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Decreased *Npas4* and *Arc* mRNA levels in the hippocampus of aged memory-impaired wild type but not memory preserved 11β-HSD1 deficient mice

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Short title: 11β-HSD1-deficiency and ageing alters *Npas4* and *Arc* mRNA

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Abstract

Mice deficient in the glucocorticoid-regenerating enzyme 11β-HSD1 resist age-related spatial memory impairment. To investigate the mechanisms/pathways involved, we used microarrays to identify differentially expressed hippocampal genes that associate with cognitive ageing and 11β-HSD1. Aged wild-type mice were separated into memory-impaired and unimpaired relative to young controls according to their performance in the Y-maze. All individual aged 11β-HSD1-deficient mice showed intact spatial memory. The majority of differentially expressed hippocampal genes were increased with ageing (e.g. immune/inflammatory response genes) with no genotype differences. However, the neuronal-specific transcription factor, *Npas4* and immediate early gene, *Arc* were reduced (relative to young) in the hippocampus of memory-impaired but not unimpaired aged wild-type or aged 11β-HSD1-deficient mice. Quantitative RT-PCR and in situ hybridization confirmed reduced *Npas4* and *Arc* mRNA expression in memory-impaired aged wild-type mice. These findings suggest that 11β-HSD1 may contribute to the decline in *Npas4* and *Arc* mRNA levels associated with memory impairment during ageing, and that decreased activity of synaptic plasticity pathways involving Npas4 and Arc may, in part, underlie the memory deficits seen in cognitively-impaired aged wild-type mice.

Introduction

Cognitive decline is a prominent feature of normal ageing in humans and rodents. However, large inter-individual differences exist ranging from little change to mild or severe impairments (1, 2). Glucocorticoids (GCs; largely cortisol in humans, corticosterone in rats and mice), released from the adrenal cortex following stress or diurnal activation of the hypothalamic-pituitary-adrenal (HPA) axis are implicated in age-related cognitive impairment. While short-term elevated GC levels are generally considered adaptive, prolonged exposure can detrimentally affect brain structure and function, particularly in the hippocampus where they decrease neurogenesis, cause dendritic atrophy and impair

memory (3, 4). Hippocampus-dependent memory impairments are associated with elevated circulating GC levels during ageing in humans and rodents (5-7).

GCs modulate episodic and working memory processes primarily via activation of 2 nuclear receptors, the high affinity mineralocorticoid receptors (MR) and low affinity glucocorticoid receptors (GR). Both are ligand-activated transcription factors and highly expressed in the hippocampus (8). Before accessing receptors, GCs may be subject to intracellular metabolism. The hippocampus highly expresses 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) which contributes to intracellular GC levels by catalysing the regeneration of cortisol and corticosterone from inert 11keto forms (cortisone, 11-dehydrocorticosterone) (9, 10). Our recent studies support a pivotal role for 11β-HSD1 generated GCs in age-related cognitive decline (11). Aged 11β-HSD1 deficient mice show preserved hippocampus dependent learning and memory throughout life, resisting age-related spatial memory deficits observed in wild-type mice as shown in both watermaze and Y-maze tasks (12, 13). Conversely, transgenic mice with forebrain specific overexpression of 11β-HSD1 show accelerated cognitive ageing (14). Short-term selective inhibition of 11β-HSD1 reverses spatial memory deficits in aged C57BL/6J mice (15, 16). Improved cognition in aged mice with 11β-HSD1 deficiency or inhibition associates with reduced intrahippocampal corticosterone (CORT) levels during learning (17), a switch from predominant activation of 'anti-cognitive' GRs to predominant 'pro-cognitive' MRs (18) and enhanced hippocampal long-term potentiation (LTP) (13). The downstream genes/pathways beyond receptor activation that underlie age-related memory deficits associated with 11β-HSD1 activity are unknown.

Acquisition of long-term memory requires gene transcription and protein synthesis (19). Gene expression microarrays have identified genes and pathways in rodent hippocampus that associate with cognitive ageing (20-23). These include down-regulated immediate early gene (e.g. *Arc, Egr-1, Vgf*) and insulin signaling pathways (e.g. *Insr, Ide, Stat5b*) and up-regulated general oxidoreductase activity genes (e.g. *Acads, Aldh9a1*) selectively in aged cognitively impaired animals (22). To dissect the pathways underlying cognitive protection

with 11β-HSD1 deficiency, the Y-maze was used to define cognitive function in aged wild-type and 11β-HSD1-deficient mice (relative to young controls) prior to microarray analysis of hippocampal gene transcript profiles. Aged wild-type mice were further separated into memory impaired and unimpaired groups with the aim of identifying differentially expressed hippocampal genes that associate with cognitive ageing.

