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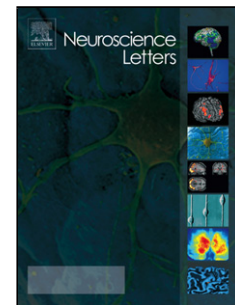
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The nuclear retention of transcription factor FOXO3a correlates with a DNA damage response and increased glutamine synthetase expression by astrocytes suggesting a neuroprotective role in the ageing brain

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Highlights

- Nuclear FOXO3a significantly correlates with glutamine synthetase expression
- FOXO3a nuclear localisation correlates with a DNA damage response
- Glutamine synthetase expression correlates with increasing Alzheimer pathology

Abstract

The accumulation of reactive oxygen species leading to oxidative damage and cell death plays an important role in a number of neurodegenerative disorders. FOXO3a, the main isoform of FOXO transcription factors, mediates the cellular response to oxidative stress by regulating the expression of genes involved in DNA repair and glutamine metabolism, including glutamine synthetase (GS). Immunohistochemical investigation of the population-based neuropathology cohort of the Medical Research Council's Cognitive Function and Ageing Study (MRC CFAS) demonstrates that nuclear retention of FOXO3a significantly correlates with a DNA damage response and with GS expression by astrocytes. Furthermore, we show that GS expression correlates with increasing Alzheimer-type pathology in this ageing cohort. Our findings suggest that in response to oxidative stress, the nuclear retention of FOXO3a in astrocytes upregulates expression of GS as a neuroprotective mechanism. However, the activity of GS may be compromised by increasing levels of oxidative stress in the ageing brain resulting in dysfunctional enzyme activity, neuronal excitotoxic damage and cognitive impairment.

Keywords: FOXO3a; glutamine synthetase; DNA damage response; astrocyte; neurone; Alzheimer's pathology; ageing brain.

Introduction

Alzheimer's disease (AD) is the most common form of dementia and is pathologically characterised by the extracellular deposition of β -amyloid ($A\beta$) protein, intracellular neurofibrillary tangles (NFT) of hyperphosphorylated tau, neuronal loss and extensive synaptic changes in the cerebral cortex. Given the currently limited success of $A\beta$ -based therapies [25], it is likely that the successful treatment of AD will also include modulation of mechanisms which protect against other processes causing neuronal dysfunction and neurodegeneration, including excitotoxicity and oxidative stress. Characterisation of these mechanisms is essential to develop novel neuroprotective targets aimed at preventing neuronal dysfunction and cognitive impairment in the ageing population.

Glial pathology occurs in the ageing brain and is a major contributor to age-related neurodegeneration [26, 38]. Astrocytes play a key role maintaining homeostasis in the CNS, including the uptake and recycling of neurotransmitters such as glutamate [20]. Extracellular glutamate levels are mainly regulated through re-uptake from the synaptic cleft via glial excitatory amino acid transporters (EAATs) [43]. A functional glutamate-glutamine metabolic cycle between astrocytes and neurones is vital for preventing excessive extracellular accumulation of the neurotransmitter leading to neuronal excitotoxicity [8, 11]. Loss of astrocyte-associated EAAT2 and glutamate excitotoxicity are features of brain ageing and neurodegenerative diseases, including AD [18, 21, 36].

Forkhead box class O (FOXO) proteins form a family of transcription factors which are phosphorylated and regulated by Akt, resulting in their nuclear exclusion and the termination of their activity [42]. Activation of FOXO, depending on cell type, regulates a wide range of biological processes including stress resistance, cell cycle regulation, development and ageing [19]. Glutamine synthetase (GS), which catalyses the conversion of glutamate to glutamine, is highly expressed in astrocytes and is transcriptionally regulated by the phosphoinositide-3-kinase (PI3K)-Akt-FOXO pathway [41]. In contrast to Akt signalling, oxidative stress induces the nuclear retention of FOXO which, depending on the severity of the stimulus, results in either apoptosis or a protective response, including the transcription of anti-oxidant genes and activation of a DNA damage response [3, 15, 17, 39]. One member of the forkhead transcription factors, FOXO3a, has been implicated in a number of neurodegenerative disorders, including AD [3, 29, 33, 45], motor neuron disease [24], Parkinson's disease [12] and stroke [13], and is expressed throughout the cortex and hippocampus [14].

