The unfolded protein response is activated in the olfactory system in Alzheimer’s disease

Helen C. Murray1,2*, Birger Victor Dieriks1, Molly E. V. Swanson1, Praju Vikas Anekal1, Clinton Turner3, Richard L. M. Faul1, Leonardo Belluscio4, Alan Koretsky2 and Maurice A. Curtis1*

Abstract

Olfactory dysfunction is an early and prevalent symptom of Alzheimer’s disease (AD) and the olfactory bulb is a nexus of beta-amyloid plaque and tau neurofibrillary tangle (NFT) pathology during early AD progression. To mitigate the accumulation of misfolded proteins, an endoplasmic reticulum stress response called the unfolded protein response (UPR) occurs in the AD hippocampus. However, chronic UPR activation can lead to apoptosis and the upregulation of beta-amyloid and tau production. Therefore, UPR activation in the olfactory system could be one of the first changes in AD. In this study, we investigated whether two proteins that signal UPR activation are expressed in the olfactory system of AD cases with low or high amounts of aggregate pathology. We used immunohistochemistry to label two markers of UPR activation (p-PERK and p-eIF2α) concomitantly with neuronal markers (NeuN and PGP9.5) and pathology markers (beta-amyloid and tau) in the olfactory bulb, piriform cortex, entorhinal cortex and the CA1 region of the hippocampus in AD and normal cases. We show that UPR activation, as indicated by p-PERK and p-eIF2α expression, is significantly increased throughout the olfactory system in AD cases with low (Braak stage III-IV) and high-level (Braak stage V-VI) pathology. We further show that UPR activation occurs in the mitral cells and in the anterior olfactory nucleus of the olfactory bulb where tau and amyloid pathology is abundant. However, UPR activation is not present in neurons when they contain NFTs and only rarely occurs in neurons containing diffuse tau aggregates. We conclude that UPR activation is prevalent in all regions of the olfactory system and support previous findings suggesting that UPR activation likely precedes NFT formation. Our data indicate that chronic UPR activation in the olfactory system might contribute to the olfactory dysfunction that occurs early in the pathogenesis of AD.

Keywords: Alzheimer’s disease, Unfolded protein response, Olfactory bulb, Anterior olfactory nucleus, PERK, eIF2α

* Correspondence: h.murray@auckland.ac.nz; m.curtis@auckland.ac.nz

1Department of Anatomy and Medical Imaging and Centre for Brain Research, Faculty of Medical and Health Science, University of Auckland, Private Bag 92019, Auckland, New Zealand

Full list of author information is available at the end of the article

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Introduction

Olfactory dysfunction is an early and prevalent symptom of Alzheimer’s disease (AD) that can precede the diagnostic memory and cognitive symptoms by many years [4, 12]. Pathologically, AD is characterised by the accumulation of beta-amyloid plaques and tau neurofibrillary tangles (NFTs), which also accumulate very early in the disease process in the olfactory bulb [23]. Beta-amyloid and NFT aggregates can disturb many cellular processes, such as protein degradation, that can lead to endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR). Under normal physiological conditions, activation of the UPR triggers a signalling cascade that restores proteostasis. However, chronic activation of the UPR can initiate apoptosis as has been shown in neurodegenerative diseases such as AD [11, 44].

The UPR consists of three parallel signalling pathways that are mediated by ER membrane proteins: Protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF-6). In this study, we focused on the PERK pathway, in which activation has previously been reported in post-mortem AD tissue [18, 20, 43, 47]. In response to an accumulation of misfolded proteins, the ER chaperone BiP/GRP78 is released from PERK and binds to these misfolded proteins. This leads to the dimerisation and autophosphorylation of PERK and subsequent phosphorylation of its downstream effector eukaryotic translation initiator factor 2α (eIF2α). Activated eIF2α inhibits protein synthesis to alleviate the accumulation of misfolded proteins, but increases translation of ATF4 which can drive apoptosis via C/EBP-homologous protein (CHOP). Prolonged activation of this proapoptotic pathway of the UPR leads to synaptic failure and neuronal loss [29].

Several post-mortem tissue studies have demonstrated that UPR activation via the PERK pathway occurs in the human hippocampus, frontal cortex and temporal cortex in AD [18, 20, 43, 47]. These studies further showed that markers of activated UPR such as phosphorylated PERK (p-PERK) and phosphorylated eIF2α (p-eIF2α) are observed in neurons that contain diffuse tau aggregates, but not those with dense NFTs [18, 32]. Therefore, it is hypothesised that UPR activation precedes tau formation in AD and is one of the early mechanisms involved in AD pathogenesis.

As olfactory dysfunction is one of the earliest symptoms in AD and tau and beta-amyloid aggregates accumulate in the olfactory bulb very early in the disease, we hypothesised that UPR activation in the olfactory system could be one of the early changes that occur in AD. To provide support for this hypothesis, we used immunohistochemistry to investigate whether the UPR markers p-PERK and p-eIF2α were present or increased in the olfactory system of AD cases with low and high grades of pathology. Our study included the bulbar and intrapeduncular segments of the anterior olfactory nucleus (AON) in the olfactory bulb and downstream cortical olfactory areas including the piriform and entorhinal cortex (Fig. 1), as well as the hippocampal CA1 region. We show that the UPR is activated throughout the olfactory system in all AD cases. We further show that markers of UPR activation infrequently co-occur in the same cell as tau aggregates, and only do so with diffuse tau and not NFTs, as described in previous studies. Our results highlight the concept that while UPR activation may initially be a neuroprotective process, chronic activation could be a mechanism of cellular dysfunction that drives aggregate formation and underlies olfactory dysfunction in early AD.

