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Progressive tauopathy in P301S tau transgenic mice is associated with a functional deficit of the olfactory system

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Running title: Tg P301S tau mice manifest olfactory dysfunctions

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Abstract

Multiple neurodegenerative disorders with tau pathology are characterized by the loss of memory and cognitive decline that can be associated with other symptoms including olfactory alterations that are often regarded as an early symptom of **the diseases**. Here we have investigated whether olfactory dysfunction is present in the P301S human tau transgenic mice and if it is associated to tau pathology. Progressive tauopathy and neurodegeneration were noticeable in the olfactory bulb and piriform cortex at early age in
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the P301S human tau transgenic mice and olfactory sensitivity for social or non-social odours was significantly impaired at 3 months of age, when the piriform cortex-dependent odour-cross habituation was also disrupted. The olfactory alterations in the P301S tau transgenic mouse line provide an *in vivo* system where to test mechanism-based therapies for the common and yet untreatable tauopathies.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder yet there is no effective treatment for it. Neurofibrillary tangles consisting of hyperphosphorylated tau protein are one of the major neuropathological hallmarks in AD (Spillantini & Goedert, 2013). The spatiotemporal progression of tau pathology correlates well with the progression of neurodegeneration (Braak & Braak, 1991) and dementia (Giannakopoulos *et al.*, 2003; Bierer *et al.*, 1995; Petersen *et al.*, 2006). The topographical distribution of neurofibrillary tangles is used for the pathological diagnosis of AD (Braak & Braak, 1991).

Olfactory dysfunction is one of the earliest symptoms of AD, often preceding the classical cognitive impairments by many years (Hawkes, 2003; Forster *et al.*, 2010). It has been reported that olfactory perceptual deficits are correlated with a higher risk of developing mild cognitive impairment and severity of cognitive dysfunction, which is also considered a prodromal symptom for early diagnosis of AD (Wilson *et al.*, 2009; Walla *et al.*, 2011). In many post-mortem studies of AD patients, tau pathology has been described in the olfactory system such as the olfactory bulb, the anterior olfactory nucleus and the piriform cortex (Attems *et al.*, 2014; Saiz-Sanchez *et al.*, 2014), that are associated with the Braak staging and indicative of a high risk of cognitive decline (Attems & Jellinger, 2006). **Non-AD human tauopathies such as frontotemporal dementia/Pick's disease, corticobasal degeneration and progressive supranuclear palsy have been reported to show olfactory alterations associated with progression of the pathology, leading to olfactory dysfunction** (Yoshimura, 1988; Luzzi *et al.*, 2007; Pardini *et al.*, 2009; Silveira-Moriyama *et al.*, 2010).

We hypothesized that **the** olfactory neural system can be affected by tau pathology in our **transgenic P301S tau mice, a model of tau aggregation and we decided to investigate whether tau pathology correlated with olfactory dysfunction and neurodegeneration. The P301S tau mice express human mutant P301S tau under the control of the murine *Thy1.2* promoter. In humans, the P301S mutation in the MAPT gene leads to a progressive tauopathy, causing frontotemporal dementia (Bugiani *et al.*, 1999). This**

mouse model presents progressive tau pathology until the age of 5-6 months when the mice have to be culled due to their motor phenotype (Allen *et al.*, 2002; Delobel *et al.*, 2008). The presence of olfactory dysfunction related to tau pathology and related neurodegeneration would provide an *in vivo* model where **one can** test mechanism-based therapies for the common and yet untreatable tauopathies.

Materials and Methods

Animals

Homozygous transgenic P301S (Tg P301S) tau (Allen *et al.*, 2002) and C57BL/6S (C57BL/6 OlaHsd; Harlan, UK) control mice were used for this study. Only male mice were used in the experiments. All procedures were performed in accordance with the UK Home Office Regulations for the Care and Use of Laboratory Animals and the UK Animals (Scientific Procedures) Act 1986 and were approved by the Cambridge University local ethical committee.

Immunohistochemistry

Sample preparations and the general procedures of immunostaining have been described previously (Yang *et al.*, 2015). Thirty µm free floating sections were immunostained with the monoclonal anti-phospho tau antibody AT8 (1:1000, Innogenetics) or NeuN antibody (1:400, Millipore). The immunostaining was visualized with an avidin-biotin system (Vectastain®ABC kit; Vector Laboratories) and 3′,3′diaminobenzidine as the chromogen (DAB kit; Vector Laboratories). Tissue sections were mounted on glass slides and examined using a light microscope and photographed using a digital camera (Leica DM6000 Microsystems). The characteristics of tau pathology that were determined in transgenic P301S tau mice were compared to age-matched wild type control mice at 1, 2 and 5 months of age (C57BL/6S, Harlan laboratory). The time course of neuronal cell loss was investigated in Tg P301S tau mice and age-matched wild type control mice at 1, 3 and 5 months of age (1 month; Control n=3-6, P301S n=3-6; 3 months; Control n=3-4, P301S n=3-4; 5 months; Control n=4-7, P301S n=6-7).