The Medical Research Council's Cognitive Function and Ageing Study (CFAS) is a well characterised prospective, longitudinal, population-based neuropathological study of the aging population (over 65yrs) [44]. Studies performed on this population-representative cohort we have previously demonstrated a reduction in EAAT2 expression [36] and down-

regulation of the PI3K-Akt pathway by astrocytes associated with increasing levels of Alzheimer-type pathology [37], and quantitated oxidative stress and the associated DNA damage response in this ageing cohort [35]. Given the proposed role of excitotoxicity in age-related neurodegeneration, we have now investigated the subcellular localisation of FOXO3a and its correlation with GS expression, astrogliosis and the DNA damage response in the ageing brain.

Materials and Methods

Human CNS cases

Human autopsy brain tissue was obtained from one centre of the Medical Research Council Cognitive Function and Ageing Study (MRC CFAS) [1, 44], following multi-centre research ethics committee (REC) approval (REC Reference number 11/H0308/2). Neuropathological lesions were assessed as part of the core CFAS neuropathology study using a modified protocol from the Consortium to Establish a Registry of Alzheimer's Disease (CERAD) [23] (www.cfas.ac.uk) and Braak neurofibrillary tangle staging [7]. The cases were categorised into groups representing entorhinal stages (Braak stages 0-II; 30 cases), limbic stages (Braak stages III-IV; 50 cases) and isocortical stages of tangle pathology (Braak stages V-VI; 17 cases). The mean age of death was 85.6 (SEM 7.4) years. Dementia status at death had been previously determined, based on all information available for each participant, including algorithmic (AGECAT) assessment in life, information from death certification and a Retrospective Informant Interview (RINI) developed by CFAS [34]. 59 participants had clinical dementia, 37 did not and in 2 cases clinical dementia status was undetermined. The median post-mortem delay was 17h (IQR 10-32h) and brain pH 6.49 (IQR 6.25-6.75). Formalin-fixed and frozen lateral temporal cortex samples (superior/middle temporal gyrus, Brodmann areas 22/21) were available for all cases and were used in the immunohistochemistry and western blotting experiments, respectively. The neuronal and astrocyte DNA damage response (DDR) (γ H2AX and DNA-PKcs nuclear immunoreactivity), astrogliosis (GFAP immunoreactivity), and local measures of AD-type pathology (A β and AT8) were previously assessed in these cases [35, 36]. A total of 98 participants were included in these analyses, where 61 are females.

Immunohistochemistry

Immunohistochemistry was performed using a standard avidin-biotin complex (ABC) method. Sections were deparaffinised, rehydrated to water and endogenous peroxidase activity quenched by placing the sections in 0.3% H₂O₂/methanol for 20min at room temperature (RT). Sections were subjected to antigen retrieval (0.01M tri-sodium citrate pH6.5, pressure cooker). Following incubation with 1.5% normal serum for 30min at RT, the

sections were incubated overnight at 4°C with the well characterised, commercially available antibodies against FOXO3a (1:100; AbCam, UK), or glutamine synthetase (1:500; Millipore, UK). As phosphorylation of FOXO3a leads to the nuclear exclusion of the transcription factor and the termination of its activity, we elected to use an antibody which was raised against the N-terminus of the protein, as opposed to an antibody to specifically detect the phosphorylated form. To visualise antibody binding, the horse-radish peroxidase avidin biotin complex was used (Vectastain Elite kit, Vector Laboratories, UK) with 3,3'-diaminobenzidine (DAB) as the chromagen (Vector Laboratories, UK; brown).

To investigate astrocyte association with FOXO3a, dual labelling with the astrocyte marker GFAP was performed. Following incubation with the avidin-biotin blocking kit (Vector Laboratories, UK), FOXO3A immunostained sections were incubated overnight at 4°C with anti-GFAP (1:500; DakoCytomation, UK), followed by the alkaline-phosphatase-conjugated avidin-biotin complex (Vectastain Elite kit, Vector Laboratories, UK), developed with alkaline phosphatase substrate 1 (Vector Laboratories, UK; red) and lightly counterstained with Mayer's haematoxylin. Negative controls, either omission of the primary antibody or isotype controls, were included in every run.

