visualise the data intuitively, a volcano plot was used (Figure 2B). The top 50 DEGs are presented organized according to LFC in a lollipop graph (Figure 2C). DEGs were then catalogued according to their identity as transcription or their physiological or pharmacological classifications (Supplementary Table S7) (Figure 2D). ORA KEGG analysis of the common genes revealed 55 enriched pathways including pathways involved in neurodegeneration (rno05022; P_{Adj}= 5.9E-23), including Parkinson’s disease (rno05012; P_{Adj}= 2.6E-22), prion disease (rno05020; P_{Adj}= 1.3E-19), and Alzheimer’s disease (rno05010; P_{Adj}= 6E-16) (Figure 2E, Supplementary Table S8).

Considering our WGCNA analysis, the “green module” is representative of DEGs in DH specifically with correlation to Plasma AVP (bicor -0.8, <0.001; Supplementary Figure S1E). This module was enriched for GO terms and KEGG pathway relating to synaptic transmission and activity (Figure 2F). *CamkV*, which encodes a pseudokinase which is expressed at the synapse (62)**,** alongside other genes expressed in the synapse, reside within the “green module” with high connectivity and links to neuronal development genes such as *Chrm1 and Gria2.*

Comparison of ageing DEGs with adult DH DEGs

In order to identify genes regulated by dehydration in adult rats that are also sensitive to ageing, we compared the list of genes significantly modulated by ageing in the SON (1283, Figure 1) to our previously published list of genes regulated by dehydration in the SON of adult rats (2246) (53). This comparison revealed 384 DEGs commonly regulated by dehydration in adult rats and by ageing in the SON (Figure 3A). These genes are displayed in a volcano plot using LFC of the ageing EH dataset (Figure 3B) and the adult DH dataset (Figure 3C). The top 50 DEGs are presented organized according to LFC during ageing in a lollipop graph (Figure 3D). DEGs were then catalogued according to their identity as transcription factors or their physiological or pharmacological classifications (Supplementary Table S9) (Figure 3E). There is a positive correlation between the common DEGs of the two datasets (Figure 3F). However, some genes upregulated by dehydration in the adult are downregulated by ageing (for example, *Igfbp2*, *Nid2* and *Pcdh12*). Conversely, some genes downregulated by dehydration in the adult are upregulated during ageing (for example, *Clic6*, *Itgb2* and *Pbx3*). ORA by KEGG of the common genes revealed pathways such as lysosomes (rno04142; P_{Adj}= 1.86E-3), ECM-receptor interaction (rno04512; P_{Adj}= 3.2E-3), protein processing in the endoplasmic reticulum (rno04141; P_{Adj}= 1.9E-2), and longevity regulating pathway (rno04211; P_{Adj}= 2.1E-2) (Figure 3G, Supplementary Table S10).

Comparison of ageing DEGs with aged DH DEGs

We found that 212 genes change in expression as a consequence of both ageing and DH in aged rats (Figure 4A). These genes are displayed in a volcano plot using LFC of the ageing dataset (Figure 4B). Different genes were highlighted when the common genes were displayed in a volcano plot using the LFC of the aged DH dataset (Figure 4C). The top 50 DEGs are presented organized according to LFC during ageing in a lollipop graph (Figure 4D). DEGs were then catalogued according to their identity as transcription factors or their physiological or pharmacological classifications (Supplementary Table S11) (Figure 4E). There is a positive correlation between the common DEGs of the two datasets (Figure 4F). However, some genes upregulated by dehydration in aged animals are downregulated by ageing (for example, *C3*, *Dct* and *Cdkl4*). Conversely, some genes downregulated by dehydration in the adult are upregulated during ageing (for example, *Tnc*, *C1ql4*). ORA KEGG analysis of the common genes revealed three enriched pathways: lysosomes (rno04142; P_{Adj}=1.7E-4), protein processing in endoplasmic reticulum (rno04141; P_{Adj}= 2E-2), and aminoacyl-tRNA biosynthesis (rno00970; P_{Adj}=2E-2) (Figure 4G). DEGs that overlapped the aged DH and ageing comparisons were best represented by the “turquoise” WGCNA module (including 25% of module genes). The ‘turquoise” module, which was the largest cluster of genes identified by WGCNA, was enriched for terms involved in the protein

folding process. Terms such as endoplasmic reticulum (GO:0005783; P_{Adj} 7.00e-10), response to endoplasmic reticulum stress (GO:0034976; P_{Adj} 3.275e-06), and protein processing in endoplasmic reticulum (KEGG:04141; P_{Adj} 2.412e-11) were all significantly enriched (Figure 4H). Genes with high connectivity in the "turquoise" module included *Atf4* and *Hspa5* which are involved in protein folding processes and ER stress pathways (63,64). Reassuringly, WGCNA analysis further reiterated the conclusions of the overrepresentation analyses. Our analysis suggests that stimulating the aged SON with DH aggravates the ageing process and the SON attempts to cope with these stresses by activation of the unfolded protein response pathway.

Comparison of adult DH DEGs with aged DH DEGs

Comparison of adult DH significant DEGs with aged DH significant DEGs revealed that 767 genes change in expression as a consequence of dehydration in both adult and aged rats, 730 genes change only in aged DH, and 1479 genes change only in adult DH (Figure 5A). Each of these gene categories will be considered separately.

Common DH DEGs

We found that 767 genes change in expression as a consequence DH in both adult and aged rats (Figure 5A). These genes are displayed in a volcano plot using LFC of the adult (Figure 5B) or aged (Figure 5C) DH datasets. There was a strong positive correlation between aged and adult DH DEGs of the two datasets (Figure 5D). Only one gene upregulated by dehydration in adult animals was downregulated in aged rats (*Mdb6*). Conversely, only three genes downregulated by dehydration in the adult were upregulated in DH aged animals (*Grifin*, *Bcas1* and *Qdpr*). Linear regression analysis comparing the common adult DH genes and aged DH DEGs (Figure 5E) revealed that ageing decreased the r^2 value from 0.407 to 0.309 and revealed a significantly smaller slope with aged compared to adult rats ($F = 119.6$, $p < 0.0001$). Further, comparing average absolute log2FC values, we found that aged rats have a much smaller SON transcriptomic response to WD (0.343) when compared to adult rats (0.405), at least for these 784 commonly DEGs ($p < 0.0001$ by Wilcoxon matched-pairs signed-rank test). Overall, these data suggest that response to DH in old animals is blunted compared to adult animals.

The top 50 DEGs are presented organized according to Aged DH LFC in a lollipop graph (Figure 5F). DEGs were then catalogued according to their identity as transcription factors or their physiological or pharmacological classifications (Supplementary Table S12) (Figure 5G). Pathways such as protein processing in the endoplasmic reticulum (rno04141; P_{Adj}= 2.7E-11), aminoacyl-tRNA biosynthesis (rno00970; P_{Adj}= 2.9E-07), and circadian entrainment pathway (rno04713; P_{Adj}= 2.4E-2) (Figure 5H, Supplementary Table S13) were amongst the enriched terms in ORA KEGG analysis.