![Fig. 1 Schematic of the human olfactory pathway. The pathway of primary odour information from the olfactory sensory neurons (OSNs) in the olfactory epithelium of the nose to the anterior olfactory nucleus (AON), piriform cortex (PIR) and entorhinal cortex (EC) via mitral cell projections. This circuitry is based on the olfactory circuitry of the mouse brain and has been extrapolated to the human brain. Abbreviations: AONb, bulbar anterior olfactory nucleus; AONi, intrapeduncular anterior olfactory nucleus; AONc, cortical anterior olfactory nucleus; CN, caudate nucleus; GL, glomerular layer; MCL, mitral cell layer](image-url)
Materials and methods
Human tissue acquisition and processing
Human post-mortem brain tissue was obtained from the Neurological Foundation Human Brain Bank and the Human Anatomy Laboratory within the Department of Anatomy and Medical Imaging, University of Auckland, New Zealand. The tissue was donated with informed consent from the family prior to brain removal and all procedures were approved by the University of Auckland Human Participants Ethics Committee (Ref: 011654). All cases used in this study were assessed by an independent neuropathologist who determined the Braak staging, thal phase and NIA-AA score. The 12 neurologically normal donor cases had no history of neurological abnormalities and no other neuropathology was noted. The mean age (± standard deviation) of the normal cases was 73 ± 12.9 years and ranged from 56 to 94 years. The average post-mortem delay was 25 ± 9.7 h with a range of 13 to 48 h (Table 1). The 16 AD donor cases had a clinical history of dementia and the clinical AD diagnosis was confirmed by an independent pathologist. The average age of AD cases was 79 ± 10 years and ranged from 60 to 94 years. The average post-mortem delay was 8.9 ± 4.8 h with a range of 3.5 to 15 h (Table 1).

The right hemisphere of each brain was fixed by perfusion of 15% formaldehyde in 0.1 M phosphate buffer through the cerebral arteries and the hemisphere was subsequently dissected into approximately 60 blocks as described in previous publications [48]. From each block, a 0.5 cm-thick section was selected for paraffin-embedding and the remaining tissue was snap frozen using dry-ice snow and stored at −80°C. The olfactory

| Case  | Pathology Diagnosis | Age | Sex | PMD | Braak Stage | B-amyloid Thal Phase | NIA-AA Score (AD severity) |
|-------|---------------------|-----|-----|-----|-------------|-----------------------|--------------------------|
| A271  | AD                  | 68  | F   | 6   | VI          | 5                     | A3 B3 C2 (high)           |
| A275  | AD                  | 94  | F   | 3.5 | V           | 5                     | A3 B3 C2 (high)           |
| A283  | AD/LBD (neocortical/diffuse) | 60  | F   | 16  | VI          | 5                     | A3 B3 C2 (high)           |
| A284  | AD/LBD (neocortical) | 82  | M   | 18.5| III         | 5                     | A3 B2 C1 (intermediate)   |
| A286  | AD/LBD (neocortical/diffuse) | 92  | M   | 8.5 | III         | 5                     | A3 B2 C2 (intermediate)   |
| A290  | AD/LBD (amygdala)   | 73  | M   | 4   | V           | 5                     | A3 B3 C2 (high)           |
| A295  | AD/LBD (amygdala)   | 69  | M   | 12  | V           | 5                     | A3 B3 C2 (high)           |
| A296  | AD/LBD (amygdala)   | 74  | F   | 8.5 | V           | 5                     | A3 B3 C2 (high)           |
| A299  | AD                  | 94  | F   | 8.5 | V-VI        | 5                     | A3 B3 C2 (high)           |
| A301  | AD                  | 75  | M   | 12.5| VI          | 4                     | A3 B3 C2 (high)           |
| A303  | AD                  | 87  | M   | 24  | IV          | 4                     | A3 B2 C2 (intermediate)   |
| A304  | AD/LBD (neocortical/diffuse) | 79  | F   | 13  | IV          | 5                     | A3 B2 C2 (intermediate)   |
| A309  | AD                  | 90  | F   | 31  | III         | 4                     | A3 B2 C1 (intermediate)   |
| A313  | AD                  | 77  | M   | 3.5 | VI          | 5                     | A3 B3 C2 (high)           |
| A3120 | AD/LBD (amygdala)   | 74  | F   | 6.5 | IV          | 5                     | A3 B2 C2 (intermediate)   |
| H251  | AD                  | 77  | M   | 11.5| III         | 5                     | A3 B2 C3 (intermediate)   |
| OFB55 | NORMAL              | 56  | M   | 26  | –           | –                     | –                        |
| OFB57 | NORMAL              | 63  | M   | 36  | –           | –                     | –                        |
| OFB59 | NORMAL              | 67  | F   | 20  | –           | –                     | –                        |
| OFB6A | NORMAL              | 82  | M   | 24  | –           | –                     | –                        |
| OFB8A | NORMAL              | 87  | M   | 48  | –           | –                     | –                        |
| H190  | NORMAL              | 72  | F   | 19  | 0           | –                     | –                        |
| H230  | NORMAL              | 57  | F   | 32  | 0           | –                     | –                        |
| H240  | NORMAL              | 73  | F   | 26.5| I-II        | –                     | –                        |
| H242  | NORMAL              | 61  | M   | 19.5| 0           | –                     | –                        |
| H243  | NORMAL              | 77  | M   | 13  | 0           | –                     | –                        |
| H246  | NORMAL              | 89  | F   | 17  | I-II        | –                     | –                        |
| H250  | NORMAL              | 94  | M   | 19  | I-II        | –                     | –                        |

Abbreviations: LBD Lewy body disease
*aonly olfactory bulbs were obtained for these cases*
bulbs were removed from the brain prior to perfusion to preserve tissue integrity and were immersion fixed in 15% formaldehyde. The olfactory bulbs and brain tissue blocks were processed for paraffin embedding as described previously [50]. The paraffin blocks were sectioned at a thickness of 7 μm using a rotary microtome (Leica Biosystems RM2335). The olfactory bulbs were sectioned in the sagittal plane, while the hippocampus was sectioned coronally. The sections were floated on a water bath set at 37 °C (Leica Biosystems, HI1210) before being mounted on Superfrost Plus slides (Menzel-Glaser) and air-dried for 18 h at room temperature.