Materials and Methods

Animals

Male mice homozygous for targeted disruption of the *Hsd11b1* gene (*Hsd11b1*$^{-/-}$), congenic on the C57BL/6J genetic background (24, 25) and age-matched C57BL/6J mice as wild-type (*Hsd11b1*$^{+/+}$) controls were bred and maintained within our biomedical research facility housed under standard conditions on a 12 h light/dark cycle (lights on at 07:00 h), with food and water ad libitum until behavioural testing in the Y-maze at either 6 m (young) or 24 m (aged). All animal procedures were performed according to local ethical guidelines of the University of Edinburgh Ethics Committee and those of the U.K. Animals (Scientific Procedures) act, 1986.

Y-maze

Young (6 months) and aged (24 months) wild-type and *Hsd11b1*$^{-/-}$ mice were tested in a two trial Y-maze task previously validated as hippocampus dependent (18, 26) for assessment of their spatial memory. All behavioural testing was carried out in the morning (between 08.00 - 11.00am) in a dimly lit room. Each mouse was placed at the end of one of the three arms of the maze designated the “start arm” and allowed to explore the maze with one arm blocked (novel arm) for 5 min (trial 1) before returning to their home cage. Fixed spatial cues (various objects such as glass bottle, pipette rack, plastic breaker etc) surrounded the maze. After an inter-trial interval (ITI) (either 1 min or 2h) the mouse was placed back in the maze start arm and allowed to explore all three arms (trial 2) for 5 min (18). The maze was wiped clean with 70% ethanol in between animals to remove olfactory cues. All mice (young, n=9/genotype;

aged WT, n=13; aged *Hsd11b1*$^{-/-}$ mice, n=8) were tested first with the 1 min ITI to ensure they responded to novelty, had no motor deficits and could see the spatial cues around the maze. Spatial memory was tested with the 2h ITI one week later. The time spent in each of the arms was calculated with the ANY-maze software (Stoelting, Dublin, Ireland). Aged mice failing to spend significantly more time in the novel arm compared to the previously visited arms were classed as cognitively impaired while mice showing a preference for the novel arm similar to young controls were cognitively unimpaired. Aged cognitively impaired (AI) and unimpaired (AU) wild-type mice were randomly selected from the groups for the microarray study (n=4/group).

Animals were culled by cervical dislocation between ~8 and 10 AM three days following the end of Y-maze testing. Brains were removed, dissected and the hippocampus was snap frozen in RNase free eppendorf tubes on dry ice and stored at -80°C. For in situ hybridization studies, brains were frozen on powdered dry ice and stored at -80°C.

RNA extraction and Affymetrix GeneChip processing

Total RNA was extracted from hippocampal tissues of young wild-type (WT_Y), young *Hsd11b1*$^{-/-}$(KO_Y), aged unimpaired wild-type (WT_AU), aged impaired wild-type (WT_AI) and aged *Hsd11b1*$^{-/-}$ (KO_A) mice using TRIzol reagent (Invitrogen, Paisley, UK) and RNeasy Mini Kit (QIAGEN, West Sussex, UK). Concentration and purity of each RNA sample was assessed using a GeneQuant RNA/DNA calculator (GE healthcare, Amersham, UK). Hippocampal RNA samples were processed through standard Affymetrix protocols, and hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips (n=4 per group, Affymetrix, Santa Clara). CEL files for all 20 chips were imported into the Affy package of BioConductor, and were processed (background subtraction and normalization) with the Robust Multichip Average (RMA) algorithm. Chip data quality control was performed by (i) visual inspection of the chip images (not shown) which showed no obvious abnormalities and (ii) histogram of raw intensities from all chips which showed no clear outliers and had the usual distribution. Quality control indicated that the data were technically good. Expression levels for each chip

and fold changes between genotype for each tissue were calculated. Genes with no or very low expression (i.e. expressed below a normalized expression value of 100 in all, or all but one, of the samples) were excluded. Data were exported in text format and imported into a MySQL database. Annotation data for the genes were obtained from NetAffx. A web accessible front-end query tool was built that allows query of the data by expression data (normalised expression, fold-changes, p-values) and by sequence annotation (gene title and symbol, Entrez gene ID, Affymetrix ID, and Gene Ontology data). Microarray data are available in the Gene Expression Omnibus (GEO) with accession number GSE68515. Microarray processing was carried out by ARK-Genomics (Roslin Institute, Edinburgh UK).

