Olfactory bulb neuroproteomics reveals a chronological perturbation of survival routes and a disruption of prohibitin complex during Alzheimer’s disease progression

Mercedes Lachén-Montes, Andrea González-Morales, María Victoria Zelaya, Estela Pérez-Valderrama, Karina Ausín, Isidro Ferrer, Joaquín Fernández-Irigoyen & Enrique Santamaría

Alzheimer’s disease (AD) is the most common form of senile dementia. In general, two subgroups are recognized, a familial early-onset form, and a sporadic late-onset form, albeit 95% of the patients develop sporadic AD. Together with typical symptoms such as memory loss and behavioral disorders, AD patients present olfactory dysfunction in 90% of the cases. Interestingly, this deficit occurs at early stages of the disease and it is considered a premotor sign of neurodegeneration. The olfactory bulb (OB) is the first central structure of the olfactory pathway in the brain. Multiple reports have evidenced neuropathological changes, and molecular alterations in the OB derived from rodent AD models, and human AD brains. Interestingly, the accumulation of beta-amyloid (Aβ) and phospho-Tau protein in the anterior olfactory nucleus and OB correlates with the...
The utility of olfactory tissue in the early diagnosis of AD. Although this unbiased technology has greatly enhanced the ability to characterize novel pathways particularly in brain areas associated with AD, few studies have examined proteome profiling of the early-affected OB region with the aim to investigate incipient neurodegenerative changes in AD phenotypes. Mass-spectrometric exploration of the OB derived from AD models has revealed a proteostasis impairment in this olfactory region. In the APP/PS1 (Amyloid precursor protein/ Preselinin 1) mouse model of AD, an early dysregulation of FAK and MEK/ERK signaling pathways precedes the β-amyloid deposits, and (++) 3–4 Aβ3 depositions. Graduation of phospho-TAU deposit: (−) negative; (+) 1–2 isolated Aβ deposits, (++) 3–4 Aβ3 depositions, and (+++) >4Aβ3 depositions. Graduation of phospho-TAU deposit: (−) negative; (+) 1–2 isolated Aβ deposits, (++) 3–4 Aβ3 depositions, and (+++) >4Aβ3 depositions.

Table 1. Subjects included in the proteomic study. The neuropathological assessment was performed according to Thal phases, CERAD score, NIA-AA guidelines and PART criteria. Aβ3 immunostaining was scored on a 4-tiered scale as: (−) negative, (+) 1–2 isolated Aβ deposits, (++) 3–4 Aβ3 depositions, and (+++) >4Aβ3 depositions. Graduation of phospho-TAU deposit: (−) negative; (+) 1–2 isolated Aβ deposits, (++) 3–4 Aβ3 depositions, and (+++) >4Aβ3 depositions. (Phb) complex. Interestingly, a cross-disease study also pointed out that Phb subunits are differentially modulated in the OB across AD-related co-pathologies, providing mechanistic clues to the intriguing divergence of AD pathology across different types of dementias.

Results

Proteostasis impairment in the OB during AD progression. To determine the OB site-specific proteomic signature during AD progression, a label-free MS-based approach was performed on OB tissue derived from AD subjects with different grading and controls with no known neurological history (Table 1). Among 1311 quantified proteins across all experimental groups, 278 proteins tend to be differentially expressed between controls and AD phenotypes (Fig. 1a, Supplementary Table 1 and Supplementary Fig. 1). Our analysis revealed
that 110 olfactory proteins are differentially expressed in early AD stages, increasing the proteome alterations as the disease progresses (125, and 158 differential proteins in intermediate and advanced stages respectively) (Fig. 1b). The distribution between up-regulated and down-regulated proteins was very similar across AD grading (Fig. 1b). Interestingly, 24 proteins overlapped between all stages (Fig. 1c), suggesting a potential role during AD evolution (Table 2). This set of proteins mainly clustered in specific biological process like growth of neurites (CLASP2, CPNE1), long-term potentiation (PPP1R1B), protein degradation (USP7, PSMD12, PSMF1), neuritogenesis (TNIK, S100B, STMN1), morphology of the nervous system (MUT, YES1), and synaptic plasticity (AP2S1, AP3D1, STXBP1). In order to evaluate the impact of AD in the OB at synaptic level, we have compared the OB differential expressed proteomes across AD staging with the information stored in three repositories containing the largest number of synapse specific proteins (G2Cdb, Synaptome DB, and SynsysNet)\(^{16-18}\). The analysis revealed that 162 out of 278 differential proteins (58% of the differential protein set) tend to localize to synaptic terminal (63, 70, and 96 differentially expressed synaptic proteins in initial, intermediate, and advanced stages respectively) (Supplementary Fig. 2). This meta-analysis verified a progressive synaptic degeneration at the level of OB during AD progression.