Piriform cortex tissue was not available on the paraffin-embedded tissue blocks and was instead obtained from the fixed-frozen tissue blocks. These blocks were sectioned at 50 μm on a freezing sliding microtome and stored at 4 °C in phosphate-buffered saline (PBS) containing 0.1% sodium azide.

Immunofluorescent labelling of paraffin sections
Paraffin sections underwent two sequential rounds of immunofluorescent triple labelling to stain four markers of interest plus either p-PERK or p-eIF2α on each section. For each brain region, three sections were labelled for each staining combination per case. For the olfactory bulb, the most central section of the bulb and two sections approximately 350 μm either side were selected. For the CA1 and entorhinal cortex (EC) analysis, three coronal sections spaced 100 μm apart from the central 2 cm of the hippocampal formation were selected.

The fluorescent triple labeling procedure follows the method described in Stevenson et al., (2020) [42]. For the olfactory bulb and hippocampus sections, the slides were heated at 60 °C for 1 h (h) to melt the paraffin wax before being cleared in xylene (2 × 30 min) and rehydrated in an ethanol series of 100%, 2 × 10 min; 95%, 5 min; 80%, 5 min; and 75% ethanol, 5 min. Heat-induced epitope retrieval was performed using 10 mM sodium citrate buffer (0.9 NA) at pH 6.0 in a pressure cooker (2100 Antigen Retriever, Aptum Biologics Ltd.) for 20 min at 121 °C and left to cool for 1.5 h. The slides were then washed in PBS and permeabilised in PBS + 0.1% Triton X-100 for 15 min at room temperature. To block for non-specific binding of the secondary antibodies, the sections were incubated in 10% normal goat serum (Gibco #16210–072) for 1 h at room temperature. The first round of primary antibodies (Table 2) included PGP9.5 and either p-eIF2α, p-PERK, CK1δ or p-IRE1α diluted in 1% normal goat serum. The sections were incubated in the primary antibody mixture overnight in a humidified chamber at 4 °C. Secondary antibodies – goat anti-rabbit Alexa Fluor 488 (ThermoFisher; A11034) and goat anti-mouse Alexa Fluor 647 (ThermoFisher; A21236) – together with Hoechst 33342 nuclei stain (1: 20,000 dilution, Molecular probes; H1399) were diluted in 1% normal goat serum and applied for 3 h at room temperature. The sections were washed and coverslipped with Prolong® Gold mounting media (Molecular Probes; P36930). After each step, the sections were washed for 3 × 5 min in PBS.

Sections were imaged using an automated fluorescence microscope (Zeiss Z2 Axioimager) equipped with MetaSystems VSlide slide scanner (MetaSystems) running MetaFer (V 3.12.1) with a 20x air objective (0.9 NA). Following imaging, the sections were immersed in PBS and carefully decoverslipped for the second round of staining. The sections were immersed in 80% formic acid for 5 min, which served to eliminate the previous fluorescent staining and retrieve the tau and beta-amyloid aggregate epitopes. The washing, permeabilisation and blocking steps were repeated as described above and the second round of primary antibodies (NeuN, Tau, and Amyloid) were added to the sections overnight at 4 °C. The secondary antibodies (goat anti-rabbit Alexa Fluor 488 (ThermoFisher A11034), goat anti-mouse Alexa Fluor 594 (ThermoFisher A11032) and goat anti-guinea pig 647 (ThermoFisher A21450) were diluted in 1% normal goat serum and Hoechst 33342 nuclear stain was added. Secondary antibodies were added for 3 h at room temperature, after which the sections were washed, coverslipped with Prolong Gold AntiFade mounting media and imaged again.

### Table 2: Primary antibodies used in this study

| Antigen         | Species     | Dilution | Manufacturer       | Catalogue Number     | RRID   |
|-----------------|-------------|----------|--------------------|----------------------|--------|
| Phospho-eIF2α (pSer51) | rabbit     | 1:500    | Millipore-Sigma    | SAB4504388            | AB_2847826 |
| Phospho-PERK (Thr981) | rabbit     | 1:500    | Santa Cruz         | sc-32,577             | AB_2293243 |
| Phospho-IRE1α (pSer724) | rabbit     | 1:500    | Novus Biologicals  | NB100–2323            | AB_10145203 |
| CK1δ            | rabbit     | 1:400    | ThermoFisher       | PAS–32129             | AB_2549602 |
| PGP9.5          | mouse      | 1:500    | Abcam              | Ab8189                | AB_2036343 |
| NeuN            | guinea pig | 1:1000   | Millipore-Sigma    | ABN90P                | AB_2341095 |
| Tau             | rabbit     | 1:2000   | Aligent            | A0024                 | AB_10013724 |
| Beta-amyloid    | mouse      | 1:100    | Aligent            | M0872                 | AB_2056966 |
Immunofluorescent labelling of free-floating piriform cortex sections