Quantitative analysis of FOXO3a and GS

Assessment of FOXO3a and GS-specific immunoreactivity was performed by capturing bright-field microscopic images in 3 adjacent 350µm-wide cortical ribbons, consisting of contiguous fields to cover the total cortical thickness through the apex of the gyrus, using a x20 objective (Nikon Eclipse Ni-U microscope, Nikon, UK) and analysed using the Analysis ^D software (Olympus Biosystems, Watford, UK). For GS, the image was thresholded and the immunoreactive area of the field determined per total area examined. The number of FOXO3a positive pyramidal neuronal nuclei was determined using a size exclusion of >450 pixels, and the number of positive glial nuclei determined by subtracting the number of pyramidal neuronal nuclei from the total number of positive nuclei.

Statistical Analysis

As our data was skewed, median and inter-quartile range (IQR) was used for descriptive analyses. To test if dementia, sex, age or post-mortem delay (PMD) were risk factors for GS and/or FOXO3a nuclear retention, linear regressions were used. Spearman's correlation coefficient (r) was calculated to verify the strength of correlations between continuous variables. All tests were 2-tailed. 95% confidence intervals (CI) were calculated for the linear regression coefficients (β). Statistical analyses were performed using statistical package STATA, version 12.

Results

Expression of FOXO3a and GS in the ageing temporal cortex

Specific nuclear immunostaining of FOXO3a was evenly distributed throughout all layers of the temporal cortex associated with cells morphologically resembling neurones and glia, and was seldom observed in the white matter (Figure 1a). In a subgroup of 14 cases, faint FOXO3a immunoreactivity was also detected in the cytoplasmic compartment of cells (Figure 1b). Dual staining with GFAP demonstrated association of FOXO3a with astrocytes (Figure 1c), but a proportion of FOXO3a⁺ glia were not GFAP⁺. GS was exclusively associated with astrocytes throughout the cohort, predominantly staining the astrocyte cell body and their primary radiating processes within the cortex (Figure 1d).

The median number of FOXO3a⁺ neurones within the temporal cortex was 28.9 (IQR=11.4-51.8), FOXO3a⁺ glia was 86.6 (IQR=62.6-118.6) and area GS immunoreactivity was 0.5 (IQR=0.23-0.97). Neither GS nor FOXO3a (neuronal or glia immunoreactivity) significantly related to age, sex or PMD, but FOXO3a neuronal immunoreactivity did relate to tissue pH (Table 1).

GS but not FOXO3a significantly correlates with Alzheimer-type pathology

Nuclear localisation of FOXO3a in glia significantly correlated with increased expression of GS (n=85, r=0.23, p=0.035, Figure 2a). Increasing levels of GS⁺ astrocytes did not significantly correlate with astrogliosis (n=92, r=0.15, p=0.163) but did with global measures of brain Alzheimer-type pathology (Braak neurofibrillary tangle stage) (n=93, r=0.23, p=0.029, Figure 2b), and with local measures of AD pathology in the temporal cortex, namely A β (n=90, r=0.24, p=0.020, Figure 2c) and tau area immunoreactivity (n=89, r= 0.24, p= 0.022, Figure 2d). In contrast, neither FOXO3a⁺ glia nor FOXO3a⁺ neurones significantly correlated with Braak stage (n=89, r=0.06, p=0.571; n=89, r=-0.08, p=0.437, respectively), A β (n=86, r=0.09, p=0.405; n=86, r=0.16, p=0.142, respectively), or tau pathology (n=84, r=0.14, p=0.194; n=84, r=0.08, p=0.482). The number of FOXO3a⁺ neurones, FOXO3a⁺ glia, and GS immunoreactive area (%) within each Braak stage is shown in Table 2.

FOXO3a nuclear expression significantly correlates with a DNA damage response

We investigated the relationships between FOXO3a neuronal and glial expression with astrogliosis and levels of a DNA damage response. FOXO3a⁺ glia did not correlate with astrogliosis (n=88, r=0.09, p=0.377), but significantly correlated with FOXO3a⁺ neurones (n=89, r=0.68, p<0.001, Figure 3b). FOXO3a⁺ glia correlated with γ H2AX⁺ glia (n=87, r=0.23, p=0.030, Figure 3c), and FOXO3a⁺ neurones significantly correlated with γ H2AX⁺ neurones (n=87, r=0.27, p=0.010, Figure 3d).