Unique Adult DH DEGS

We found that 1479 genes significantly change in expression as a consequence DH in adult rats, but not in aged rats (Figure 6A). These genes are displayed in a volcano plot using LFC of the adult DH dataset (Figure 6B). The top 50 DEGs are presented organized according to LFC in a lollipop graph (Figure 6C). DEGs were then catalogued according to their identity as transcription factors or their physiological or pharmacological classifications (Supplementary Table S14) (Figure 6D). ORA KEGG analysis of the adult DH transcripts identified multiple enriched pathways such as ribosomes (rno03010; P_{Adj}=3.02E-08), MAPK signalling pathway (rno04010; P_{Adj}=6.5E-3), and aldosterone regulated sodium reabsorption (rno04960; P_{Adj}= 1.7E-2) (Figure 6E, Supplementary Table S15).

Unique Aged DH DEGS

We found that 730 genes change in expression as a consequence DH in aged rats, but not in adult rats (Figure 7A). These genes are displayed in a volcano plot using LFC of the aged DH dataset (Figure 7B). The top 50 DEGs are presented organized according to LFC in a lollipop graph (Figure 7C). DEGs were then catalogued according to their identity as transcription factors (57) or their physiological or pharmacological classifications using the IUPHAR- BPS (58) (Supplementary Table S16) (Figure 7D). ORA KEGG analysis revealed enrichment of pathways related to neurodegenerative diseases, including Parkinson's (rno05012; P_{Adj}= 1.9E-14), Alzheimer's (rno5010; P_{Adj}= 2.73E-14), prion (rno05020; P_{Adj}= 9.37E-14), and spinocerebellar ataxia (rno05017; P_{Adj}= 3.48E-6). Also, multiple signal pathways were affected such as cAMP (rno04024; P_{Adj}= 7.2E-3) and GMP-PKG (rno04022; P_{Adj}= 7.9E-3) (Figure 7E, Supplementary Table S17).

Discussion

The precise regulation of salt and water balance is essential for survival and good health, and when threatened, is aggressively defended. Integrated neuroendocrine mechanisms function to control the excretion and consumption of water and salt in order to maintain the optimal bodily composition required for good health (8,9). These mechanisms deteriorate with age, and dehydration is a common cause of morbidity and mortality in the elderly. Here we show that the process of natural ageing restructures the basal transcriptome of the SON of the rat, the core hypothalamic osmoregulatory control centre, and profoundly alters the response of the SON to dehydration.

We compared the transcriptomes of the SON in adult and aged euhydrated rats and found massive changes in gene expression associated with ageing. Functional ORA and WGCNA revealed enrichment for genes involved in extracellular matrix (ECM) organisation and cell adhesion. It is known that the SON has a complex and dynamic ECM that has been implicated in its physiological functioning. The SON undergoes dramatic activity-dependent

structural plasticity as a consequence of sustained physiological stimulation (34-36). This structural plasticity is characterised by synaptic remodeling, increased direct neuronal membrane apposition, and dendritic bundling. Intrinsic glial cells actively participate in these plastic changes in addition to the MCNs themselves. Crucial to these processes are cell adhesion molecules, belonging to immunoglobulin superfamily proteins and extracellular matrix glycoproteins, which participate in neuronal-glial, neuronal-neuronal, and glial-glial recognition and guidance (65). That the expression of genes encoding these classes of proteins changes with ageing suggests that intercellular interactions maybe altered in the aged SON, perhaps related to the reported hypertrophy of glial perikarya and cell processes (66).

We have previously described how the adult SON transcriptome responds to denydration (53). Here we report how the transcriptome of the aged SON responds to dehydration. Interestingly, ORA analysis revealed that gene sets were enriched in terms related to neurodegenerative processes and diseases. This is perhaps paradoxical, given that the SON has been described as being resistant to deterioration consequential to Alzheimer's disease (AD) (23-33), but might suggest that the process of dehydration itself provokes degenerative consequences.

Inappropriate release of the antidiuretic hormone AVP has been highlighted as one of the major causes of irregular water homeostasis in ageing (67). AVP MCNs undergo numerous morphological changes as they age, indicative of hyperactivity, including increased size of perikarya, nucleoli and Golgi apparatus in humans and rodents (68), analogous to morphological changes in these neurones with dehydration (69). 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. It has been suggested that such hyperactivity of MCNs may in itself lead to electrolyte disorders in the elderly (67). Transcriptional changes have also been reported in the aged SON (20), some of which are indicative of neuronal activation. For example, the expression of the neuronal plasticity marker gene *Fos* is increased in the aged SON (20,70). We examined the relationship between ageing and physiological activation at the whole transcriptome level by comparing ageing DEGs with both adult and ageing DH DEGs. We found that 384 genes that change in expression as a consequence of DH in adult rats are also altered by ageing. ORA revelead that these genes are involved in lysosomal function, ECM-receptor interaction, protein processing in the endoplasmic reticulum, and longevity. Comparison of the ageing DEGS with the aged DH DEGs revealed 212 common genes with enrichment for pathways related to lysosomal function, endoplasmic reticulum protein processing and stress, and the recruitment of amino-acyl tRNAs. Thus, it would appear that ageing in the SON involves the regulation of genes networks that are common to both adult

and aged DH. These networks are involved in the synthesis and delivery of considerable quantities of secretory protein products, consistent with the previously reported hyperactivity of aged SON neurones (67,68,70).

Comparison of the adult SON DH DEGS with those of the aged SON revealed that the transcriptomic response to DH is very different. It is noteworthy that a large number of genes that are significantly altered in expression as a consequence of dehydration in the adult animal do not respond to dehydration in the aged rat. These genes are enriched for functionalities related to translation (ribosomes), the MAPK signalling pathway, and aldosterone actions that are presumably lost in the aged animal. A smaller number of genes are altered in expression as a consequence of DH only in the aged SON. ORA again revealed enrichment for terms involved in neurodegeneration that are not evident in that adult animal as well as terms associated with signalling pathways involving cAMP and GMP-PKG.

When placed under stress, the capabilities of the AVP system have been shown to decrease with age (71-73). In contrast to increased activity under basal conditions in adult animals, reports have described an attenuation of the AVP system in response to dehydration with ageing (74). This has led some to suggest that deficits in mechanisms controlling transcription, mRNA stability or translation in the ageing SON MCNs may be responsible (75). Indeed, we have previously shown that the steady-state response to dehydration of a number of selected gene transcripts (*Fos, Creb3l1, Giot1, Caprin2, Rasd1, Slc12a1*) is attenuated in aged animals (20). This appears to be a transcriptome level effect, with many of the common genes regulated by dehydration showing a blunted response in aged animals compared to adults (Figure 5). This generalised attenuation of the transcriptomic response to dehydration is likely to greatly affect SON function and overall osmoregulatory effectiveness under these challenging physiological and pathophysiological conditions.