Real-Time Quantitative RT-PCR

Total RNA (1.5 µg) from hippocampal samples from the experimental groups (WT_Y, KO_Y, WT_AU, WT_AI and KO_A) (n=5-8/group including overlap with animals used in microarray) was reverse transcribed into cDNA with oligo(dT) primers using the QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer's instructions. Gene specific mRNA levels were determined in cDNA samples incubated in triplicate with gene specific primers and fluorescent probes (using pre-designed assays from Applied Biosytems, Warrington, UK) in 1 x Roche LightCyclerR 480 Probes mastermix. PCR cycling and detection of fluorescent signal was carried out using a Roche LightCyclerR 480 (Roche Applied Science, Burgess Hill, U.K.). A standard curve was constructed for each primer probe set using a serial dilution of cDNA pooled from all samples. The primers (Invitrogen) were designed for use with intron-spanning probes from the Roche Universal Probe Library. LightcyclerR 480 Software was used to analyse the data produced after RT-PCR. All results were corrected by normalization to the expression level of the reference (housekeeping) gene *Gapdh,* which did not differ between groups and expressed arbitrarily as an adjusted ratio.

In situ hybridization

PCR products corresponding to nucleotides 1859-2349 of the mouse *Arc* cDNA and 1055-1607 of the mouse *Npas4* cDNA were generated from control C57BL/6J mouse hippocampus cDNA and subcloned into the pGEM-T Easy vector (Promega). ^{35}S-UTP (Perkin Elmer Life) labeled sense and antisense cRNA probes were generated using restriction enzyme-linearized plasmid as template for *in vitro* transcription using either T7 or SP6 RNA polymerase as appropriate. Cryostat coronal brain sections at the level of the dorsal hippocampus from young and aged WT_AI (WT_AU were omitted due to small sample size) and *Hsd11b1*$^{-/-}$ (KO) mice (n=6-9/group, including overlap with animals used in microarray) were post-fixed in 4% paraformaldehyde, acetylated (0.25% acetic anhydride in 0.1M triethanolamine, pH 8.0), washed in phosphate-buffered saline, dehydrated in graded ethanol and air-dried. Sections were hybridized with probe overnight at 50°C, followed by RNaseA treatment and standard saline citrate (SSC) buffer washes. Slides were dehydrated in graded ethanol and air-dried before exposure to Biomax MR-1 film (Amersham) for 5-8 days at RT. The slides were then dipped in NTB-2 emulsion (Eastman Kodak Co, Rochester, NY) and exposed for 3 weeks at 4°C before developing and counterstaining with 1% pyronine. Hybridization signal was assessed by computer-assisted grain counting using Zeiss KS300 image analysis software. Silver grains were counted within a fixed circular area under bright-field illumination using the X40 magnification objective, over individual cells by an investigator blinded to age and genotype. For each animal, 15-18 cells/subregion of hippocampus or cortical layer were assessed and background, counted over areas of white matter was subtracted. The labeled RNA probes (antisense and sense) were first hybridized onto control brain sections from adult mice to test their specificity at the autoradiographic level. No binding was detected with labeled sense probes of Npas4 and Arc (data not shown).

Data Analysis

Statistical analysis of the microarray gene expression data was carried with the Limma R/Bioconductor software package (27) yielding multiple testing corrected p-values for each comparison of interest. A non-parametric statistical test (Rank Products, RP) was also used. The RP approach has been shown to be reliable for identifying biologically relevant expression differences even with highly noisy data (28, 29). Genes were considered differentially expressed between groups when rank product P values were less than 0.05. The Y-maze (2h ITI time spent in novel arm) and *in situ* gene expression data across the groups were analyzed using two-way analysis of variance (ANOVA) with age and genotype as the independent variables followed by Tukey's multiple comparison's test as appropriate for between group comparisons. Comparisons of time spent in the arms of the Y-maze within each group were analyzed by Student's paired t-tests. The quantitative RT-PCR data with the aged impaired and unimpaired wild type mice as separate groups were analyzed by one-way analysis of variance with Tukey's multiple comparison's test as appropriate for between group comparisons. Data are shown as means ± SEM.