As the piriform cortex tissue was not available on paraffin-embedded tissue blocks, it was processed from the fixed-frozen tissue blocks. Three sections spaced 200 μm apart were selected for each staining combination. The human piriform cortex traverses the junction of the temporal and frontal lobes and in this study, we used the temporal aspect of the piriform cortex located anterior to the amygdala which shows distinct three-layered cytoarchitecture. These sections were labelled using free-floating fluorescent immunohistochemistry as previously described [31]. The two sequential rounds of fluorescent staining could not be performed on free-floating sections so the co-labelling of p-PERK and p-eIF2α with tau was not investigated in this region. Sequential sections were stained for p-PERK and NeuN, p-eIF2α and NeuN, and tau, amyloid and NeuN. The sections labelled for p-PERK and p-eIF2α underwent heat-induced epitope retrieval using 10 mM sodium citrate buffer with 0.05% Tween 20, pH 6.0 and rapid heating to 100 °C using a microwave for 30 s, followed by cooling at room temperature for 1 h. The sections labelled for tau and beta-amyloid underwent antigen retrieval using 80% formic acid for 5 min at room temperature. All sections were blocked for non-specific secondary antibody binding by incubating in 10% normal goat serum (Gibco #16210–072) for 1 h at room temperature. Primary antibodies (Table 2) were diluted in 1% normal goat serum and sections were incubated in this solution for 48 h at 4 °C. Subsequently, the relevant species-specific secondary antibodies and nuclear counterstain (as above) were applied for 24 h at room temperature. Between steps, the sections were washed in PBS with 0.1% Triton X-100. Finally, the sections were mounted onto slides and coverslipped using Prolong Gold AntiFade mounting media (Molecular Probes #P36930). Imaging was carried out as for the paraffin sections.

Alignment and segmentation of images

The fluorescent images from the paraffin sections were separated into the individual channels as 8-bit greyscale images. The Hoechst labelling was present for every staining and imaging round so nuclei were used as the intrinsic markers for image registration between staining rounds. We used a custom-designed registration code in Python to automatically register the nuclei images. We found that the accuracy of the registration was improved when the images were pre-processed before the registration. We applied a 50-pixel rolling background subtraction and a 5-pixel median filter to the nuclei images to smooth out any nuclei staining fluctuations. The processed nuclear images were registered with each other using a Jupyter Notebook to implement an AKAZE affine registration and a transformation matrix was extracted for each image set [2]. Subsequently, we applied this transformation matrix to all the individual images for the set. The individual images were merged together into a single file and pseudo-coloured using ImageJ v1.52p.

Semi-quantitative analysis of p-eIF2α, p-PERK, tau and NeuN+ cells

All measurements and cell counts were performed using ImageJ v1.52p. The area of the region of interest (ROI) was first measured using the polygon selection tool on the merged image. The olfactory bulb AON is subdivided into a series of compartments throughout the olfactory bulb and tract. These compartments can be delineated based on the high PGP9.5 immunoreactivity and an abundance of diffuse neuronal nuclei which are evident with Hoechst staining (Fig. 2a-b). The AON compartments are grouped based on their location: the bulbular AON (AONb) encompasses all compartments within the bulb, while the intrapuduncular AON (AONi) encompasses all compartments within the olfactory tract [37]. The piriform cortex, entorhinal cortex and CA1 regions of the hippocampus were delineated according to the Allen Human Brain Atlas [3, 13].

Once the ROI was delineated, for each image channel the background fluorescence intensity was measured from a 15 μm² area of the ROI in triplicate and averaged. Starting with the p-eIF2α or p-PERK channel, cells with positive staining were selected using the multipoint region tool in ImageJ. One multipoint marker was placed on the brightest p-eIF2α or p-PERK spot in the cell. For all remaining cells, those that were tau+ and NeuN+ were selected by placing a multipoint marker. As all three antibody labels are cytoplasmic, this method resulted in each cell within the image containing one multipoint marker at the same coordinate for all three image channels. At each marker coordinate the point intensity was measured for each image channel as grey values between 0 and 255 and subtracted from the background average for that channel. To be considered a positively stained cell, the point intensity needed to be above a determined threshold for that channel (grey values above background: 35 for tau, 15 for NeuN and 30 for p-eIF2α or p-PERK). Therefore, if a cell was positive for more than one marker then it would be indicated by point intensities above threshold for each image channel at that marker coordinate. A subset of the images were counted by a second investigator to ensure the counting method was repeatable. The data are presented as the percentage of neurons (NeuN+ cells) containing p-eIF2α or p-PERK or the number of p-eIF2α or p-PERK cells per mm². To investigate the amount of co-labelling between tau and p-eIF2α or p-PERK we have also presented the data as the percentage of tau+ cells.
that also contain p-eIF2α or p-PERK and the percentage of p-eIF2α or p-PERK+ cells that also contained tau. We investigated the correlation between the percentage of p-PERK+ and p-eIF2α+ neurons and the age or post-mortem delay for each case using a Spearman’s correlation coefficient. There was no significant correlation with age in any of the regions assessed (Additional file 1: Supplementary Figure 1A – E). There was no significant correlation with post-mortem delay in the CA1 regions. However, there were moderate inverse correlations with post-mortem delay in other regions (Additional file 1: Supplementary Figure 1F – J). This is likely due to the normal cases used in this study having longer post-mortem delays than the AD cases. To control for this difference, we investigated the correlation for cases that had a post-mortem delay between 11.5 and 36 h as this range overlapped between the two groups. There was no significant correlation between the percentage of p-eIF2α+ or p-PERK+ neurons and post-mortem delay for these cases, indicating that our results are unlikely to reflect a loss of phospho-epitopes in control cases due to post-mortem delay (Additional file 1: Supplementary Figure 1K – O).

**Semi-quantitative analysis of tau and beta-amyloid load**

To measure the total load of tau and beta-amyloid, the area of the ROI was first measured using the polygon selection tool. The threshold tool was then used to determine the area of the ROI covered by tau or beta-amyloid immunoreactivity. A threshold of at least 40 grey values was used to detect true labelling above background. The thresholded area was normalised to the total ROI area to obtain the percentage area of aggregate labelling.