Relationship to dementia status

Neither GS (OR=2.37, 95%CI (OR)=0.96; 5.86, $p=0.061$), FOXO3a neuronal (OR=0.99, 95%CI (OR)= 0.98;1.01, $p=0.591$) nor FOXO3a glial expression (OR=1.00, 95%CI(OR)= 0.99; 1.01, $p=0.279$) were significant predictors of dementia when the analyses were controlled for age and sex.

Discussion

FOXO transcription factors control various biological functions, including apoptosis, and the expression of genes involved in the regulation of glutamine metabolism, DNA repair and resistance to oxidative stress [4, 19, 41]. In the present study we demonstrate that nuclear retention of FOXO3a significantly correlates with a DNA damage response and with GS expression by astrocytes, but not with Alzheimer-type pathology in the ageing brain. Furthermore, we show that GS expression correlates with local burdens of A β plaques and tau pathology in this ageing cohort.

The generation of reactive oxygen species (ROS) leading to oxidative damage and neuronal cell death plays an important role in the pathogenesis of neurodegenerative disorders, suggesting that anti-oxidant defence mechanisms are unable to cope with increasing ROS levels in these diseases [22]. Glutamate and A β are two oxidative stressors: at high concentrations glutamate elevates intracellular calcium levels and increases the formation of ROS [30]; while A β induces mitochondrial dysfunction resulting in the generation of ROS [2]. FOXO3a, the main isoform of FOXO transcription factors, mediates cellular responses to oxidative stress and modulates adaptive responses. Post-translational phosphorylation of FOXO3a regulates the translocation of FOXO3a from the nucleus to the cytosol, resulting in the repression of the transcription of genes associated with protection against oxidative stress, DNA repair and anti-apoptosis [42]. The redox potential of neurones and glia is essential to protect against neurotoxic ROS levels which result in neuronal dysfunction and are associated with cognitive impairment. In the present study we demonstrate increased nuclear retention of FOXO3a by neurones and glia significantly correlates with a DNA damage response suggesting that, in response to oxidative DNA damage, FOXO3a may play a key role regulating the expression of neuroprotective anti-oxidant genes.

Cognitive decline is associated with synaptic loss and impaired synaptic connectivity, which may occur as a result of impaired neurotransmitter recycling and associated neuronal excitotoxicity [27]. We previously showed a reduction in astrocyte expression of the glutamate transporter EAAT2 associated with increasing levels of Alzheimer pathology, which likely results in the accumulation of excitotoxic levels of glutamate [36]. Levels of glutamate, the major excitatory neurotransmitter in the CNS, are primarily regulated by GS

which is expressed by astrocytes in all cortical layers [32]. An increase in GS expression has been reported in the prefrontal cortex in AD and in the CSF of vascular dementia, motor neuron disease and AD patients [5, 9, 40]. In support of these findings, we demonstrate a significant increase in GS expression associated with increasing levels of Alzheimer-type pathology in the ageing brain. In contrast, other studies have reported a reduction in the astrocytic expression of this enzyme in AD which shows no association with A β pathology [16, 32]. Complicating the interpretation of these conflicting reports GS is significantly oxidised in both AD and mild cognitive impairment cases [10], with oxidation significantly reducing its activity [31]. Furthermore, while A β has been shown to induce astrocyte expression of GS *in vitro* [28], proteomic analysis has identified GS as being susceptible to oxidation after exposure to A β_{1-42} [6]. Our findings suggest that in response to oxidative stress, the nuclear retention of FOXO3a increases the expression of GS as a neuroprotective mechanism by astrocytes to maintain homeostasis of the synaptic environment and protect against accumulating levels of glutamate and neuronal excitotoxicity in the ageing brain. However while levels of GS may rise, its activity may be compromised by increasing levels of oxidative stressors, including glutamate and local A β pathology, resulting in dysfunctional enzyme activity, neuronal excitotoxic damage and cognitive impairment.

Astrocytes play a key role in maintaining homeostasis within the synaptic and neuronal environments. Understanding which factors control and modulate FOXO3a-mediated neuroprotection is crucial to identify potential therapeutic targets in the treatment of AD.