Results

Spatial memory status of aged wild-type and 11β-HSD1-deficient mice in the Y-maze

All young and aged wild-type and *Hsd11b1*$^{-/-}$ mice spent significantly more time in the novel arm than previously visited arms of the Y-maze ($P<0.01$, paired t-tests) following a 1 min ITI (Fig. S1) confirming the aged mice had no impairment of vision or motor deficits. There were little inter-individual differences within the aged mice groups (both wild-type and *Hsd11b1*$^{-/-}$) following the 1 min ITI with all mice performing equally well regardless of their later cognitive status following the 2h ITI. Analysis of the 2h ITI Y-maze data, as a measure of spatial memory, revealed a significant effect of genotype ($F_{1,33}$ =8.4, $P< 0.01$) and genotype x age interaction ($F_{1,33}$= 5.7, $P<0.05$) (Fig. 1A). Aged (24 months) wild-type mice spent less time in the novel arm during the retention trial after a 2h ITI compared to young (6 months) wild-type

controls (P<0.05) and to aged *Hsd11b1*$^{-/-}$ mice (P<0.01) (Fig. 1A), recapitulating previous findings (13, 18). Aged wild-type mice (n=13) showed an overall impairment in spatial memory (Fig. 1A), but examination of individual animals revealed 4 unimpaired mice with intact spatial memory similar to young mice i.e. spending significantly more time in the novel arm (P<0.05) than previously visited arms of Y-maze (Fig. 1B). Aged memory-unimpaired wild-type (WT_AU) mice spent significantly (P<0.001) greater % time in the novel arm than aged memory-impaired wild-type mice (WT_AI) (Fig.1B). Mice from each age and genotype group were used for microarray analysis of hippocampal gene expression (Fig.1B).

Differentially expressed hippocampal genes associated with 11β-HSD1 deficiency, memory impairment and ageing

Data from aged wild-type mice were examined as two subgroups [aged memory-impaired (WT_AI, n=4) and aged memory unimpaired (WT_AU, n=4)] and also as a combined group (WT_A, n=8). These were compared with aged *Hsd11b1*$^{-/-}$ mice, which showed no cognitive impairment. Most differentially expressed hippocampal transcripts were increased with age, but did not differ between genotypes (~1.5 fold increased, <0.05 RP score compared to corresponding young controls). These include inflammatory/immune response genes (*C1qb, C1qbc, B2m, Aif1, Fcgr2b, Fcgr3, Trem2, Lyz2, Mpeg1*), glial/structural genes (*Gfap, Vim, Dmp1*), cholesterol/lipid metabolism (*Apod*), signal transduction/transport (*Anxa3, Cyba, Abca8a*), protein binding (*Rtp4, Tyrobp*) (Table S1). Some genes were increased with age (~1.5 fold, P<0.05 RP score) in wild-type mice regardless of cognitive status (WT_A) but not in *Hsd11b1*$^{-/-}$ mice including the rate-limiting retinoic acid-synthesizing enzyme (*Aldh1a2*) and the GABA transporter 2 (*Slc6a13*) (Fig. 2). Only three genes were differentially expressed significantly between WT_AI and WT_AU (Fig. 2 and Fig. S2) and between WT_AI and KO_A (Fig. 2 and Fig. S3). Of these, the transcription factor *Npas4* and immediate early gene *Arc*, are of particular interest because of their link with learning and memory (30, 31). *Npas4* and *Arc* mRNA levels were selectively decreased with age in the hippocampus of WT_AI (P<0.01 and P<0.05, RP score respectively) but not WT_AU or

KO_A mice (Fig. 2 and Fig. 3). *Npas4* mRNA levels were also lower in KO_Y compared to WT_Y ($P<0.05$, Fig. 2). Interestingly, the level of *Agxt2l1* (alanine-glyoxylate aminotransferase 2-like 1), a gene whose function is poorly characterized but a dysregulation in prefrontal cortex has been associated with mood disorders (32), differed significantly between AU and AI WT mice ($P<0.01$, RP score) and was increased with age in the hippocampus of both WT-AU and KO_A ($P<0.001$, RP score) but not WT_AI mice (Fig. 2 and Fig. 3).