**Statistical analysis**

Data visualisation and statistical analysis were performed using GraphPad Prism Version 8.03. All data are presented as mean ± standard deviation (SD) from the total values across three different sections per case. To test for differences between AD and normal cases, a parametric unpaired t-test was used for regions where the
data fit the assumptions of normal distribution and equality of variance. If the data did not satisfy these assumptions then a non-parametric Mann-Whitney test was performed. To test for differences in the percentage of neurons containing p-eIF2α or p-PERK across Braak stages, a non-parametric Kruskal-Wallis test with Dunn’s multiple comparisons test was performed for each region, as not all groups fit the assumptions for parametric testing. To investigate the statistical significance of correlations between the percentage of neurons containing p-eIF2α or p-PERK and tau or beta-amyloid load, a Spearman’s correlation coefficient was used. Statistical significance was set as $p < 0.05$.

**Results**

**p-PERK and p-eIF2α are located in areas of the olfactory bulb affected by tau and beta-amyloid aggregation in AD**

To study UPR activation in the olfactory system of AD patients and normal individuals, we performed immunohistochemistry with p-PERK and p-eIF2α antibodies on post-mortem human brain sections. We used antibodies that have been used in previous studies of human brain tissue [17, 18, 32] and co-labelled with antibodies for tau and beta-amyloid on the same section to determine co-localisation. For consistency, we used the same tau and beta-amyloid antibodies that are used by our clinical pathologist for Braak staging in our comparison of olfactory regions to the other brain regions. The tau antibody labels amino acid 243–441 at the C-terminal of human tau protein, independently of phosphorylation sites and the beta-amyloid antibody epitope corresponds to amino acids 8–17.

Tau and beta-amyloid aggregates were detected in olfactory bulbs from AD cases. These aggregates were most abundant within the AON but also scattered throughout the external plexiform layer and mitral cell layer (Fig. 2a). As it is difficult to delineate the external plexiform layer from the mitral cell layer in the human bulb they have been labelled together in Fig. 2a and b. We also observed that cells expressing p-PERK and p-eIF2α were predominantly located in the AON. These p-PERK+ and p-eIF2α+ cells did not appear to be clustered around beta-amyloid plaques in the AON (Additional file 2: Supplementary Figure 2). Furthermore, p-PERK and p-eIF2α immunoreactivity was identified within large neurons (approx 20 μm diameter) with large diffuse nuclei and located along the border of the granule cell layer or within the external plexiform layer (Fig. 2b, c, h). The size and location of these cells are consistent with previous descriptions of mitral cells [5, 24, 28, 41].

To determine whether the IRE1α branch of the UPR pathway was also activated in the olfactory bulb we labelled for p-IRE1α. In AD cases only, we found very few p-IRE1α+ neurons within the AON (Additional File 2: Supplementary Figure 3). We did not observe any p-IRE1α+ mitral cells.

**Increase in p-PERK+ and p-eIF2α+ cells throughout the human olfactory system in AD compared to neurologically normal cases**

We investigated whether UPR activation was present in downstream olfactory regions in AD and normal cases. We found p-PERK and p-eIF2α immunoreactivity as punctate granular staining in the cell cytoplasm resembled granulovacuolar degeneration, as described in other studies [18, 32, 40, 43, 49]. Labelling for the granulovacuolar degeneration marker CK1δ was also observed in these regions of the olfactory bulb and hippocampus (Additional file 2: Supplementary Figure 3) In all regions we assessed, nearly all cells with p-PERK and p-eIF2α immunoreactivity also co-labelled with the neuronal marker NeuN and were therefore considered to be p-PERK+ neurons or p-eIF2α+ neurons. We observed p-PERK+ neurons (Fig. 2c – G) and p-eIF2α+ neurons (Fig. 2h – I) in all the olfactory and hippocampal regions in AD cases.

We subsequently quantified the number of neurons and total cells expressing p-PERK and p-eIF2α. The percentage of p-PERK+ neurons was significantly increased in AD cases for all the olfactory and hippocampal regions assessed in this study (Fig. 3a, Additional file 3: Supplementary Table 1). The analysis of p-PERK cell density showed that the number of p-PERK+ cells per mm² was also significantly increased in AD cases compared to normals for all regions except the piriform cortex (Additional file 4: Supplementary Figure 5). Within AD cases, there was no significant difference in the amount of p-PERK between the olfactory regions (AONb, AONi, piriform cortex) and all regions except the AONi had significantly more p-PERK+ neurons than the entorhinal cortex (Additional file 3: Supplementary Table 2).

The quantification of p-eIF2α immunoreactivity showed a significant increase in the percentage of p-eIF2α+ neurons for AD cases for all regions (Fig. 3b, Additional file 3: Supplementary Table 1). The number of p-eIF2α+ cells per mm² was also significantly increased in AD cases compared to normals for all regions (Additional file 4: Supplementary Figure 5). Similar to p-PERK, there was no significant difference in the amount of p-eIF2α across the olfactory regions within AD cases. However, the CA1 region had significantly more p-eIF2α+ neurons than the AONb, AONi and EC ((Additional file 3: Supplementary Table 2).

This quantification showed little to no p-PERK+ or p-eIF2α+ neurons in normal cases. Taken together, these data indicate that UPR activation is substantially increased throughout the olfactory and hippocampal regions of AD cases. The amount of activation is also relatively consistent throughout the AONb, AONi and piriform cortex.
Tau and beta-amyloid load is increased in the human olfactory system in AD compared to neurologically normal cases

We next evaluated whether tau and beta-amyloid load were increased in the regions where UPR activation was identified. We found that tau load was significantly increased in all the regions we assessed in AD cases compared to normals (Fig. 3c, Additional file 3: Supplementary Table 3). Similarly, the beta-amyloid load was significantly increased in all regions, except the AONi in AD cases compared to normals (Fig. 3d, Additional file 3: Supplementary Table 3). Within AD cases, tau load in the AONb and AONi was significantly higher than that of the entorhinal cortex, and beta-amyloid load in the AONi was significantly lower than all other regions (Additional file 3: Supplementary Table 4). Together with the p-PERK and p-eIF2α labelling, these data indicate that tau and beta-amyloid pathology are significantly increased in the olfactory regions of AD cases where UPR activation also occurs.