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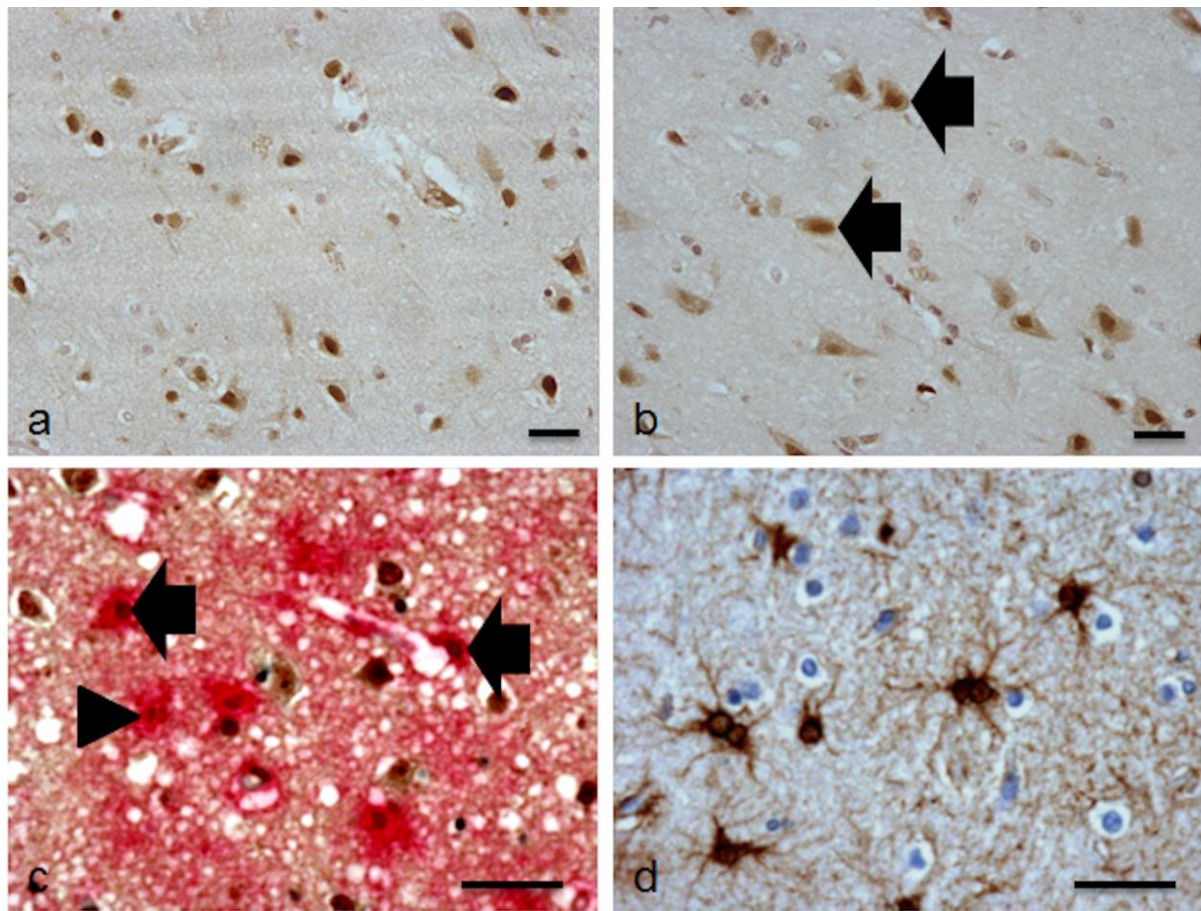
Figure Captions

Figure 1. Expression of FOXO3a and GS in the lateral temporal cortex. (a) Specific nuclear immunostaining of FOXO3a was associated with cells morphologically resembling neurones and glia. (b) In a subgroup of cases increased levels of FOXO3a were detected in the cytoplasmic compartment, examples are indicated by the arrow. (c) FOXO3a co-localised with some (arrow), but not all (arrow head) GFAP⁺ astrocytes. (d) GS was exclusively associated with cells with an astrocytic morphology. Scale bar represents 100µm in (a) and (b), and 50µm in (c) and (d).