Decreased Npas4 and Arc but increased Hsd11b1 mRNA levels in the hippocampus of aged memory-impaired wild type mice

Differential expression of *Npas4* and *Arc* mRNA levels was validated by quantitative real-time PCR (qPCR) using total RNA from the hippocampus of WT_AI, WT_AU, KO_A, and corresponding young mice of each genotype (n=5-8/group). The microarray changes in *Agxt2l1* mRNA levels were, however, not fully validated by qPCR with no significant increase in WT_AU compared to WT_AI ($P=0.11$) or compared to WT_Y ($P=0.47$) (Fig. 3). Although the microarray data analysis did not reveal differences in *Hsd11b1* mRNA levels between young and aged wild-type mice, qPCR showed significantly higher levels of *Hsd11b1* mRNA in the WT_AI group compared to WT_Y controls ($P<0.001$) (Fig. 3) confirming our previous findings (14). Both hippocampal *Npas4* and *Arc* mRNA levels differed significantly between the groups ($F_{4,27}=5.1$, $P<0.01$, and $F_{4,26}=5.9$, $P<0.01$, respectively, Fig. 3). Hippocampal *Npas4* and *Arc* mRNA levels were decreased selectively in WT_AI but not WT_AU mice (WT_Y vs WT_AI, $P<0.05$ and $P<0.01$, respectively Fig. 3). *Npas4* mRNA levels were lower in the hippocampus of WT_AI compared to WT_AU mice ($P<0.01$, Fig. 3). In contrast, both hippocampal *Npas4* and *Arc* mRNA levels were not significantly altered with age in *Hsd11b1*$^{-/-}$ mice (Fig. 3). The lower Npas4 mRNA levels in KO_Y vs WT_Y from the microarray data (n=4/genotype) was however not evident by qPCR in the larger sample size (n=8/genotype) (Fig. 3).

Decreased Npas4 and Arc mRNA expression selectively in hippocampal CA1 cells of aged memory-impaired wild type but not 11β-HSD1-deficient mice.

We performed *in situ* hybridization to gain a regional resolution of the reduced hippocampal *Npas4* and *Arc* mRNA expression in aged memory-impaired wild-type mice. In young WT mice, *Npas4* mRNA expression was greatest in the cortical region (layers 2/3 and 5) and hippocampus, particularly in the CA1 and CA3 cell layers (Fig. 4A). Within the CA1 subregion of the hippocampus, *Npas4* mRNA levels were decreased with age (~40% reduction, $F_{1,26}$=11.8, P<0.01) in WT_AI but not KO_A mice (Fig. 4B). Levels of *Npas4* mRNA in the CA1 region showed a non-significant trend to be lower in WT_AI mice compared to KO_A mice ($F_{1,26}$ =3.2, P=0.08) (Fig. 4B). CA3 *Npas4* mRNA levels were decreased with age regardless of genotype (~40% reduction, $F_{1,24}$ =19, P<0.001, Fig. 4B). In the cortex, levels of *Npas4* mRNA were affected by age but not genotype (cortical layer 2/3, $F_{1,25}$= 12.9, P<0.01; cortical layer 5, $F_{1,25}$= 6.8, P<0.05, Fig. 4B); post hoc analysis showed a decrease with age in WT_AI (~37% decrease, P<0.05) but not KO_A mice (Fig. 4B).

Levels of *Arc* mRNA were highest in the CA1 cell layer of the hippocampus and layers 2/3 and 5 of the cortex (Fig. 5A). In the hippocampus, there was an age ($F_{1,27}$=14.8, P<0.001) and age x genotype interaction ($F_{1,27}$=5.0, P<0.05) selectively in CA1 (Fig. 5B). *Arc* mRNA levels were decreased with age in hippocampal CA1 cells of WT_AI mice (~ 50% reduction, P<0.001) and CA3 (~33% reduction, P<0.05) but not KO_A mice (Fig. 5B). In the cortex, *Arc* mRNA levels were decreased with age but not genotype (cortical layer 2/3, $F_{1,30}$= 32, P<0.001; cortical layer 5, $F_{1,30}$= 40, P<0.001), Fig. 5B. Post hoc analysis revealed a decrease with age in both WT_AI (~52-57% decrease, P<0.001) and KO_A mice (~41-46% decrease, P<0.05)(Fig. 5B).