Stereology

Stereological techniques were used in quantifying cells, and the counts were made in 2-dimensional space. For cortical counting, a total number of 5 sections per animal taken every 12th brain sections were used after immunostaining with NeuN antibody. NeuN-positive neurons were counted in layer I-II and layer III of the piriform cortex using the optical fractionator method of stereological counting with commercially available stereological

software (Stereo Investigator; MBF Bioscience) as previously described (Yang *et al.*, 2015). The **piriform cortex was divided into anterior and posterior divisions and the lateral olfactory tract (LOT) was used as a landmark (Ekstrand *et al.*, 2001).** The total number of mitral cells in the olfactory bulb was similarly estimated using Nissl staining to identify the cap-shaped cell soma in the mitral cell layer. Sample counting windows were randomly identified using the computer software after setting the parameters for **the** counting frame and the counting grid at 40 µm x 40 µm and 200 µm x 200 µm (cortex), 60 µm x 60 µm and 120 µm x 120 µm (olfactory bulb), respectively. The number of cells in each window was counted **using a 20x objective**.

Olfactory sensitivity test

For consistency only male mice were investigated, and they were singly housed for at least 2 weeks prior to the test and their home cages were used as a testing chamber. The mice were repeatedly exposed to filter paper saturated with mineral oil 5 times (habituation). Each presentation lasted for 2 min with an interval of 1 min among presentations. The filter paper with olfactory stimulus diluted in mineral oil (1-butanol and vanilla) or **distilled water (female urine)** was hung on the grid of their home cage. **Female urine was pooled from five non-cagemates. Urine was collected by holding the mouse by the scruff of the neck and stored in 50 µl aliquots at -80 °C till use.** The olfactory stimulus was presented in an ascending order of concentration. The concentration of odour at which the animals showed snout-oriented sniffing within 1 cm from the origin of the odour was recorded. Tg P301S tau mice and wild type control mice were tested at 1 month (1-butanol: Control n=5, P301S n=7, vanilla: Control n=5, P301S n=6), 3 months (1-butanol: Control n=7, P301S n=7, vanilla: Control n=6, P301S n=5) and 5 months (1-butanol: Control n=13, P301S n=10, vanilla: Control n=7, P301S n=6) of age. Four month-old animals were used to test the behaviour for the social odour (female urine: Control n=17, P301S n=12).

Odour cross-habituation test

The olfactory memory was tested in 3 month-old P301S and control mice using an odour cross-habituation test, which was modified from the previously published protocol (Wesson *et al.*, 2010). Odours used in this study (n=3; limonene, pentanol and heptanone; Sigma-Aldrich) were diluted 1x 10^{-3} into mineral oil and 20µl of odour was applied to 3MM filter paper (15cm x 1.5cm; Whatman). This test was performed during the dark phase of 12 hour light/dark cycle under **a** red light in order to minimise any visual attraction or anxiety during the test. Odours were presented three successive times for 2 minutes, separated by 30

seconds intervals by hanging the filter paper to the cage grid. The presentations of odours were counterbalanced in testing groups. The time for active investigation of odours was defined as snout-oriented sniffing within 1cm of the origin of the odour. The raw investigatory values were normalised to the maximum investigatory duration during three trials for each odour. Discrimination index was calculated by subtracting the normalised duration of the previous trial odour investigation (3rd trial) from that of the following 1st trial presentation.

Statistical analyses

Statistical analyses were performed by unpaired two-tailed *t* tests using GraphPad Prism version 5.0 and one-way ANOVA was used for group analysis. **Results of odour-cross habituation test were analyzed in SAS v9.1 by a three-way repeated measure ANOVA implemented in a mixed model using *Genotype* as the between-subject factor and *Trial* and *Odour* as within-subject factors, respectively. Power transformation was applied on residuals to obtain normality. *Post-hoc* analysis was conducted using Tukey–Kramer to correct for multiple comparisons. Correlation coefficient analysis was performed in MATLAB R2015b using the following equation:**

$$\rho(A,B) = \frac{1}{N-1}\sum_{i=1}^{N}\left(\frac{A_i-\mu_A}{\delta_A}\right)\left(\frac{B_i-\mu_B}{\delta_B}\right)$$

Where μ_A and μ_B are the means of the groups A and B; δ_A and δ_B are the standard deviations of A and B. When the *p* value was 0.05 or less it was regarded as statistically significant.