Limitations of this study

Although we have previously employed microarrays to catalogue and compare differences in the transcriptomic response to dehydration of adult male and female SON (39), in this ageing study we only examined male rats. Given that age-related sex differences in osmoregulation in the rat have been described (75,76) this is a shortcoming of this study. In this context it is important to note that in young humans, both sexes have the same number of AVP neurones (77), but these neurones are larger in males compared to females (78). This perhaps contributes to the elevated levels of circulating AVP in young males compared to females. With age, the size of AVP neurones increases in females, but not in males, and this results in increased circulating AVP levels in females with age (79,80).

Rats were harvested at a single time point in the daytime (10 am-12 noon). In young humans, AVP is secreted with a diurnal rhythm with a strong peak during the night (81,82). This nocturnal peak diminishes during the course of healthy ageing (81-84). In the rat, a similar diurnal rhythm has been observed (88,89). Plasma AVP levels peak at dusk and reach a nadir at dawn, with pituitary content showing an inverse pattern (85,86). The effect of ageing on this rhythm in rats has not been studied and was not addressed in our study.

We note that whilst the genome sequencing projects have revealed that approximately 22,000 protein coding genes are needed to make a mammal, a mere 5% of genes dominate 70% of neuroscience publications (87). The remaining 95% of genes have been defined as the ignorome (88). The extent of the ignorome, by definition, impacts upon the biological value of GO analyses. Given that 58% of annotations are represented by only 16% of human genes (89), it is clear that GO and pathway analysis is limited by the fact that most genes are not annotated with any functional information. It is amusing to note that this bias is evident from the inclusion of the Coronavirus disease-COVID-19 pathway (rno05171) in one of our OR-KEGG analyses (Figure 6E), which simply reflects the extent of recent research in this area (90).

Whilst transcriptomics can provide considerable global information regarding global steady state mRNA levels, it must be conceded that it is the proteome that is the engine of the cell. Physiological extrapolations from transcriptome data must therefore be interpreted with caution.

Finally, we acknowledge that this bulk RNAseq analysis, whilst focussed on the SON, does not address the cellular heterogeneity of this discrete hypothalamic nucleus. We note that one study has carried out a single nuclei RNAseq analysis of the ageing female mouse hypothalamus (91). However, limited data on the SON precluded deconvolution of our transcriptomes. Remining of these data revealed only 7 genes as being significantly changed as a consequence of aging in the small number (only ~100 in each age group) of identified female mouse MCNs. None of these genes overlapped with our ageing SON dataset, possibly due to sex or species differences, or technical issues and limitations related to different methodologies. For example, our bulk SON transcriptome analyses revealed the over-expression of *Oxt* in response to ageing. On the other hand, Hajdarovic *et al.* (91) found a reduction in *Oxt* derived RNA in the aged hypothalamus when treating their single-nucleus RNAseq as bulk. This apparent discrepancy might be related to many factors, such as differences in RNA processing, changes in nucleus-to-cytoplasm transportation, sex dimorphism of hypothalamic transcriptomic responses to ageing, amongst others.

Conclusions

It is now well recognised that disorders of body salt and water composition become more commonplace as we age. Changes in the vasopressin system with ageing, as described here at the transcriptome level, are thus potentially of great importance clinically, and may be of potential prognostic and therapeutic value. We reveal that healthy ageing results in numerous robust changes in the transcriptomic landscape of the SON, with extracellular matrix components being particularly prominent. Overall, the response to dehydration in aged animals appears to be blunted, but intriguingly DEGs associated with neurodegenerative processes become apparent, raising the possibility that long-life episodes of dehydration may impact the development of neurodegenerative diseases such as Alzheimer's or Parkinson's.

Materials and Methods

Animals

All experiments were performed under the auspices UK Home Office Project Licence held under, and in strict accordance with, the provisions of the UK Animals (Scientific Procedures) Act (1986); they were also approved by the University of Bristol Animal Welfare and Ethical Review Board. We choose to use male Wistar Han rats from the international standardisation programme (IGS) in our study (Charles River). This carefully managed breeding program minimises the impact of genetic drift so that colonies bred in different locations around the world are not significantly divergent from each other giving a level continuity in studies performed in laboratories worldwide. A total of 24 rats aged (18 months old) were purchased for this study. Rats were housed in groups of 3 at a constant temperature (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. Cages contained sawdust, bedding material, and cardboard tubing for enrichment. Rat cages were randomly assigned to two groups of 12 animals: EH (free access to drinking water) and DH (removal of drinking water for 72 hours). All rats were humanely killed by striking of the cranium (stunning), and then immediately decapitated with a small animal guillotine (Harvard Apparatus). Brains were rapidly removed from the cranium and placed into a chilled rat brain matrix for separation of the forebrain from the hindbrain. The forebrain was placed cut edge down onto aluminium foil resting on pellets of dry ice and covered with powdered dry ice (within 3 minutes of stunning). Animal experiments were performed between 10 am-12 pm.

Plasma AVP measures

Trunk blood plasma for AVP measures was collected in 1 ml aliquots and snap frozen in liquid nitrogen before being stored at -80C. Plasma was extracted with two sample volumes

of ice-cold acetone, 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). Plasma AVP concentrations were determined by specific radioimmunoassay (92).

Plasma Osmolality measures

Trunk blood was collected in heparin-coated tubes and centrifuged at 1,600×g for 15 minutes at 4 °C. Plasma osmolality was measured by freezing point depression using a Roebling micro-osmometer (Camlab).

SON RNA extraction

Bilateral punches of the SON were collected from coronal slices with a 1 mm sample corer (Fine Scientific Tools) using the optic chiasm as a reference as described. The microtubes containing SON punches were maintained on dry ice prior to re-suspension by continuous vortexing for 1 minute in 400 µL of QIAzol lysis reagent (Qiagen). After a 10-minute incubation at room temperature, debris was pelleted by centrifugation at 12,000xg for 3 minutes. Then, 350 µL was removed and mixed with an equal volume of absolute ethanol. This mix was applied to a Zymo-Spin IIC column and total RNA was extracted using a Direct-zol RNA MiniPrep extraction kit (Zymo Research, Irvine, CA, USA). Total RNA was eluted in a volume of 25 µL.

RNA sequencing (RNAseq)

RNA went through rigorous quality control checks to assess purity and integrity (Agilent BioAnalyzer; RNA TapeStation) The RIN value range was 8.4-8.8 (see Supplementary Table 18). Poly(A)-selected mRNA was sequenced (Source Bioscience). Poly(A) enriched bulk RNA-sequencing libraries were constructed using Illumina TruSeq Stranded mRNA kits. Libraries were loaded onto lanes of an Illumina NextSeq flowcell and sequenced using 75bp paired-end (PE) runs. Each sample generated >35million PE reads. Data was analysed as described in detail (53). The analysis was sufficiently powered (n = 5 per group) to reduce the false discovery rate, and to enable systems level analysis (93). Differentially expressed genes (DEGs) with p adjusted (padj) values of <0.05 are considered significant.