Discussion

Lifelong deficiency or short-term inhibition of 11β-HSD1 consistently preserves or improves spatial memory in aged mice (12, 13, 15, 18). Here we identified two hippocampal genes, the brain-specific activity-dependent transcription factor *Npas4* (neuronal Per-Arnt-Sim domain protein 4) and the immediate early gene *Arc* (activity-regulated cytoskeleton-associated protein) as differentially expressed with ageing, cognitive decline and 11β-HSD1 deficiency. Given the crucial roles of Npas4 and Arc in the regulation of learning and memory (31, 33-37), their decreased mRNA levels in the hippocampus of aged memory-impaired (AI) but not unimpaired (AU) wild-type or 11β-HSD1-deficent mice, suggests that these proteins may lie in pathways that are important for preservation of hippocampus-associated memory in ageing and which are maintained by 11β-HSD1 deficiency/inhibition.

Several studies have used microarrays to identify hippocampal gene transcripts associated with cognitive ageing under basal (home cage) and memory-activated (1h after watermaze training) conditions in rats (22, 23, 38-40) and mice (41, 42). The number of hippocampal genes identified as differentially expressed between aged cognitively impaired and unimpaired animals vary with some studies revealing more genes altered than others. Importantly, several of the transcripts elevated with ageing regardless of genotype or cognitive status in our study were also identified as genes regulated by ageing and not memory decline in cognitively tested aged rats, including inflammatory/immunity genes (*C1qc, C1qb, B2m, Aif1, Fcgr2b*), structural (*Gfap, Vim*), cholesterol/lipid metabolism (*Apod*) and signal transduction (*Anxa3*) (21, 22, 43). This affords some confidence that these reflect ageing *per se* rather than the processes underlying cognitive variation with age.

In contrast to previous studies in rats that examined memory-activated hippocampal gene expression profiles (22, 23), only three notable hippocampal genes were differentially expressed between the AI and AU wild-type mice as characterized in the Y-maze spatial recognition memory task. This low number of differentially expressed hippocampal genes suggests that the AI and AU characterization based on a single “non-aversive” Y-maze trial

may not be as reliable as previous methods which use multiple “aversive” watermaze trials as shown in aged mice (42) and rats (6, 44, 45). The AU wild-type mice would benefit with further characterization in the watermaze to demonstrate a consistent phenotype of preserved memory function. Thus the implications of the altered gene transcript levels in AU wild-type mice are less clear and could simply reflect within-subject variability rather than a consistent distinct subject cognitive phenotype. In contrast, the impaired and preserved cognitive phenotype of the key comparisons between AI wild-type and aged 11β-HSD1-deficient mice, respectively, have been reliably confirmed in previous studies following both Y-maze and watermaze protocols (12, 13, 18). Among the differentially expressed genes, *Arc* transcript levels were reduced selectively in AI wild-type mice, a finding consistent with the correlation of hippocampal *Arc* mRNA levels with spatial memory (46) and previous studies in aged rats with memory deficits (21, 22, 47, 48). Acutely, both a memory-enhancing dose of CORT and learning have been shown to increase *Arc* mRNA and protein expression (43, 46, 49) indicating a plausible pathway linking stress and its GC mediators through life, cognition and individual differences in cognitive decline with age. Moreover, hippocampal *Arc* expression was reduced in CA1 and CA3 but not dentate gyrus of AI wild-type mice in line with sub-regional changes in basal levels of *Arc* mRNA in aged rats (47). Decreased *Arc* mRNA levels have been reported during both “off line” periods of rest and following spatial behavior in the aged hippocampus (47). Indeed, *Arc* mRNA levels under resting home cage conditions is thought to reflect active information processing in cells that previously transcribed *Arc* in response to behaviour (50). Thus, reduced levels of *Arc* mRNA in CA1 and CA3 pyramidal cells of AI wild-type mice may reflect impaired memory consolidation, as in $Arc^{-/-}$ mice (51), during the rest (home cage) period. Importantly, aged 11β-HSD1-deficient mice and AU wild-type mice do not show reduced *Arc* mRNA levels in CA1 and CA3 cells and are not impaired in the Y-maze.