Results

Early appearance of hyper-phosphorylated tau in olfactory system of Tg P301S tau mice

Tau pathology in olfactory structures including **the** olfactory bulb and piriform cortex was investigated by immunohistochemistry in homozygous Tg P301S tau mice at 1, 2 and 5 months of ages using monoclonal antibody AT8 **which** detects hyper-phosphorylated tau (Fig 1). Progressive tauopathy in other parts of the nervous system such as cortex and spinal cord has been well documented throughout the life span of Tg P301S tau mice but tauopathy in olfactory structures has not been thoroughly described. **In the olfactory bulb of Tg P301S tau mice the mitral cell layer** was dominantly affected by tau pathology already at 1 month of age, displaying neuronal cell bodies containing hyperphosphorylated tau protein and abnormal dystrophic neurites (Fig 1A-D). No staining was observed with AT8 antibody in age-matched wild type control mice (Fig 1E). The mitral cell layer was more

severely affected at 2 months of age in P301S tau mice, showing more neuronal cell bodies and the processes were intensely stained by AT8 antibody (Fig 1F-I). At 2 months of age hyper-phosphorylated tau also appeared in the frontal association cortex. This was not consistent at 1 month of age (Fig 1J). At 5 months of age, P301S tau mice presented AT8 staining throughout all layers of the olfactory bulb including **the** granule cell layer, mitral cell layer and external plexiform layer (Fig 1K-M and P). **A small proportion of tuft cells, as well as cells in the anterior olfactory nucleus were also labeled with AT8 antibody at 5 months of age (data not shown).** The frontal association cortex was heavily stained with many AT8-positive neurons, consistent with progressive tauopathy found throughout the cortex of 5 month-old P301S mice (Fig 1N) (Delobel *et al.*, 2008). No AT8 immunostaining was observed in age-matched wild type control mice also at 2 and 5 months of age (Data not shown). These observations indicate that hyperphosphorylated tau appeared early in the olfactory bulb in Tg P301S human tau mice. **These findings in P301S tau mice thus reflect in part the neuropathology in the human AD brain, showing neurofibrillary tangles in all layers of the olfactory bulb including the mitral cell layer and external plexiform layer (Kovacs *et al.*, 1999). However there was no significant neuronal reduction in mitral cell counts in the olfactory bulb compared to aged-matched control mice at the time of observation (5 months, Control $3.1x10^4 \pm 1612$ *vs* P301S $2.9x10^4 \pm 1425$, unpaired *t*-test, $p>0.05$).**

Progressive neurodegenerative tauopathy in the piriform cortex of Tg P301S mice

The most affected area in the olfactory bulb seemed to be the mitral cell layer and external plexiform layer, which consist of primarily glutamatergic neurons that project to the main olfactory cortex such as piriform cortex. The excitatory signals are transmitted to the amygdala and hippocampus via the piriform cortex. Anatomically, the piriform cortex locates at the base of the latero-ventral area of the rodent brain and consists of two subdivisions, the anterior piriform cortex (aPC) and the posterior piriform cortex (pPC) (Fig 2A). The **anterior piriform cortex was distinguished from the posterior cortex based on the division made where the lateral olfactory tract (LOT) disappears on the ventral surface of the brain (Fig 2A)(Ekstrand *et al.*, 2001).**

Progressive tau pathology was detected in the piriform cortex with AT8 antibody at 1 month, 3 months and 5 months of age in Tg P301S tau mice (Fig 2A-F). The AT8-postive neurons were mostly found in layer II while they were sparse in layer III of the piriform cortex. They were more abundant in the posterior part than the anterior part throughout the ages tested (Fig 2). At 5 months of age P301S tau mice revealed severe tau pathology with intense AT8 staining in the piriform cortex (Fig 2C and F). This was associated **with** neuronal cell loss as

shown by stereological quantification of NeuN-positive neurons in the piriform cortex (Fig 3). The quantification was separately performed in layer I-II and layer III in the anterior and posterior piriform cortex at 1, 3 and 5 months of age and comparing P301S tau and wild type control mice (Fig 3E and J). The neuronal loss in **the** piriform cortex was noticeable from the age of 3 months in P301S tau mice, **showing a 25% reduction** compared to age-matched control mice (Fig 3E and J). Interestingly, the progression of neuronal loss did not proceed in the same manner in **the** anterior and posterior piriform cortex. While no further reduction was observed in layer I-II in the anterior part, an increase from 25% to 42% was observed in the same layers in the posterior piriform cortex at 5 months of age (Fig 3E and J).

The piriform cortex layer III was in general, less affected by tau pathology compared to layer II, since AT8-positive neurons were sparsely found in layer lll even at the advanced stage of disease (Fig 3A-D). Moreover, no significant neuronal loss was found in layer lll of **the** anterior piriform cortex at any age tested, **whilst** 13% of neuronal loss was found in layer III of the posterior piriform cortex in mice at 5 months of age. Immunohistochemistry using NeuN antibody showed that layer II and III in the posterior piriform cortex became thinner due to the neurodegeneration in the P301S tau mice compared to the control mice at 5 months of age (Fig 3F-I). However, the corresponding area in the anterior piriform cortex was largely intact (Fig 3A-D). The neuronal loss was more pronounced in the posterior piriform cortex compared to the anterior part and suggests that the posterior piriform cortex could be more vulnerable to neuropathological changes as seen also in human tauopathy (Saiz-Sanchez *et al.*, 2014). **Only a low level of AT8-postive staining was observed in other olfactory structure such as the olfactory tubercles (data not shown).**

Impairment of olfactory functions in Tg P301S tau mice

Olfactory sensitivity

The presence of hyperphosphorylated tau and subsequent reduction in neuronal cell counts in the piriform cortex lead us to question whether the Tg P301S tau mice had normal olfactory functions. Therefore, we tested two different aspects of olfaction, olfactory sensitivity and odour-cross habituation behaviour.