Neurons with p-PERK and p-eIF2α are found in olfactory regions of AD brains with low-grade NFT pathology

To investigate whether UPR activation was related to the disease stage, we grouped the data according to the Braak stage for each case. We found that the percentage of p-PERK+ neurons (Fig. 4a) and p-eIF2α+ neurons (Fig. 4b) were increased in cases with Braak stage III-IV and V-VI pathology compared with Braak stage 0 or I-II. This increase was statistically significant in the piriform cortex and CA1 region for p-PERK and in the AONb, entorhinal cortex and CA1 region for p-eIF2α. There was no significant difference in the percentage of p-PERK+ or p-eIF2α+ neurons between cases with Braak stage III-IV and Braak stage V-VI in any region (Additional file 3: Supplementary Table 5).

A similar result was observed for the percentage of tau+ neurons, which was increased in cases with Braak stage III-IV and V-VI pathology compared to Braak stage 0 or I-II (Fig. 4c). This increase was statistically
significant in the AONb, AONi, piriform cortex, and CA1 region. There was no significant difference in the percentage of tau+ neurons between cases with Braak stage III-IV and Braak stage V-VI in any region (Additional file 3: Supplementary Table 5).

Fig. 4 Quantification of p-PERK+, p-eIF2α+ and tau+ neurons in each region at different stages of pathology. Cases were grouped based on their Braak stage, with stage 0 and stage I-II indicating normal cases with no or very minimal tau pathology respectively, stage III-VI indicating AD cases with mild, low-level tau pathology and stage V-VI indicating AD cases with advanced, high-level tau pathology. Across all regions assessed, the percentage of p-PERK+ neurons (a) and p-eIF2α+ neurons (b) was increased in cases with Braak stage III-VI pathology compared to Braak stage 0 or I-II. This increase was statistically significant only in the piriform cortex and CA1 region for p-PERK+ and in the AONb, entorhinal cortex and CA1 region for p-eIF2α+. There was no significant difference in p-PERK+ or p-eIF2α+ neurons in cases with low Braak stage AD pathology (III-IV) compared to high Braak stage AD pathology (V-VI) in any region. (c) Similar results were observed for the percentage of tau+ neurons. *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001 compared to Braak stage 0 in the same region.

p-PERK+ and p-eIF2α+ neurons contain diffuse tau aggregates rather than NFTs

Previous studies have shown UPR activation is limited to neurons that contain diffuse tau rather than dense NFTs and we sought to determine if this was the case for the
olfactory regions. In all the regions assessed, NeuN+ cells that contained both tau and p-PERK or p-eIF2α contained diffuse tau aggregates rather than dense NFTs (Fig. 5a - d). The percentage of p-PERK+ cells (Fig. 5e) or p-eIF2α+ cells (Fig. 5f) that contained tau was relatively low and was not significantly different across the regions assessed (Additional file 3: Supplementary Table 6 and 7).

The co-labelling of tau and UPR markers was also examined as the percentage of total tau+ cells that contained either p-PERK or p-eIF2α. A very low proportion of the tau+ cells contained p-PERK (Fig. 5g) or p-eIF2α (Fig. 5h). Across the regions, the percentage of total tau+ cells that contained either p-PERK or p-eIF2α was significantly higher in the CA1 region compared to the AONi and EC for p-PERK, and the AONi alone for p-eIF2α.
The amount of p-PERK and p-eIF2α in olfactory regions is not associated with the amount of tau or beta-amyloid

We further investigated whether there is a correlation between tau or beta-amyloid load and the amount of UPR activation in AD cases. There was no significant correlation between the percentage of p-PERK+ or p-eIF2α+ neurons and tau load in any of the regions assessed (Fig. 6a – e). Furthermore there was no significant correlation between percentage of p-PERK+ or p-eIF2α+ neurons and the percentage of tau+ neurons in any region, other than the entorhinal cortex where there was a significant positive correlation between the percentage of p-eIF2α+ neurons and tau+ neurons (Additional file 4: Supplementary Figure 4). There was also no significant correlation between the percentage of p-PERK+ or p-eIF2α+ neurons and beta-amyloid load in the AONb, AONi, EC, and CA1 (Fig. 6f – j). However, there was a significant moderate positive correlation between the percentage of p-eIF2α+ neurons and beta-amyloid load in the piriform cortex (Fig. 6h). Overall it was determined that for AD cases there was no consistent relationship between aggregate load and UPR activation in the regions assessed.

Discussion

In this study, we provide the first evidence that the UPR is activated throughout the human olfactory system in AD. As olfactory deficits are an early and prevalent symptom of AD and pathological tau and beta-amyloid aggregates accumulate in the olfactory bulb very early in the disease progression, our data implicate the UPR as a potential mechanism contributing to the first pathological changes in AD. It is important to acknowledge that like all post-mortem studies, we have reported findings at the end-stage of the disease and our conclusions on the temporal relationship between UPR activation and AD progression are based on the assumption that early or pre-symptomatic AD is reflected by lower stages of brain pathology as indicated by Braak staging.