Npas4 mRNA levels were also decreased in the hippocampal CA1 region of AI wild-type but not aged 11β-HSD1-deficient mice. Reduced levels of *Npas4* mRNA in hippocampus have

previously been noted in aged rats (52) but this has not been specifically associated with the subset of animals showing cognitive decline. However, recent evidence indicates a key role for Npas4 in memory formation (33, 34). Moreover, Npas4 influences the survival of neurons, development and maintenance of synapses, and regulation of synaptic plasticity (30) via downstream target genes, including brain derived neurotrophic factor (BDNF)(53). Indeed, in a separate study we found hippocampal levels of *Bdnf* mRNA to be reduced in aged wild-type but not aged 11β-HSD1-deficient mice (Yau et al, unpublished data). This is consistent with the lower *Npas4* mRNA levels in AI wild-type mice, decreased mRNA levels of both *Bdnf* and *Npas4* in the hippocampus of aged rats (52), and reduced transcription of multiple *Bdnf* isoforms in *Npas4*$^{-/-}$ mice (54).

Putative negative glucocorticoid response elements (GREs) found upstream of the *Npas4* transcription initiation site suggest regulation by CORT (55). Indeed, *in vivo* treatment with high CORT doses reduce *Npas4* mRNA and protein expression in mouse hippocampus and frontal cortex (55, 56). This suggests the maintained *NPas4* mRNA levels in AU wild-type mice and aged 11β-HSD1-deficient mice may, at least in part, be due to lower brain intracellular CORT levels as a consequence of decreased 11β-HSD1 activity (17). In support of this notion, hippocampal *Hsd11b1* mRNA levels were selectively increased in AI but not AU wild-type mice compared to young wild-type mice.

It may be speculated that the greater rise in hippocampal CORT levels induced by learning/training in aged wild-type mice (17), activates GRs which in turn reduces *Npas4* transcription directly by binding to negative GREs in its promoter (55). A decrease in *Npas4* mRNA expression may contribute to reduced transcription of its target gene, *Bdnf* (52) which regulates neuroplasticity and memory mechanisms (30, 52). Lower *Npas4* expression may also affect the expression of *Arc* indirectly. Indeed, memory-activated expression of *Npas4 mRNA* in the dorsal hippocampus of mice appears upstream of several other immediate early genes including *Arc* (33). Moreover, conditional deletion of *Npas4* in cultured mouse hippocampal neurons abolished the depolarization-induced expression of *Arc* mRNA (33).

Thus, a reduced *Npas4* expression could contribute to lower levels of *Arc* transcripts in the hippocampus of AI wild-type mice.

These findings implicate both Npas4 and Arc in the pathways that may underlie the impairment and maintenance of spatial memory associated with ageing and 11β-HSD1 deficiency. However, any causal relationship between 11β-HSD1 deficiency, resistance to ageing-associated decline of *Npas4* and *Arc* mRNA levels, and ageing-associated spatial memory deficits remains to be determined. If 11β-HSD1 deficiency causes resistance to age-related decline of hippocampal *Npas4* and/or *Arc* mRNA levels then short-term selective inhibition of 11β-HSD1 in aged C57BL/6J mice, which reverses spatial memory impairments (16), would be anticipated to associate with increased *Npas4* and/or *Arc* mRNA levels. Future studies could examine this in aged mice during 11β-HSD1 inhibitor treatment when spatial memory is improved and after stopping treatment when memory reverts back to impaired to test if *Npas4* and/or *Arc* mRNA levels are increased and decreased, respectively. Furthermore, intra-hippocampal administration of high CORT levels to aged 11β-HSD1 deficient mice (to levels equivalent to those found in aged wild type mice), could be carried out to establish whether it is the lower hippocampal CORT levels as a consequence of 11β-HSD1 deficiency (17) that prevents the decreased *Npas4* and *Arc* mRNA levels and/or memory deficits. It is likely that there are other hippocampal synaptic plasticity genes modulated by 11β-HSD1 activity that play a role in the variable cognitive decline with ageing. Examination of learning-activated gene transcripts and proteins in the hippocampus and functional characterization of selected genes *in vivo* could help gain further insight into the mechanisms whereby 11β-HSD1 activity contributes to age-related memory decline.

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The authors of the manuscript have no conflicts of interest to declare.