Olfactory sensitivity was performed using non-social odours (e.g. 1-butanol and vanilla) and social odour (e.g. female urine) using the ascending staircase method to test P301S tau mice and age-matched control mice (Fig 4A-D). Tg P301S tau mice showed a significant reduction in olfactory sensitivity towards both social and non-social odours at 3 months of age. For non-social odour **using** 1-butanol, which is a less favoured scent, P301S tau mice presented a clear deficit at 5 months of age (Fig 4A, unpaired *t*-test, ***p<0.001) **whilst with**

non-social odour vanilla, which is a neutral scent, the P301S tau mice showed progressive impairment in odour sensitivity starting at 3 months (unpaired *t*-test, **p*<0.05) and established a more robust deficit at 5 months of age (Fig 4B, unpaired *t*-test, ****p*<0.001). In order to confirm the de-sensitised olfaction, male P301S tau mice were exposed to female urine, which is generally a strong social odour for male mice. Olfactory sensitivity declined dramatically in P301S tau mice at the age of 4 months (Control 21 ± 0.39 *vs* P301S 14.2 ± 2.1, unpaired *t*-test, ****p*<0.001, Fig 4C). Olfactory exploratory behaviour clearly demonstrated that P301S tau mice displayed no significant explorative behaviour towards the weak stimulus represented **by highly diluted urine (0.0001% v/v) compared to wild type control mice (Control 7.1 ± 2.2 *vs* P301S 1.16 ± 0.47, unpaired *t*-test, **p*<0.05).** Whereas the P301S tau mice spent the same amount of time exploring high concentration of female urine (1% v/v) compared to the wild type control mice (Fig 4D).

Odour-cross habituation was disrupted in Tg P301S tau mice

Neuronal cell loss and **the** heavy load of tau pathology were significant in the piriform cortex, especially in its posterior part in the P301S tau mice (Fig 3). Therefore the Tg P301S tau mice with their age-matched controls were subjected to the odour-cross habituation test, which has been suggested to be a piriform cortex-dependent task for rodents (Linster *et al.*, 2009). When the wild type control mice with intact olfactory function were repeatedly exposed to the same scent their olfactory explorative behaviour was dramatically reduced at the second and third exposure to the scent (habituation status) and when a novel scent was introduced to them they showed a high level of explorative behaviour to the scent (dis-habituation status) (Fig 4E). Three different odours were counterbalanced for testing each animal (e.g. limonene, heptanone, pentanol). **There was a significant genotype effect in response to new odour exposure ($F_{2, 48}$=4.61, *p*=0.015). This was supported by the discrimination index (Control 0.72 ± 0.07 *vs* P301S 0.14 ± 0.19, *p*=0.043), which indicates that the normal pattern of odour-cross habituation was disturbed in the Tg P301S tau mice (Fig 4E). P301S tau mice showed an incomplete habituation for each odour and compromised dis-habituation and were less able to discriminate a novel scent from the previously exposed scent (Fig 4E). In order to compare the similarity of odour-cross habituation between the wild type control and the Tg P301S tau mice, we performed a correlation coefficient analysis and found no significant association between the two genotypes (*r*=0.61, *p*>0.05). In line with this, a three-way repeated measure ANOVA was used to examine the effect of odour, trial, and genotype on the time for active investigation and there was a significant three-way interaction ($F_{4, 48}$=2.99, *p*=0.028).** This abnormal odour-cross habituation behaviour is also indicative of impairment in olfactory memory, which can be attributed to tau pathology and neuronal

dysfunction in the piriform cortex, amygdala and perirhinal/entorhinal cortex present in the P301S tau mice.

Discussion

There are several studies reporting that the olfactory neural circuit is affected very early on and could be a disease-starting point in major brain disorders such as AD and PD (Doty, 2012; Alves *et al.*, 2014). Since the loss of olfactory function is one of the common symptoms in neurological brain disorder and often regarded as a preclinical symptom in demented diseases, this area has been also considered as a therapeutic target (Wilson *et al.*, 2009; Forster *et al.*, 2010; Alves *et al.*, 2014). This study presents an *in vivo* model of human tauopathy with olfactory neural circuitry displaying tau aggregation, neural degeneration and olfactory dysfunction. In an animal model overexpressing a mutated form of the human amyloid-beta precursor protein, a correlation between the extracellular loads of amyloid and the olfactory deficit assessed by olfactory cross-habituation test has been described (Wesson *et al.*, 2010). Similarly, in a transgenic mouse overexpressing human tau, Tα1-3RT mouse line, an olfactory functional deficit has been shown using olfactory habituation test (Macknin *et al.*, 2004). Our studies **with** the Tg P301S tau mice overexpressing human mutant tau (4R0N P301S) **under the murine *Thy1.2* promoter,** suggest that their olfactory system is vulnerable to hyperphosphorylated tau and it **could** be one of the brain regions targeted early **on** by tau pathology.