Reverse transcription (RT) quantitative polymerase chain reaction (RT-qPCR)

For cDNA synthesis, 40 ng of total RNA was reverse transcribed using the QuantiTect reverse transcription (RT) kit (Qiagen). Primers for rat genes used in this study can be found

in the Supplementary Table 19 (Eurofins MWG Operon). The optimization and validation of primers were performed using standard Applied Biosystems protocols. The cDNA from RT reaction was diluted 1:4 with H_2O and used as a template for subsequent polymerase chain reactions (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 used (94). The internal control housekeeping gene used for these analyses was the housekeeping gene *Gapdh*.

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Figure Legends

Figure 1 Transcriptome dynamics of the ageing rat SON. PCA plot illustrating distinct differences between the adult and ageing SON (A). Volcano plot showing the distribution of DEGs (B). Top 50 ageing DEGs (C). Classification of top 50 DEGs; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (D). Dot plot showing pathway analysis against the KEGG database and their associated DEGs. The top 5 most significant DEGs by P_{adj} associated with each overrepresented KEGG terms are shown as dots on the x-axis; colour denotes the direction of changed expression (LFC), size denotes expression level. Enriched KEGG terms are plotted on the y-axis. Expression is color coded (E). WGCNA analysis showing the “blue” module and its associated enriched GO pathways (MF, BP, CC, and KEGG) (F).

Figure 2: Transcriptome dynamics evoked by DH in the aged rat SON. PCA plot illustrating differences between the aged EH and DH groups (A). Volcano plot showing the distribution of DEGs (B). Top 50 aged DH DEGs (C). Classification of to 50 DEGs by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (D). ORA KEGG pathway analysis of the DEGs (E). WGCNA analysis showing the “green” module and its associated enriched GO pathways (MF, BP, CC, and KEGG) (F).

Figure 3: Comparison of adult DH DEGs with ageing DEGs. Venn comparison of adult DH and ageing DEGs (A). Volcano plot showing the distribution of DEGs using LFC of the ageing DEGs (B) or using LFC of the adult DH DEGs (C). Lollipop graph highlighting the top 50 DEGs common between the two datasets (D). Classification of the top 50 DEGs by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (E). Correlation analysis (F), and ORA KEGG pathway analysis of the DEGs (G).

Figure 4: Comparison of aged DEGs with aged DH DEGs. Venn comparison of aged DH and ageing DEGs (A). Volcano plot showing the distribution of the DEGs using LFC of the ageing DEGs (B) or using LFC of the aged DH DEGs (C). Lollipop graph highlighting the top 50 DEGs common between the two datasets (D). Classification of the top 50 DEGS by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (E).

Correlational analysis of the common DEGs (F). ORA KEGG pathway analysis of the common DEGs (F). WGCNA analysis showing the turquoise module and its associated enriched GO pathways (MF, BP, CC, and KEGG) (G).

Figure 5: Comparison of adult DH DEGs with aged DH DEGs: common DEGs. Venn comparison of adult DH and aged DH DEGs highlighting the common genes (n=767) (A). Volcano plot showing the distribution of the common adult DH and aged DH DEGs using LFC of the adult DH DEGs (B) or using LFC of the aged DH DEGs (C). Correlation analysis (D) and linear regression analysis (E) comparing the adult responses to DH (E). Lollipop graph highlighting the top 50 DEGs common between the two datasets (F). Classification of the top 50 DEGS by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (G). ORA KEGG pathway analysis of the common DEGs (H).

Figure 6: Comparison of adult DH DEGs with aged DH DEGs: unique adult DH DEGs. Venn comparison of adult DH and aged DH DEGs highlighting the unique adult DH DEGs (n=1479) (A). Volcano plot showing the distribution of the unique adult DH DEGs (B). Lollipop graph highlighting the top 50 unique adult DH DEGs (C). Classification of the top 50 DEGS by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (G). ORA KEGG pathway analysis of the unique adult DH DEGs (H).

Figure 7: Comparison of adult DH DEGs with aged DH DEGs: unique aged DH DEGs. Venn comparison of adult DH and aged DH DEGs highlighting the unique aged DH DEGs (n=730) (A). Volcano plot showing the distribution of the unique aged DH DEGs (B). Lollipop graph highlighting the top 50 unique aged DH DEGs (C). Classification of the top 50 DEGS by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (G). ORA KEGG pathway analysis of the unique agedDH DEGs (H).

Supplementary Figure S1. Body weight was documented before and after DH and plasma osmolality and plasma AVP were measured (A). Analysis by two-way ANOVA with Tukey's post hoc test. A Grubbs' test identified a significant outlier for aged DH AVP with an Alpha = 0.05 which was removed (22.9 pg/ml). PCA plot illustrating the distinct differences between all four groups in this study (B). RT-qPCR validation of DEGs in ageing EH SON (comparing adult EH and aged EH) (C) and DH in SON of aged animals (comparing aged EH and aged DH) (D). Analysis by unpaired *t*-test. Spearman correlations comparing the RT-qPCR and RNAseq expression data from the ageing EH SON (comparing adult EH and aged EH) (E) and DH in SON of aged animals (comparing aged EH and aged DH) (F). WGCNA module-trait association matrix (G). Each row corresponds to a module labeled with a colour, and

each column corresponds to a physiological trait. Dark red shows strong poitive correlation whilst dark blue shows strong negative correlation. Dot plot showing GO ORA analysis of the MF terms (H) and Reactome pathway analysis (I) and their associated DEGs derived from the ageing DEGs. Values are means +/- SEM of n=5. *p<0.05, **p<0.01, ***P<0.001.