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Figure legends

Figure 1. Spatial memory status of aged wild-type and 11β-HSD1-deficient mice in the Y-maze (A) Aged (24 months) wild-type (WT) mice as a group (n=13) showed impaired spatial memory retention in the Y-maze after a 2h inter-trial interval (ITI) (less time in novel arm) compared to young (6 months) WT controls (n=9) and aged (24 months) *Hsd11b1*$^{-/-}$ (KO) mice (n=8). (B) Spatial memory of mice selected for microarray analysis from (A) showing the 5 groups used: WT_Y (young wild-type), KO_Y (young *Hsd11b1*$^{-/-}$), KO_A (aged *Hsd11b1*$^{-/-}$), WT_AI (aged wild-type memory-impaired) and WT_AU (aged wild-type memory-unimpaired) (n=4/group). *P<0.05, **P<0.01 significant difference between comparisons. Data shown are mean ± SEM.

Figure 2. Microarray mean chip intensity of a selection of differentially expressed hippocampal genes in wild-type and 11β-HSD1-deficient mice. (A) Increased with age in WT but not in KO. (B) Decreased with age in WT_AI but not in KO. (C) Increase with age in WT_AU and in KO but not in WT_AI. Comparisons between young and aged wild-type unimpaired and impaired mice [(WT_Y) and (WT_AU) or (WT_AI), n=4/group] and between aged wild-type and *Hsd11b1*$^{-/-}$ mice [WT_AI and KO_A, n=4/group] differed by ~1.5 fold (*P<0.05, **P<0.01 RP score). § P<0.05 compared to WT_Y. Data shown are mean ± SEM.

Figure 3. Quantitative real time PCR measurement of *Hsd11b1*, *Agxt2l1*, *Npas4* and *Arc* mRNA levels in the hippocampus of wild-type and 11β-HSD1-deficient mice. Levels of *Hsd11b1, Agxt2l1, Npas4* and *Arc* mRNA in the hippocampus of young wild-type (WT_Y), young *Hsd11b1*$^{-/-}$ (KO_Y), aged wild-type impaired (WT_AI), aged wild-type unimpaired (WT_AU) and aged *Hsd11b1*$^{-/-}$ mice (KO_A) were measured relative to *Gapdh* and expressed as a ratio (n=5-8/group). *P<0.05, **P<0.01, ***P<0.001 significance difference between groups. Data shown are mean ± SEM.

Figure 4. Differentially expressed *Npas4* mRNA in hippocampus and cortex of aged wild-type and 11β-HSD1-deficient mice. (A) Representative *in situ* hydridization autoradiograms showing *Npas4* mRNA expression in coronal mouse brain sections at the level of the dorsal hippocampus from young wild-type (WT_Y), young *Hsd11b1*$^{-/-}$ (KO_Y), aged memory-impaired wild-type (WT_AI) and aged *Hsd11b1*$^{-/-}$ mice (KO_A) (n=6-8/group). Mice were previously tested in the Y-maze to confirm spatial memory status as in figure 1 with only WT_AI mice included for in situ hybridization analysis. (B) Quantification of *Npas4* mRNA levels in hippocampus sub-regions (dentate gyrus, DG, CA1 and CA3) and cortex (layers 2/3 and V) of wild type and *Hsd11b1*$^{-/-}$ mice. *P<0.05 significant difference between groups. Data shown are mean ± SEM.

Figure 5. Differentially expressed *Arc* mRNA in hippocampus and cortex of aged wild-type and *11β-HSD1-deficient* mice. (A) Representative in situ hybridization autoradiograms showing *Arc* mRNA expression in coronal mouse brain sections at the level of the dorsal hippocampus from young wild-type (WT_Y), young *Hsd11b1*$^{-/-}$ (KO_Y), aged memory-impaired wild-type (WT_AI) and aged *Hsd11b1*$^{-/-}$ mice (KO_A) (n=6-9/group). Mice were previously tested in the Y-maze to confirm spatial memory

status as in figure 1 with only WT_AI mice included for in situ hybridization analysis. (B) Quantification of *Arc* mRNA levels in hippocampus sub-regions (dentate gyrus, DG, CA1 and CA3) and cortex (layers 2/3 and V) of wild-type (WT) and *Hsd11b1*$^{-/-}$ mice. *P<0.05, **P<0.01, ***P<0.001 significant difference between groups. Data shown are mean ± SEM.