Our immunohistochemical studies with the anti-hyperphosphorylated tau antibody AT8 in Tg P301S tau mice, show that the mitral cells are the most affected neuronal population in the olfactory bulb, with intracellular tau deposits in the soma, as well as dystrophic neurites projecting through in the external plexiform layer. The synaptic processing in the external plexiform layer, between the glomerular layer and the mitral cell layer is known to be critical in the piriform cortex for perception of odour (Wilson *et al.*, 2014). Therefore the early tau hyperphosphorylation in these areas of the olfactory bulb could initiate the synaptic dysfunction that progresses together with neuronal cell loss in the piriform cortex of the Tg P301S tau mice. **It is possible that the *Thy* 1.2 promoter drives higher expression in mitral cells in this P301S tau model, however, neurofibrillary tangles have been described in mitral cells in Alzheimer's disease and also in normal aging** (Kovacs *et al.*, 1999)**, indicating that these cells are susceptible to develop tau pathology also in human brain.**

Functional impairments in odour sensitivity and discrimination became more severe when the neuronal cell loss in the piriform cortex co-exists with a synaptic deficit in the olfactory

bulb. The glutamatergic mitral cells mainly project to the primary cell layer of the piriform cortex (layer II), where discrete neurodegeneration and tau pathology was observed, reflecting the selective vulnerability of different neuronal populations in this mouse model of human tauopathy. **It was reported that strong afferent inputs from olfactory bulb are found in layer II principal neurons in the piriform cortex, whereas neurons in layer III received considerably more intra-cortical inhibition (Large *et al.*, 2016). In fact, different subsets of inhibitory GABAergic interneurons have been described in the piriform cortex. The interneurons in layer II express predominantly calretinin and vasoactive intestinal peptide, whilst those in layer III mainly express calbindin and parvalbumin (Suzuki & Bekkers, 2007).**

Moreover, the neurodegeneration seemed to progress more rapidly in the posterior piriform cortex compared to the anterior part, suggesting that the posterior piriform cortex could be more vulnerable to the neuropathological changes occurring in human tauopathy. The impaired odour quality coding, leading to olfactory deficits in AD correlates with neurological lesions in **the** posterior piriform cortex based on functional magnetic resonance imaging performed during odour cross-habituation test (Li *et al.*, 2010). Recent neuropathological studies in brains of AD patients have also demonstrated the preferential vulnerability of layer II among the three layers of piriform cortex and of the posterior piriform cortex (in human brain, temporal lobe of piriform cortex) for AD's pathology (Saiz-Sanchez *et al.*, 2014). The posterior piriform cortex presents complex connections to major adjacent cortices including perirhinal cortex, entorhinal cortex and amygdala, and these **are the** regions **in the brain** most affected in AD. The 40% reduction in NeuN positive neuronal cells in the piriform cortex was more extensive than what we have observed in other cortices of P301S tau mice such as **the** motor cortex (Hampton *et al.*, 2010) and the perirhinal cortex, where approximately 25% of neuronal loss was found at 5 months of age, presenting an advanced stage of pathology in P301S tau mice (Yang *et al.*, 2015).

The olfactory deficit was present at 3 months of age, in parallel with impaired object recognition memory, which is perirhinal cortex-dependent (Yang *et al.*, 2015) and declined motor coordination (Scattoni *et al.*, 2010), at the time when hippocampal-dependent spatial learning was normal in P301S tau mice (Scattoni *et al.*, 2010). The object-placement memory, which is also hippocampal-dependent, was not impaired in 3 month-old P301S tau mice (SY's unpublished data). Thus, the olfactory dysfunction including impaired odour discrimination observed in 3 month-old P301S tau mice are likely to be associated with the neurodegenerative tauopathy in the piriform cortex than hippocampus.

These results together with the early detection of the pathological form of tau protein in the olfactory bulb suggest that the olfactory system is more susceptible to tau pathology when compared to the other cerebral cortices in the P301S tau mice. In particular the posterior piriform cortex of Tg P301S tau mice will be a good model where functional deficits, observed also in patients with AD, can be correlated with progressive tau-related neurodegeneration. The olfactory tau pathology may contribute to the early alterations of olfactory functions that constitute the classical disease symptoms of early AD (Wilson *et al.*, 2009; Attems *et al.*, 2014). To our knowledge this is the first study on the effect of progressive tau pathology and neurodegeneration in the olfactory system and its association with olfactory functional impairment. Previous studies had reported on the influence of Aß on interneurons in the piriform cortex of APPxPS1 transgenic mice (Saiz-Sanchez *et al.*, 2013). Future studies will further examine the different susceptibility of neuronal populations to tau pathology in our mouse model of human tauopathy.