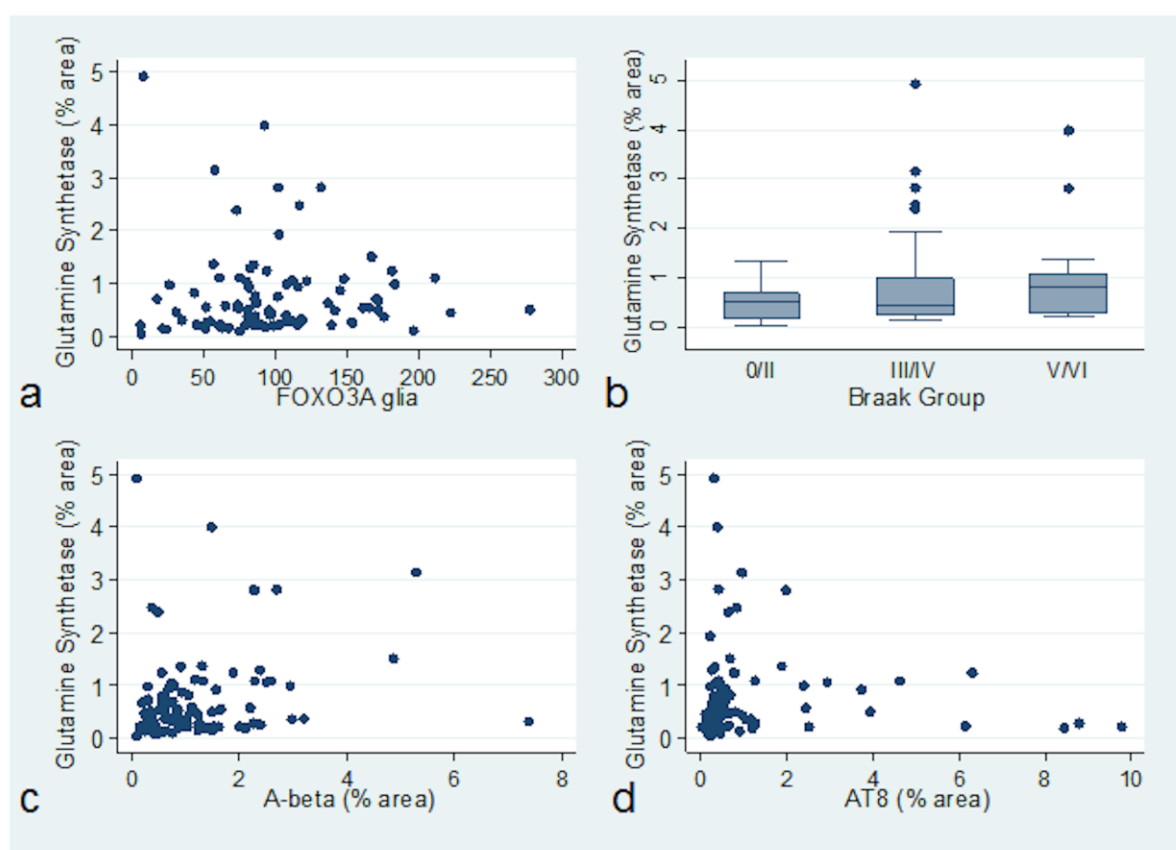


Figure 2. GS expression associates with Alzheimer-type pathology. Increasing GS⁺ astrocytes associated with (a) FOXO3a⁺ glia, (b) Braak stage, (c) β -amyloid plaques and (d) levels of tau (AT8 immunoreactivity).