In the olfactory bulb, we observed that markers of UPR activation p-PERK and p-eIF2α were predominantly clustered in the AON and mitral cells. The AON is easily delineated as a cluster of large nuclei within a distinct region of PGP9.5 immunoreactivity [42]. The mitral cell layer is more difficult to delineate due to the irregular layer structure of the human bulb, the discontinuity of the mitral cell layer and absence of markers to distinguish mitral from tufted cells in human tissue [5, 21, 27]. However, we identified p-PERK and p-eIF2α immunoreactivity in cells that matched the regional and
morphic description of mitral cells [5, 24, 28, 41]. These regions where the UPR activation was clustered is also where tau and amyloid aggregates accumulate in the AD bulb. The specificity of this distribution suggests that UPR activation and pathological aggregate deposition are linked, with the AON neurons and mitral cells being particularly sensitive to this form of ER stress and aggregate production.

The AON is a structure of particular interest in neurodegenerative diseases such as AD, Parkinson’s disease (PD), dementia with Lewy bodies, amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Olfactory deficits are a feature of all these diseases and protein aggregates including tau, beta-amyloid, alpha-synuclein and TDP43 specifically accumulate in the AON of the olfactory bulb [30, 36, 45, 46]. The reason for this specificity of aggregate accumulation is unknown, but it suggests the AON might be particularly vulnerable to dysfunction and that it might underlie olfactory symptoms. Indeed, aggregate accumulation in the AON is postulated to occur in the same presymptomatic window that the olfactory deficits arise in AD and PD [7, 8, 23]. There is also evidence that AON cell loss occurs in AD and PD; however, these studies should be repeated using modern volumetric stereological counting techniques [14, 24, 34]. The degree of cell loss is correlated with the severity of olfactory deficits in PD and while this has not been investigated in AD, it indicates that AON neurodegeneration could be a physical underpinning of olfactory dysfunction [34]. Our results show tau and beta-amyloid load in the AON is similar between AD cases with Braak stage III-IV and Braak stage V-VI pathology. This adds support to previous evidence showing aggregate deposition in the AON early in AD progression.

The specificity of UPR activation in regions affected by disease aggregates is corroborated in other post-mortem studies. For example, UPR activation was identified in the brainstem of progressive supranuclear palsy (PSP) cases, but not AD cases. It was also identified in the hippocampus and frontal cortex of AD cases, but not PSP cases [43]. Similarly in PD, UPR activation has been demonstrated in the substantia nigra where alpha-synuclein aggregates accumulate [17]. Our data similarly show that the UPR is activated in olfactory areas of the AD brain that are also affected by tau and amyloid aggregation. However, while the olfactory and hippocampal areas we studied show similar tau and amyloid load in AD cases, significantly more CA1 neurons contained p-eIF2α than AON neurons. This suggests that additional cell stress pathways may contribute to UPR activation in CA1 neurons. CA1 neurons are particularly vulnerable to energy deprivation, which is a stress factor that can induce eIF2α phosphorylation [33, 38]. This regional vulnerability to UPR activation may contribute to the pattern of cell loss and degeneration observed in AD.

When we assessed p-PERK and p-eIF2α across the olfactory regions in AD cases with different Braak stages of tau pathology, we found low grade (stage III-IV) AD cases had equivalent amounts of UPR activation to high grade (stage V-VI) AD cases. Braak staging is an indication of disease severity based on the assumption of a conserved pattern of progressive aggregate deposition and atrophy across the brain [6]. As Braak stage III and IV indicate cases where the neocortex is mostly spared from aggregate pathology, our results indicate that olfactory regions are affected by aggregates very early in the disease progression. Our identification of UPR activation in low Braak stage cases agrees with previous evidence of UPR activation in the AD hippocampus of low Braak stage cases and supports the idea that UPR activation occurs early in the disease progression [18]. While initial activation of the UPR may have a neuroprotective role to restore proteostasis in response to misfolded protein accumulation, evidence suggests that prolonged activation could contribute to neurodegeneration [19]. This sustained activation of the UPR produces a chronic inhibition of protein translation by p-eIF2α that leads to reduced production of synaptic proteins and a subsequent loss of synapses [29, 39, 40]. In addition, p-eIF2α increases the translation of specific mRNAs, such as BACE1 which is involved in the production of beta-amyloid and ATF4 which induces the expression of genes related to ER chaperone production, autophagy and apoptosis [16]. PERK activation has also been linked to tau phosphorylation by increased activity of GSK-3β, and p-PERK colocalisation with GSK-3β is observed in neurons in AD [10, 18, 26, 35]. It remains to be investigated whether chronic UPR activation leads to synaptic dysfunction in OB regions. Therefore, identifying substantial UPR activation in olfactory regions of cases with low Braak stage pathology suggests a role for this cellular stress response in both the neurodegenerative process and the formation of pathological aggregates in AD.