The olfactory system has been of huge interest since olfactory dysfunction is a prodromal symptom of many neurodegenerative disorders such as AD and PD. Moreover, the olfactory system has been proposed as a potential site for disease propagation, either anterogradely or retrogradely, in PD (Hawkes *et al.*, 2007). This means **that** the olfactory neural circuitry is the appropriate target for **the** early diagnosis or treatment of the diseases. In this report, we provide the evidence that the olfactory system of Tg P301S tau mice presents early tau pathology, **progressive neurodegeneration in the piriform cortex and olfactory functional deficits.** The olfactory alterations in the P301S tau transgenic mouse line provide an *in vivo* system where to test mechanism-based therapies for the common and yet untreatable tauopathies.

Figure Legends

Figure 1. Early appearance of hyperphosphorylated tau in the olfactory bulb of Tg P301S tau mice.
Immunostaining with phosphorylation dependent AT8 antibody was performed in the mice at 1, 2 and 5 months of age to determine the progression of olfactory tau pathology. AT8 staining in olfactory bulb of (A-D) 1 month-old P301S tau mice (E) 1 month-old wild type control mouse (F-J) 2 month-old P301S tau mice (K-P) 5 month-old P301S tau mice. White dotted lines define the regions. The boxed areas in A, C, F, H, K and N were enlarged in panels B, D, G, I, J, L, M, O and P respectively. GRL: granule cell layer; MCL: mitral cell layer; EPL: external plexiform layer; GL: glomerular layer; ONL: olfactory nerve layer; FrA: frontal association cortex; Scale bar; 100 µm (A, F); 30 µm (B, D, E, G, I, J, L, M, O, P); 1 mm (C, H, K, N)

Figure 2. Progressive tauopathy in the piriform cortex in Tg P301S tau mice.
(A) Anatomical illustration of a mouse brain indicating anterior (aPC) and posterior (pPC) piriform cortices. Areas indicated by asterisks are the corresponding locations of panels B and C. Immunohistochemistry profile of AT8-positive tau pathology at the coronal view of (B) aPC, bregma 0.86 mm (C) pPC, bregma -1.46 mm. White dotted line defined the regions of aPC and pPC. AT8 staining of tau pathology in aPC (D-F) and pPC (G-I) is shown at different ages: 1 month (1M, D, G), 3 months (3M, E, H) and 5 months (5M, F, I). Scale bar; 1 mm for B-C, 50 µm for D-I. OB: olfactory bulb; LOT: lateral olfactory tract; rf: rhinal fissure.

Figure 3. Progressive neuronal cell loss in the piriform cortex in Tg P301S tau mice.
Representative images of NeuN staining and quantification of NeuN-positive cells in aPC (A-E) and pPC (F-J) of 5 months-old mice. Wild type control mice (A, B, F, G) and P301S tau mice (C, D, H, I). Panels B, D, G and I are enlarged images of the boxed areas in panel A, C, F and H, respectively. The neuronal counts of layer I-II and layer III were performed using stereology. (E) There was a significant decrease, 25% in neurons number in layer I-II of aPC at both **3 and 5 month-old P301S** tau mice compared to age-matched control mice. (J) Progressive neuronal cell loss was observed in pPC of P301S tau mice where a 25% and 44% reduction in neurons was observed at 3 and 5 months of age respectively compared to age-matched control mice. Scale bar; 1 mm for A, C, F, H; 50 µm for B, D, G, I. Layer I-II counting: 1M: control n=6, P301S n=6; 3M: control n=4, P301S n=4; 5M: control n=5, P301S n=6. Layer III counting: 1M: control n=3, P301S n=3; 3M: control n=3, P301S n=3; 5M: control n=4, P301S n=6. White bar: Control, black bar: P301S, *p<0.05, **p<0.01 by two-tailed t-test analysis.

Figure 4. Olfactory functional deficit in Tg P301S tau mice.
Olfactory sensitivity test for non-social odours (A) Odour: 1-butanol, 1M: control n=5, P301S n=7; 3M: control n=7, P301S n=7; 5M: control n=13, P301S n=10. (B) Odour: Vanilla, 1M: control n=5, P301S n=6; 3M: control n=6, P301S n=5; 5M: control n=7, P301S n=6. *p<0.05, ** p<0.01, *** p<0.001 by two-tailed t-test analysis. Olfactory sensitivity test for social odour (C) Odour: female urine, control n=17, P301S n=12 (D) Time spent for investigation of female urine **at low (Low, 0.0001% v/v) and high (High, 1% v/v) concentration** by mice at 4 months of age. There were significant deficits in response to highly diluted urine while there was a normal response towards high concentration of urine, control n=4, P301S n=5. White bar: Control, black bar: P301S. (E) Piriform cortex dependant odour-cross habituation test. Odours (n=3; limonene, heptanone, pentanol) were presented in blocks of three successive trials. The raw investigatory values were normalised to the maximum