Figures

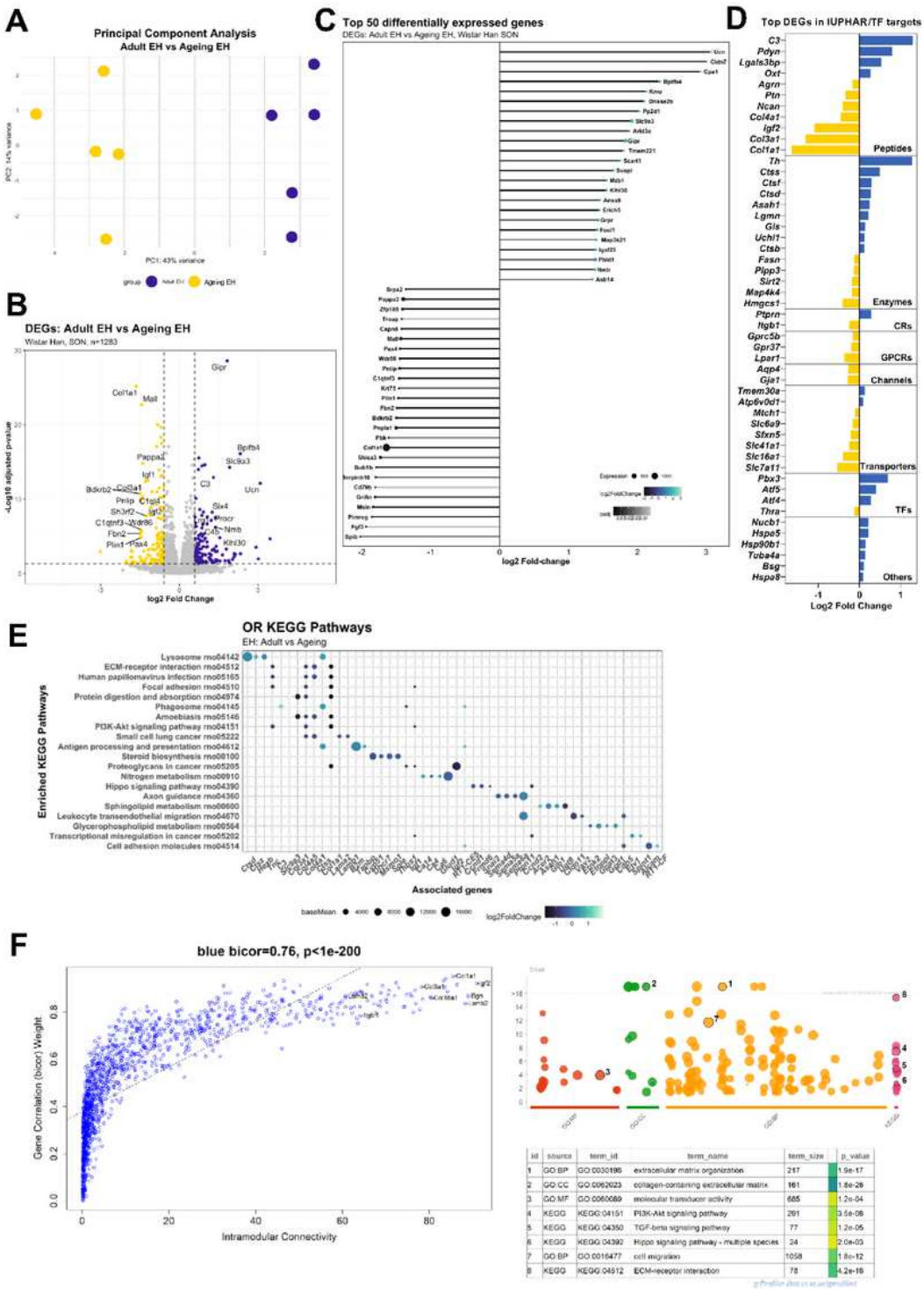


Figure 1

Transcriptome dynamics of the ageing rat SON. PCA plot illustrating distinct differences between the adult and ageing SON (A). Volcano plot showing the distribution of DEGs (B). Top 50 ageing DEGs (C). Classification of top 50 DEGs; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription

factors (D). Dot plot showing pathway analysis against the KEGG database and their associated DEGs. The top 5 most significant DEGs by Padj associated with each overrepresented KEGG terms are shown as dots on the x-axis; colour denotes the direction of changed expression (LFC), size denotes expression level. Enriched KEGG terms are plotted on the y-axis. Expression is color coded (E). WGCNA analysis showing the “blue” module and its associated enriched GO pathways (MF, BP, CC, and KEGG) (F).

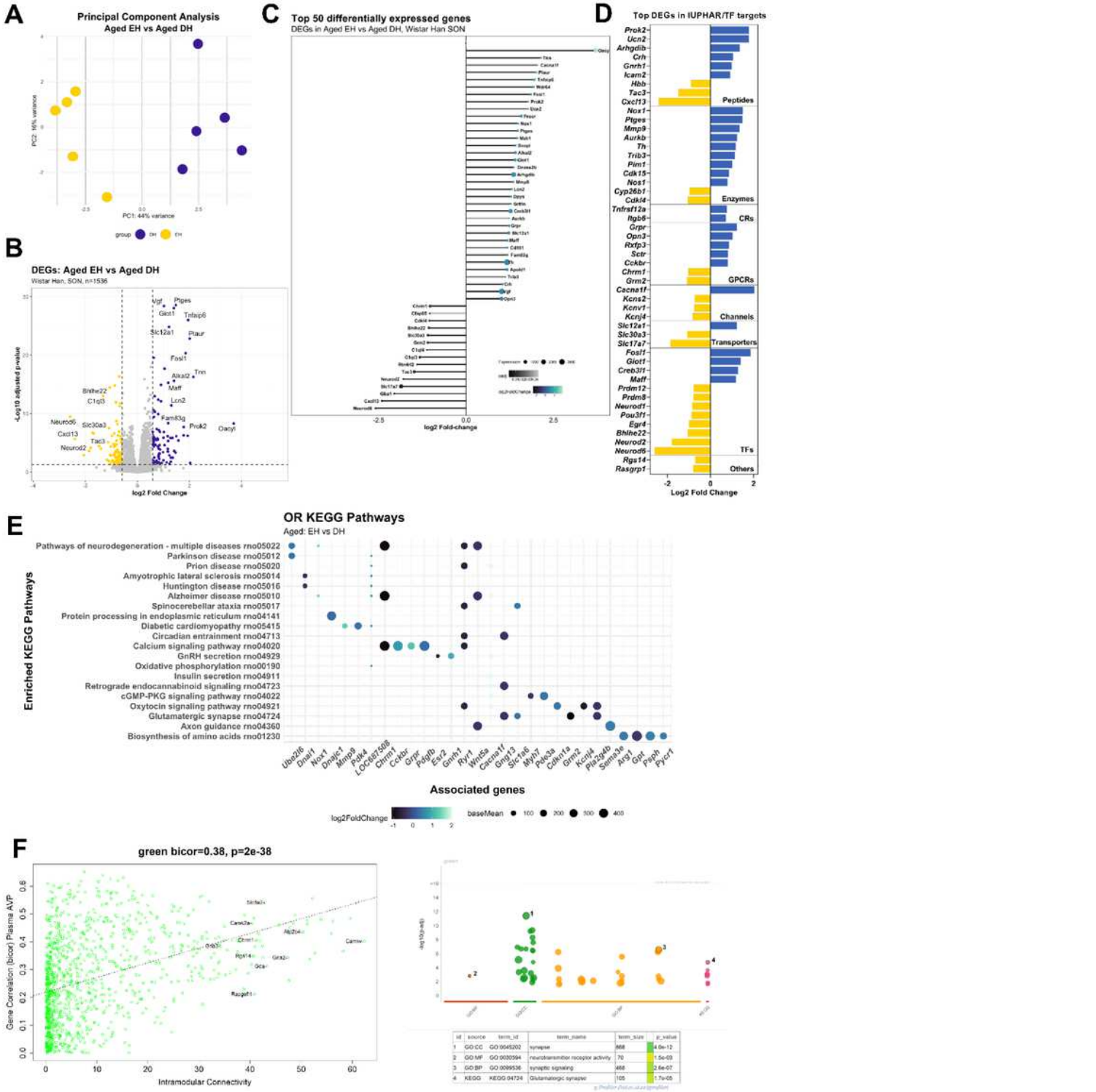
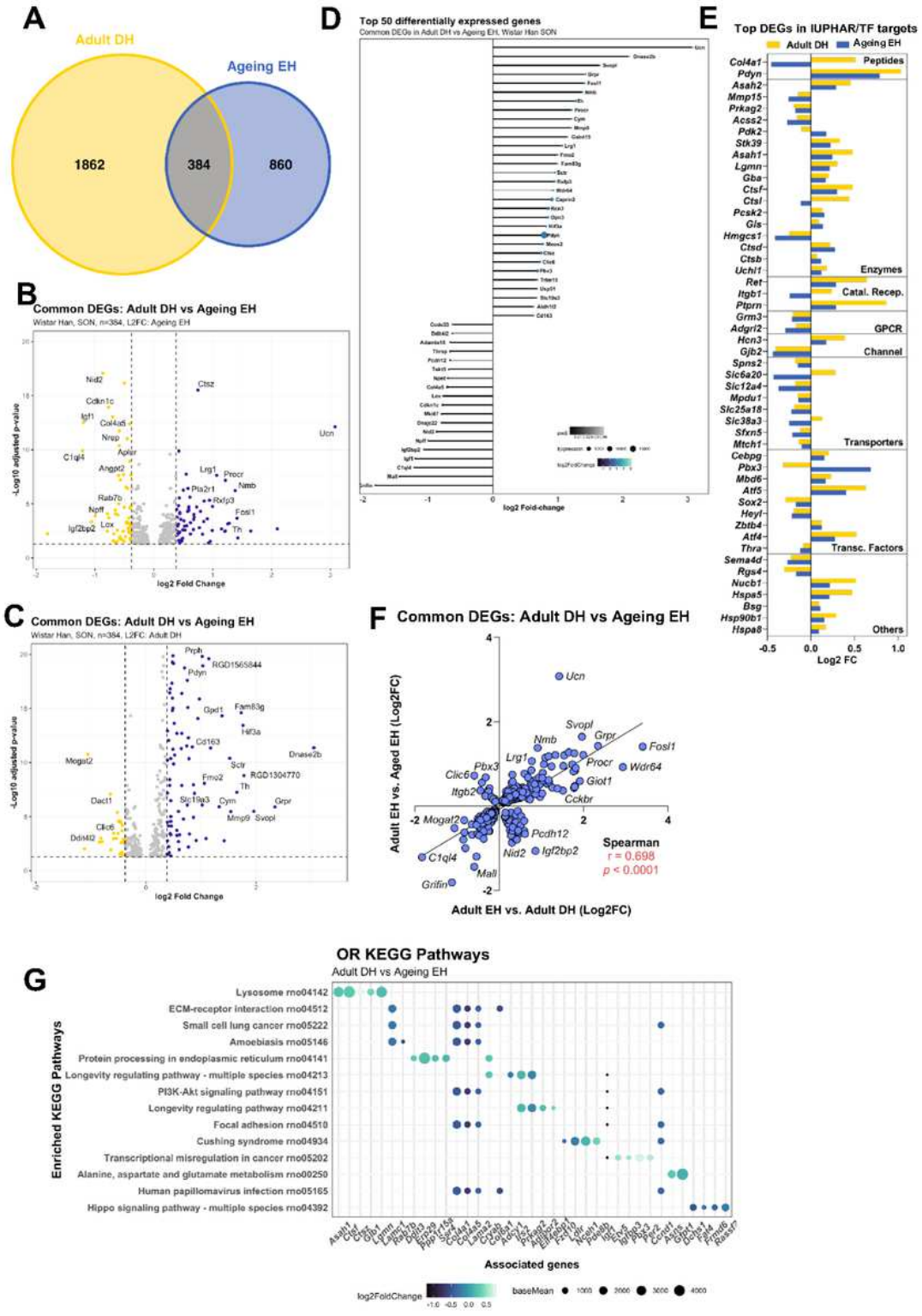