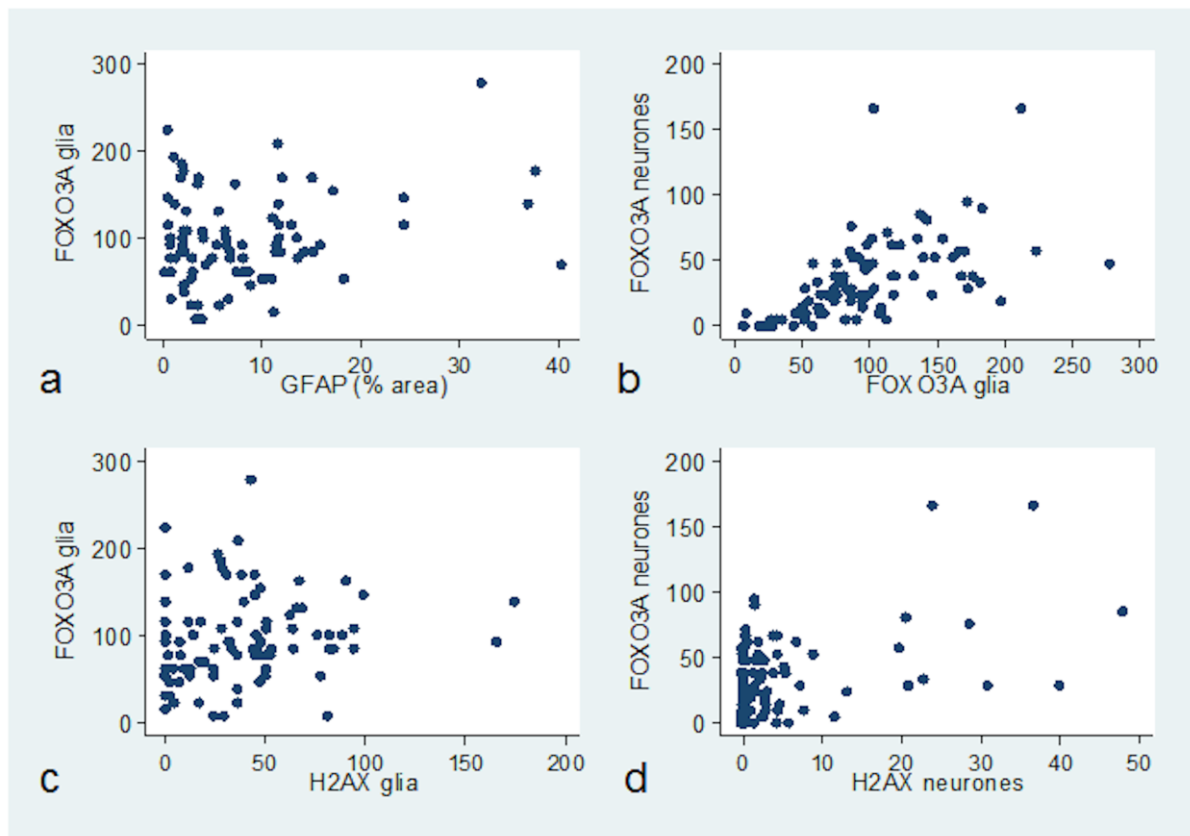


Figure 3. FOXO3a expression correlates with a DNA damage response. (a) FOXO3a⁺ glia showed no association with astrogliosis, (b) but did correlate with FOXO3a⁺ neurones. (c) FOXO3a⁺ glia significantly correlated with γ H2AX⁺ glia, while (d) FOXO3a⁺ neurones significantly correlated with γ H2AX⁺ neurones.

Tables

Table 1. Linear regression analyses investigating the relationship between GS and FOXO3a (glial or neuronal immunoreactivity) with demographics and brain pH

		β	95%CI(β)	p
GS	Age	0.01	(-0.01; 0.03)	0.440
	pH	-0.12	(-0.46; 0.22)	0.474
	PMD	0.00	(-0.01; 0.01)	0.448
	Sex	-0.23	(-0.59; 0.12)	0.191
FOXO3a glial	Age	-0.25	(-1.72; 1.23)	0.741
	pH	20.15	(-8.00; 48.29)	0.158
	PMD	0.09	(-0.49; 0.68)	0.749
	Sex	7.22	(-15.11; 29.56)	0.522
FOXO3a neuronal	Age	-0.44	(-1.32; 0.44)	0.321
	pH	25.16	(8.10; 42.22)	0.004
	PMD	-0.16	(-0.52; 0.19)	0.357
	Sex	5.30	(-8.07; 18.68)	0.433

Table 2. Number of FOXO3A⁺ neurones, FOXO3A⁺ glia, and GS immunoreactive area (%) within each Braak group

Braak group		Entorhinal	Limbic	Isocortical
FOXO3A ⁺ neurones	Median (IQR)	31.0 (14.5-58.7)	28.9 (11.9-45.5)	20.2 (9.7-52.3)
FOXO3A ⁺ glia	Median (IQR)	86.8 (51.9-149.7)	85.9 (61.5-118.0)	98.4 (68.2-115.3)
GS	Median (IQR)	0.5 (0.1-0.6)	0.4 (0.2-1.0)	0.8 (0.3-1.2)

IQR: inter-quartile range