investigatory duration during three trials for each odour. P301S tau mice displayed an abnormal pattern in habituation and dis-habituation towards novel scents. ***p*<0.05, ** *p*<0.01 by three-way repeated measure ANOVA followed by *post-hoc* analysis using Tukey–Kramer.** Odour A, B, C represent limonene, heptanone, pentanol. Solid line: Control n=3, Dashed line: P301S n=5.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Allen, B., Ingram, E., Takao, M., Smith, M.J., Jakes, R., Virdee, K., Yoshida, H., Holzer, M., Craxton, M., Emson, P.C., Atzori, C., Migheli, A., Crowther, R.A., Ghetti, B., Spillantini, M.G. & Goedert, M. (2002) Abundant tau filaments and nonapoptotic neurodegeneration in transgenic mice expressing human P301S tau protein. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **22**, 9340-9351.

Alves, J., Petrosyan, A. & Magalhaes, R. (2014) Olfactory dysfunction in dementia. *World journal of clinical cases*, **2**, 661-667.

Attems, J. & Jellinger, K.A. (2006) Olfactory tau pathology in Alzheimer disease and mild cognitive impairment. *Clinical neuropathology*, **25**, 265-271.

Attems, J., Walker, L. & Jellinger, K.A. (2014) Olfactory bulb involvement in neurodegenerative diseases. *Acta Neuropathol*, **127**, 459-475.

Bierer, L.M., Hof, P.R., Purohit, D.P., Carlin, L., Schmeidler, J., Davis, K.L. & Perl, D.P. (1995) Neocortical neurofibrillary tangles correlate with dementia severity in Alzheimer's disease. *Archives of neurology*, **52**, 81-88.

Braak, H. & Braak, E. (1991) Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol*, **82**, 239-259.

Bugiani, O., Murrell, J.R., Giaccone, G., Hasegawa, M., Ghigo, G., Tabaton, M., Morbin, M., Primavera, A., Carella, F., Solaro, C., Grisoli, M., Savoiardo, M., Spillantini, M.G., Tagliavini, F., Goedert, M. & Ghetti, B. (1999) Frontotemporal dementia and

corticobasal degeneration in a family with a P301S mutation in tau. *Journal of neuropathology and experimental neurology*, **58**, 667-677.

Delobel, P., Lavenir, I., Fraser, G., Ingram, E., Holzer, M., Ghetti, B., Spillantini, M.G., Crowther, R.A. & Goedert, M. (2008) Analysis of tau phosphorylation and truncation in a mouse model of human tauopathy. *The American journal of pathology*, **172**, 123-131.

Doty, R.L. (2012) Olfactory dysfunction in Parkinson disease. *Nature reviews. Neurology*, **8**, 329-339.

Ekstrand, J.J., Domroese, M.E., Johnson, D.M., Feig, S.L., Knodel, S.M., Behan, M. & Haberly, L.B. (2001) A new subdivision of anterior piriform cortex and associated deep nucleus with novel features of interest for olfaction and epilepsy. *The Journal of comparative neurology*, **434**, 289-307.

Forster, S., Vaitl, A., Teipel, S.J., Yakushev, I., Mustafa, M., la Fougere, C., Rominger, A., Cumming, P., Bartenstein, P., Hampel, H., Hummel, T., Buerger, K., Hundt, W. & Steinbach, S. (2010) Functional representation of olfactory impairment in early Alzheimer's disease. *Journal of Alzheimer's disease : JAD*, **22**, 581-591.

Giannakopoulos, P., Herrmann, F.R., Bussiere, T., Bouras, C., Kovari, E., Perl, D.P., Morrison, J.H., Gold, G. & Hof, P.R. (2003) Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer's disease. *Neurology*, **60**, 1495-1500.

Hampton, D.W., Webber, D.J., Bilican, B., Goedert, M., Spillantini, M.G. & Chandran, S. (2010) Cell-mediated neuroprotection in a mouse model of human tauopathy. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **30**, 9973-9983.

Hawkes, C. (2003) Olfaction in neurodegenerative disorder. *Movement disorders : official journal of the Movement Disorder Society*, **18**, 364-372.

Hawkes, C.H., Del Tredici, K. & Braak, H. (2007) Parkinson's disease: a dual-hit hypothesis. *Neuropathology and applied neurobiology*, **33**, 599-614.

Kovacs, T., Cairns, N.J. & Lantos, P.L. (1999) beta-amyloid deposition and neurofibrillary tangle formation in the olfactory bulb in ageing and Alzheimer's disease. *Neuropathology and applied neurobiology*, **25**, 481-491.