Figure 2

Transcriptome dynamics evoked by DH in the aged rat SON. PCA plot illustrating differences between the aged EH and DH groups (A). Volcano plot showing the distribution of DEGs (B). Top 50 aged DH DEGs (C). Classification of to 50 DEGs by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (D). ORA KEGG pathway analysis of the DEGs (E). WGCNA analysis showing the “green” module and its associated enriched GO pathways (MF, BP, CC, and KEGG) (F).



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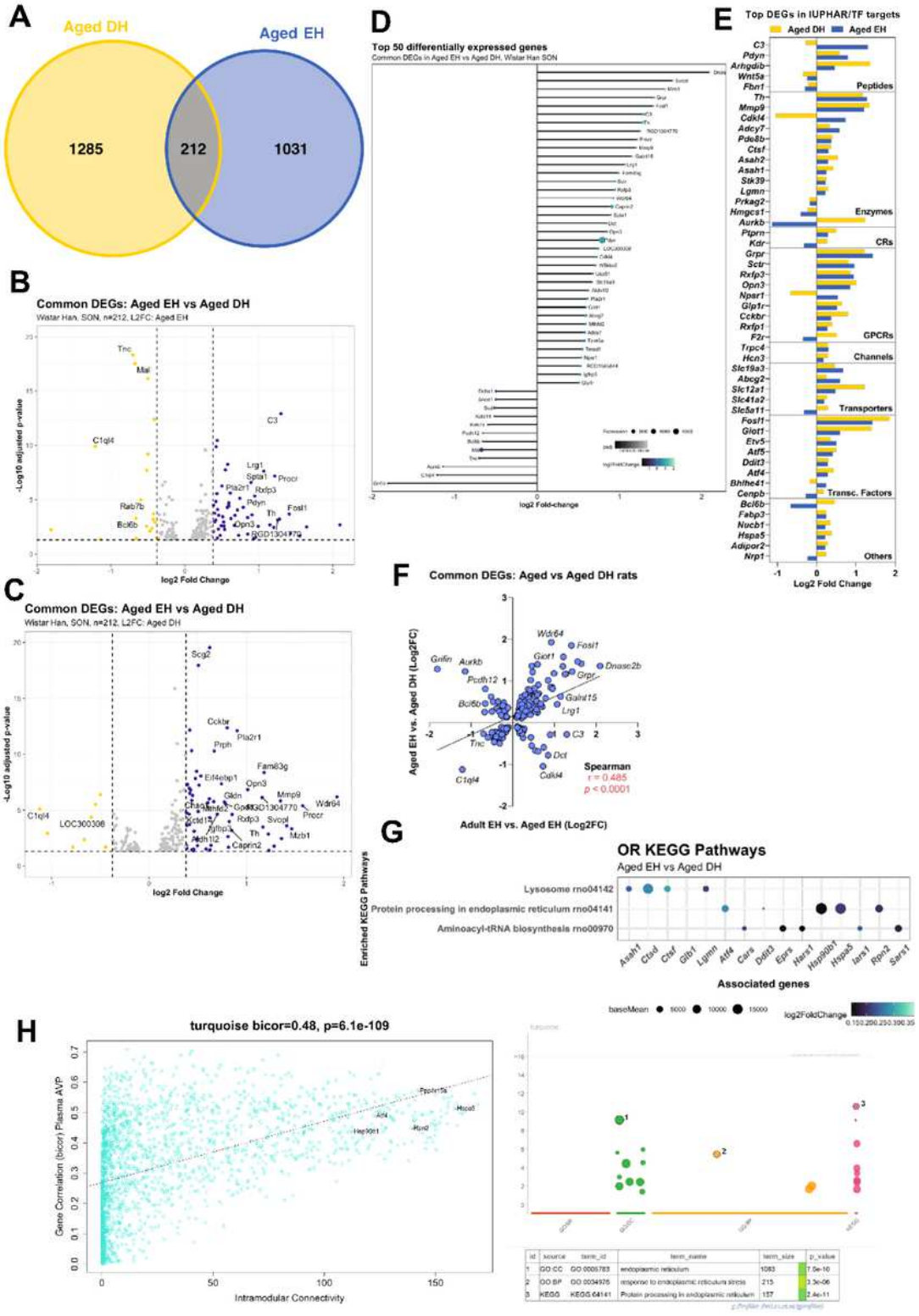