Many aspects of the relationship between the UPR and AD aggregate pathology remain unclear. While the evidence above indicates that the production of tau and beta-amyloid pathology can be a down-stream consequence of UPR activation, tau may also play an upstream role in UPR activation. A study by Wiersma et al., 2019, used in-vitro and and in-vivo tau seeding models to show that the development of tau pathology induces the formation of granulovacuolar degeneration bodies (GVBs) which contain p-PERK and p-eIF2α, similar to what is seen in the human AD hippocampus [49]. They show a strong positive correlation between the amount of tau pathology developed as a consequence of increased tau seed treatment and the amount of GVBs.
that developed, therefore providing strong evidence that
GVBs containing UPR activation markers develop as a
consequence of intracellular tau pathology. Similarly, a
recent report shows that a 35 kDa C-terminal fragment
of tau containing all four microtubule-binding repeats
can induce UPR activation through PERK in Chinese
hamster ovary cells, unlike the full-length 4R tau isoform
[15]. There are also reports that oligomeric beta-amyloid
is able to activate the UPR in-vitro [9, 25]. In contrast,
our data indicate that co-expression of activated UPR
markers and tau within the same cell is low, and when it
was observed, those cells contained diffuse tau immuno-
reactivity rather than dense NFTs. This observation was
consistent across all the olfactory and hippocampal re-
regions we assessed and is in agreement with previous
studies [18, 32, 43]. The low incidence of co-expression
specifically with diffuse tau suggests that UPR activation
precedes tau pathology, particularly the development of
diffuse tau aggregates into NFTs. It also implies that
UPR must be no longer be activated in cells with dense
NFTs or that it never activated in cells with NFTs
[43]. Furthermore, our quantitative assessment did not
find significant correlations between UPR activation and
tau or beta-amyloid load in the olfactory regions of AD
cases, indicating that cases with higher aggregate loads
did not necessarily have more UPR activation. In accord-
ance with current hypotheses, this result could be inter-
preted in two ways. Firstly, that UPR activation occurs
early in the development of tau and beta-amyloid path-
ology and declines or plateaus as aggregate loads in-
crease or secondly, that soluble tau load contributes to
UPR activation in the AD olfactory system rather than
aggregated tau. There is indeed evidence that soluble
tau may indirectly activate the UPR through cytoplasmic
processes that lead to inhibition of protein degradation
and therefore the accumulation of misfolded proteins [1,
40, 43]. These interpretations, in line with current litera-
ture, support the conclusion that UPR activation occurs
in the earliest stages of tau and beta-amyloid pathology,
but it remains unclear whether tau aggregates activate
the UPR directly.

UPR activation in the olfactory system may also be a
mechanism of degeneration that is common to other
tauopathies where olfactory dysfunction occurs. Tau ag-
gregates occur abundantly alongside alpha-synuclein
pathology in the AON of cases with PD and Dementia
with Lewy bodies [30]. Olfactory deficits are also an early
and prevalent symptom in these diseases (reviewed by
[36]). In other tauopathies such as PSP, corticobasal de-
genation, ALS and FTD, olfactory symptoms are preva-
lent but mild, and tau pathology occurs throughout the
brain yet there is very little or none in the olfactory bulb
[36, 46]. Post-mortem studies have demonstrated that
UPR activation occurs throughout the brain in these
diseases including PSP, PD, ALS, and FTD variants with
tau pathology [17, 22, 32, 43]. In our study, we noted that
neurologically normal cases with Braak stage I-II show
low levels of tau load and UPR activation in the olfactory
regions, whereas those Braak stage 0 cases did not show
any tau or UPR activation. Thus, UPR activation appears
to occur in diseases with concomitant tau pathology and
diseases where tau accumulation occurs in the olfactory
bulb tend to show a greater severity of olfactory deficits.
Therefore, it would be interesting to investigate whether
UPR activation in the olfactory bulb is a common process
in these diseases and if the amount of activation is relative
to the AON tau burden in each disease.

In conclusion, we have demonstrated that UPR activa-
tion occurs in the olfactory system of AD cases with
low-level tau pathology, indicative of the early disease
stage. Within the olfactory bulb, UPR activation pre-
dominantly occurred within the anterior olfactory nu-
cleus which is heavily affected by tau and beta-amyloid
pathology from the earliest stages of AD. Together, these
data indicate that UPR activation in the olfactory system
may contribute to the earliest changes in AD.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40478-020-00986-7.

Additional file 1. Correlation between percentage of p-PERK+ or p-
eIF2α+ neurons with age at death and post-mortem delay
Additional file 2. Distribution of p-PERK+ and p-eIF2α+ neurons relative
to beta-amyloid plaques in the AON. Immunohistochemistry for p-IRE1α
and CK1δ in the AON and CA1 region.
Additional file 3. Summary of quantitative results and statistics
Additional file 4. Correlation between percentage of p-PERK+ and p-
eIF2α+ neurons and percentage of tau+ neurons in AD cases. Graph of
p-PERK+ and p-eIF2α+ cell density in regions of the human olfactory sys-
tem in normal and AD cases.

Abbreviations
AD: Alzheimer’s disease; ALS: Amyotrophic lateral sclerosis; AON: Anterior
olfactory nucleus; ATF4: Activating transcription factor 4; ATF6: Activating
transcription factor 6; BiP/GRP78: Binding immunoglobulin protein; CHOP: C/
EBP-homologous protein; eIF2α: Eukaryotic Initiation Factor 2α; ER:
Endoplasmic reticulum; FTD: Frontotemporal dementia; IRE1α: Inositol-
requiring enzyme 1α; NFT: Neurofibrillary tangle; PD: Parkinson’s disease;
PERK: Protein kinase R (PKR)-like endoplasmic reticulum kinase;
PGRP9.5: Protein Gene Product 9.5 (also known as ubiquitin C-terminal hy-
drolyase 1, (UCHL-1)); PSP: Progressive supranuclear palsy; UPR: Unfolded protein
response

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Authors’ contributions
H.C.M and L.B. contributed to the conception and design of the experiments. Tissue processing was performed by H.M., M.A.C., B.V.D., and R.L.M.F. Pathological examination was performed by C.T. Image analysis was carried out by H.M. and M.E.V.S. Image analysis was carried out by H.M. and M.E.V.S. with critical revision by A.K. The manuscript was prepared by H.M. with critical revision by M.A.C. and feedback from all authors. All authors have approved the final manuscript.

Availability of data and materials
The data used for this study is available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

Author details
1Department of Anatomy and Medical Imaging and Centre for Brain Research, Faculty of Medical and Health Science, University of Auckland, Private Bag 92019, Auckland, New Zealand. 2Laboratory of Functional and Molecular Imaging, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA. 3Department of Anatomical Pathology, LabPlus, Auckland City Hospital, Auckland, New Zealand. 4Howard Hughes Medical Institute, Chevy Chase, MD 20815, USA.

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