Large, A.M., Vogler, N.W., Mielo, S. & Oswald, A.M. (2016) Balanced feedforward inhibition and dominant recurrent inhibition in olfactory cortex. *Proceedings of the National Academy of Sciences of the United States of America*, **113**, 2276-2281.

Li, W., Howard, J.D. & Gottfried, J.A. (2010) Disruption of odour quality coding in piriform cortex mediates olfactory deficits in Alzheimer's disease. *Brain : a journal of neurology*, **133**, 2714-2726.

Linster, C., Menon, A.V., Singh, C.Y. & Wilson, D.A. (2009) Odor-specific habituation arises from interaction of afferent synaptic adaptation and intrinsic synaptic potentiation in olfactory cortex. *Learning & memory*, **16**, 452-459.

Luzzi, S., Snowden, J.S., Neary, D., Coccia, M., Provinciali, L. & Lambon Ralph, M.A. (2007) Distinct patterns of olfactory impairment in Alzheimer's disease, semantic dementia, frontotemporal dementia, and corticobasal degeneration. *Neuropsychologia*, **45**, 1823-1831.

Macknin, J.B., Higuchi, M., Lee, V.M., Trojanowski, J.Q. & Doty, R.L. (2004) Olfactory dysfunction occurs in transgenic mice overexpressing human tau protein. *Brain research*, **1000**, 174-178.

Pardini, M., Huey, E.D., Cavanagh, A.L. & Grafman, J. (2009) Olfactory function in corticobasal syndrome and frontotemporal dementia. *Archives of neurology*, **66**, 92-96.

Petersen, R.C., Parisi, J.E., Dickson, D.W., Johnson, K.A., Knopman, D.S., Boeve, B.F., Jicha, G.A., Ivnik, R.J., Smith, G.E., Tangalos, E.G., Braak, H. & Kokmen, E. (2006) Neuropathologic features of amnestic mild cognitive impairment. *Archives of neurology*, **63**, 665-672.

Saiz-Sanchez, D., De La Rosa-Prieto, C., Ubeda-Banon, I. & Martinez-Marcos, A. (2013) Interneurons and beta-amyloid in the olfactory bulb, anterior olfactory nucleus and olfactory tubercle in APPxPS1 transgenic mice model of Alzheimer's disease. *Anatomical record*, **296**, 1413-1423.

Saiz-Sanchez, D., De la Rosa-Prieto, C., Ubeda-Banon, I. & Martinez-Marcos, A. (2014) Interneurons, tau and amyloid-beta in the piriform cortex in Alzheimer's disease. *Brain structure & function*.

Scattoni, M.L., Gasparini, L., Alleva, E., Goedert, M., Calamandrei, G. & Spillantini, M.G. (2010) Early behavioural markers of disease in P301S tau transgenic mice. *Behavioural brain research*, **208**, 250-257.

Silveira-Moriyama, L., Hughes, G., Church, A., Ayling, H., Williams, D.R., Petrie, A., Holton, J., Revesz, T., Kingsbury, A., Morris, H.R., Burn, D.J. & Lees, A.J. (2010) Hyposmia in progressive supranuclear palsy. *Movement disorders : official journal of the Movement Disorder Society*, **25**, 570-577.

Spillantini, M.G. & Goedert, M. (2013) Tau pathology and neurodegeneration. *Lancet Neurol*, **12**, 609-622.

Suzuki, N. & Bekkers, J.M. (2007) Inhibitory interneurons in the piriform cortex. *Clinical and experimental pharmacology & physiology*, **34**, 1064-1069.

Walla, P., Duregger, C., Deecke, L. & Dal-Bianco, P. (2011) Dysfunctional Incidental Olfaction in Mild Cognitive Impairment (MCI): An Electroencephalography (EEG) Study. *Brain sciences*, **1**, 3-15.

Wesson, D.W., Levy, E., Nixon, R.A. & Wilson, D.A. (2010) Olfactory dysfunction correlates with amyloid-beta burden in an Alzheimer's disease mouse model. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **30**, 505-514.

Wilson, D.A., Xu, W., Sadrian, B., Courtiol, E., Cohen, Y. & Barnes, D.C. (2014) Cortical odor processing in health and disease. *Progress in brain research*, **208**, 275-305.

Wilson, R.S., Arnold, S.E., Schneider, J.A., Boyle, P.A., Buchman, A.S. & Bennett, D.A. (2009) Olfactory impairment in presymptomatic Alzheimer's disease. *Annals of the New York Academy of Sciences*, **1170**, 730-735.

Yang, S., Cacquevel, M., Saksida, L.M., Bussey, T.J., Schneider, B.L., Aebischer, P., Melani, R., Pizzorusso, T., Fawcett, J.W. & Spillantini, M.G. (2015) Perineuronal net digestion with chondroitinase restores memory in mice with tau pathology. *Experimental neurology*, **265**, 48-58.

Yoshimura, N. (1988) Olfactory bulb involvement in Pick's disease. *Acta Neuropathol*, **77**, 202-205.