Figure 4

Comparison of aged DEGs with aged DH DEGs. Venn comparison of aged DH and ageing DEGs (A). Volcano plot showing the distribution of the DEGs using LFC of the ageing DEGs (B) or using LFC of the aged DH DEGs (C). Lollipop graph highlighting the top 50 DEGs common between the two datasets (D). Classification of the top 50 DEGS by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (E). Correlational analysis of the common DEGs (F). ORA KEGG pathway analysis of the common DEGs (F). WGCNA analysis showing the turquoise module and its associated enriched GO pathways (MF, BP, CC, and KEGG) (G).

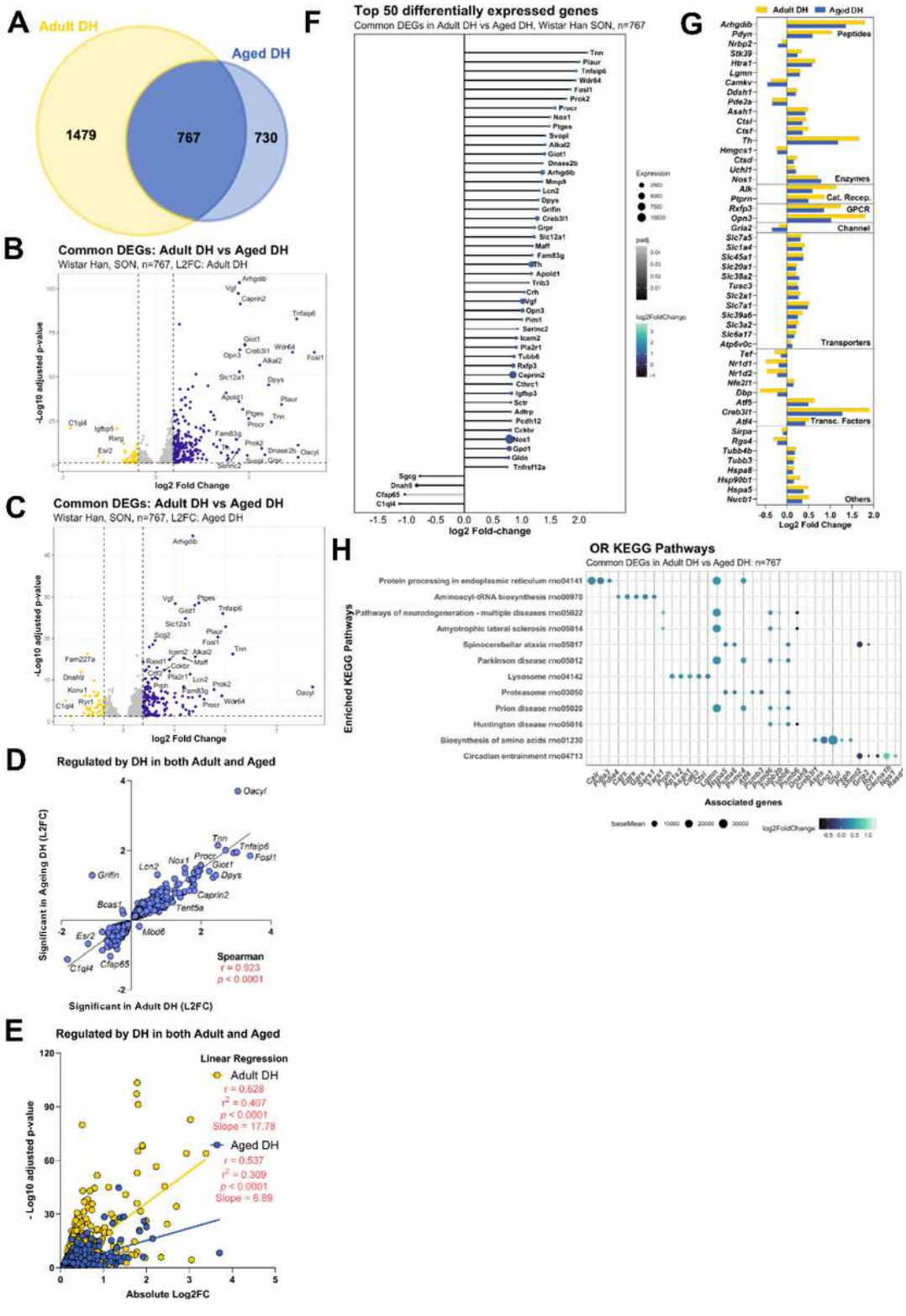

Figure 5

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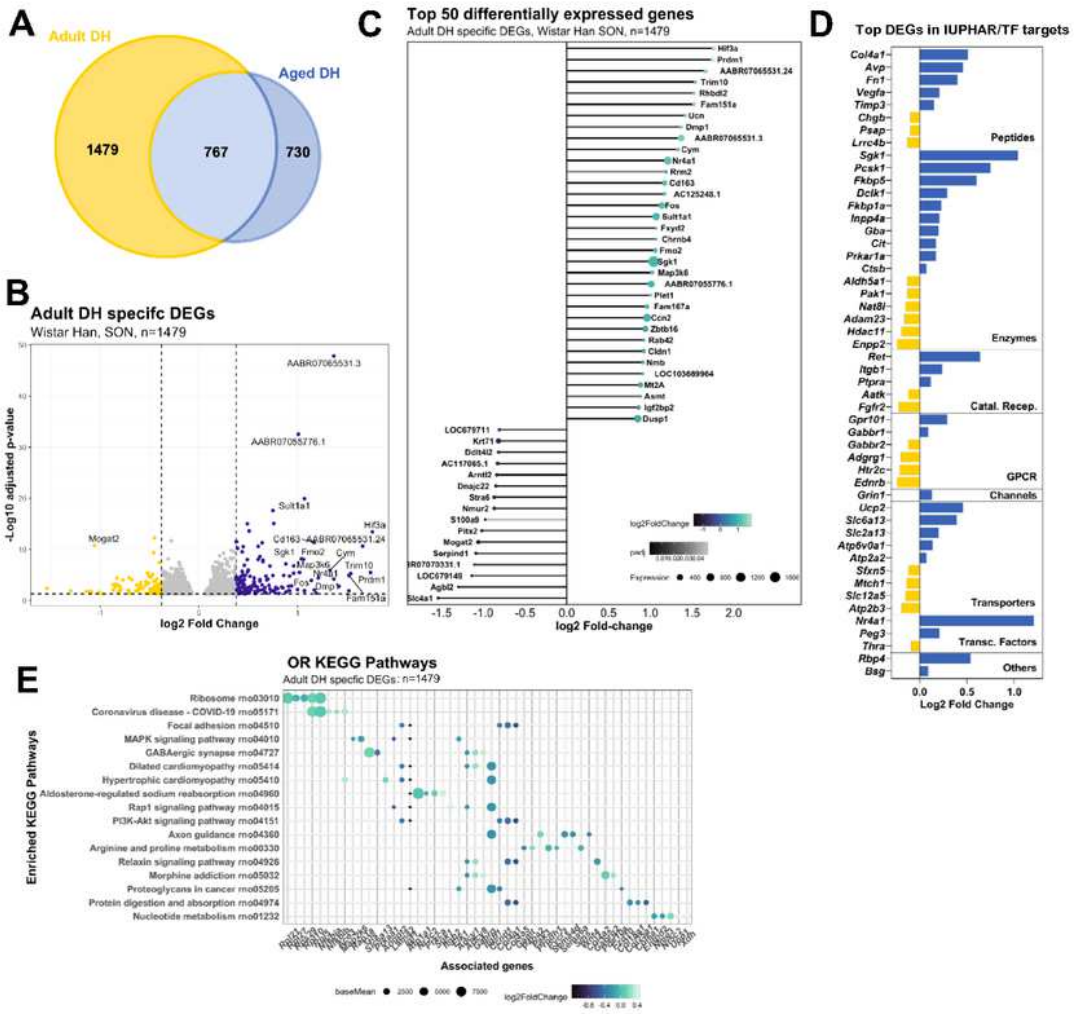

Figure 6

Comparison of adult DH DEGs with aged DH DEGs: unique adult DH DEGs. Venn comparison of adult DH and aged DH DEGs highlighting the unique adult DH DEGs (n=1479) (A). Volcano plot showing the distribution of the unique adult DH DEGs (B). Lollipop graph highlighting the top 50 unique adult DH DEGs (C). Classification of the top 50 DEGS by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (G). ORA KEGG pathway analysis of the unique adult DH DEGs (H)

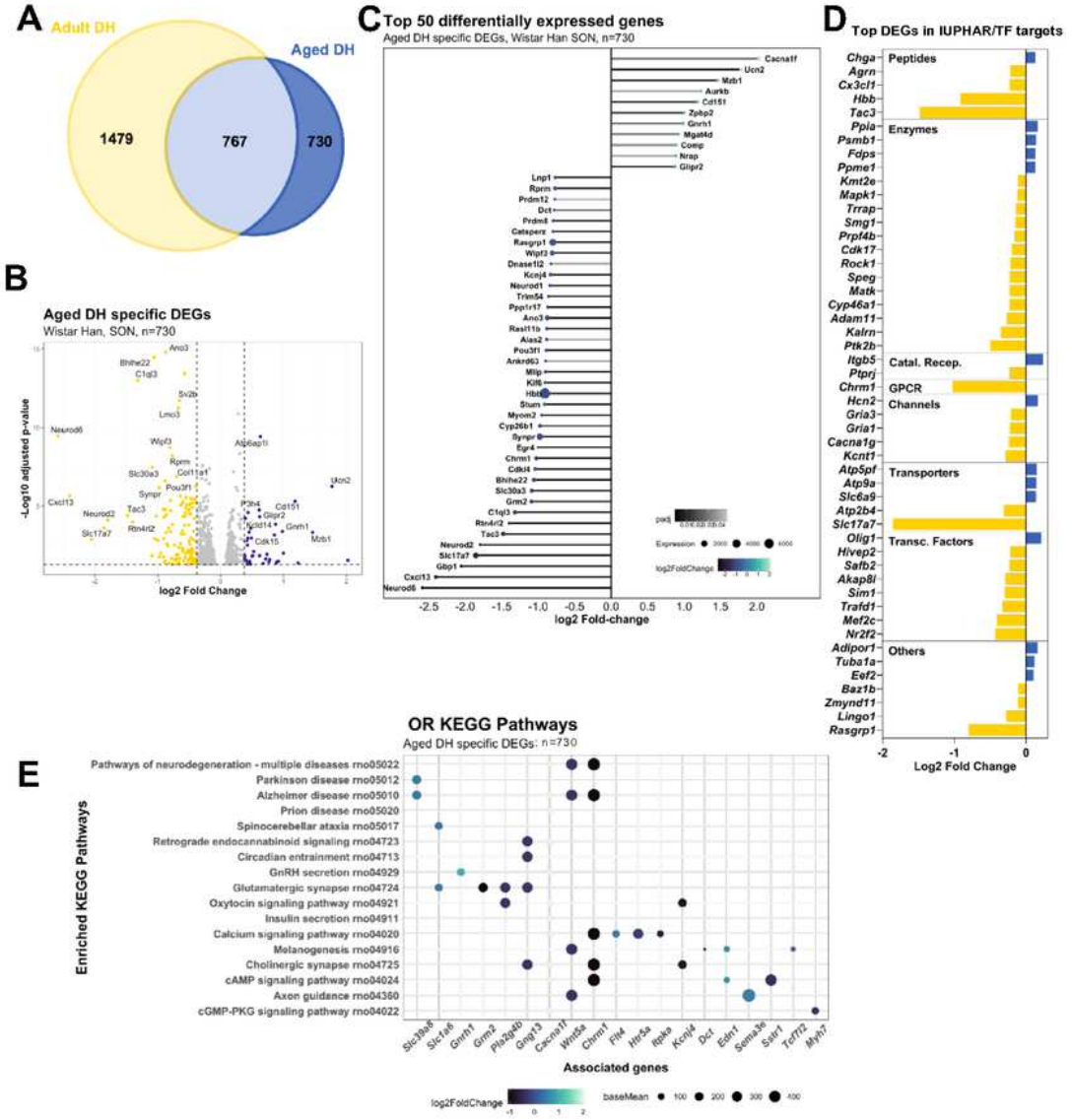

Figure 7

Comparison of adult DH DEGs with aged DH DEGs: unique aged DH DEGs. Venn comparison of adult DH and aged DH DEGs highlighting the unique aged DH DEGs (n=730) (A). Volcano plot showing the distribution of the unique aged DH DEGs (B). Lollipop graph highlighting the top 50 unique aged DH DEGs (C). Classification of the top 50 DEGS by LFC; CR: catalytic receptors, GPCRs: G protein coupled receptor, TF: transcription factors (G). ORA KEGG pathway analysis of the unique agedDH DEGs (H).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFig1.png
- SupplementarytableS18.docx
- SupplementaryTableS19.docx