**Pathway-specific alterations during AD progression.** In order to perform a proteome mapping analysis of the stage-dependent protein profiles across specific-neuronal processes, we used the IPA information of
experimental and predictive origin regarding central nervous system, in order to be confident about the potential affected signaling pathways. As shown in Fig. 2, neuronal processes such as neuritogenesis, growth and outgrowth of neurites, axonogenesis, and growth of axons are compromised across AD stages. Moreover, our results pointed out a stage-dependent deregulation of specific biological processes (Fig. 2 and Supplementary Table 2). Protein clusters involved in synaptic transmission, and morphology of neuroglia were specifically mapped in initial stages while protein groups involved in formation of OB and cell death of oligodendrocytes were exclusively detected in intermediate stages. Interestingly, a de-regulation in protein clusters related to branching of neurites and axons, astrocytosis, vesicle trafficking, and myelination appears throughout initial and advanced stages (Fig. 2).

Network-driven proteomics reveals an imbalance in the olfactory MKK3-6/p38 MAPK and PDK1/PKC signaling across AD grading. To enhance the analytical outcome of proteomic experiments, we have performed proteome-scale interaction networks merging the olfactory proteins that tend to be de-regulated across stages of AD. To that end, a protein interactome map has been constructed for each stage using the IPA software (Fig. 3). In initial stages, a deregulation of cross-linkers between plasma membrane and actin-based cytoskeleton such as the protein complex ezrin-moesin-radixin (EZR-MSN-RDX) and regulators of the interaction between components of cell-cell junctions (L1CAM, CD9, CD81, CTTN, cadherin) suggested an imbalance in the cellular assembly and morphology at early AD stages (Fig. 3a). The functional clustering also suggested a central function of ERK1/2 mediating the structural stabilization at the level of OB (Fig. 3a). In accordance with these data, our group has previously demonstrated an early hyperactivation of ERK1/2 in the OB of AD subjects7. In intermediate stages, the proteome-scale interaction network reflected an impaired

| Protein names | Gene | Uniprot code | Unique peptides | P-val Initial AD | P-val Int. AD | FC Initial AD | FC Int. AD | APP Functional Interactor | Tau Functional Interactor |
|---------------|------|--------------|----------------|-----------------|--------------|--------------|------------|--------------------------|--------------------------|
| TRAK2 and NCK-interacting protein kinase | TRAK | Q9UK55 | 0.022 0.015 0.010 | 0.63 0.29 0.45 | ✓ |
| CLIP-associated protein 2 | CLASP2 | D7S122 | 0.002 0.001 0.002 | 0.51 0.51 0.48 | ✓ |
| Copine-1 (fragment) | COPM1 | F2Z2V0 | 0.026 0.009 0.010 | 0.65 0.76 0.40 | ✓ |
| Protein S100-B | S10OB | P0A271 | 0.006 0.020 0.050 | 0.46 0.68 0.69 | ✓ ✓ |
| VPS10 domain-containing receptor SorCS2 | VPS10 | B5M1D8 | 0.010 0.002 0.029 | 0.40 0.41 0.47 | ✓ |
| Importin subunit alpha-4 | IMPA3 | A0056S | 0.047 0.006 0.000 | 0.74 0.59 0.60 | ✓ |
| Protein phosphatase 1 regulatory subunit 1B | PPP1R1B | Q9U071 | 0.042 0.009 0.010 | 0.55 0.42 0.43 | ✓ |

Table 2. Common differentially expressed OB proteins across AD stages. The fold-change (FC) of differential proteins with associated p-values from the pair-wise comparisons of control vs each AD stage, together with Protein/gene names, protein code by Uniprot, and unique peptides used for quantitation are shown. The significant downward or upward trend is represented in green or red color respectively. Proteins that are functional interactors of APP and Tau proteins are also indicated.
mitochondrial function and an imbalance in redox signaling, due to dysregulation of subunits of mitochon-
drial respiratory chain complexes I, and IV (COX5A, COX7A2, COX6B1, NDUFV2) and protein components
involved in antioxidant defense mechanisms (HSPE1, GSTM1, TXN2) (Fig. 3b). In advanced stages, the func-
tional interactome network indicated an alteration in HNRNP complexes (FUS, HNRNPA1, HNRNPH1)
and specific RNA binding proteins (EFTUD2, and MATR3), suggesting an impairment in RNA stability, and
pre-mRNA splicing processes. Moreover, the alteration of COP9 signalosome complex subunit 1 (GPS1), and
cullin-3 (CUL3) pointed out an alteration in the proteasomal degradation pathway in advanced stages of the dis-
ease (Fig. 3c). Both p38 MAPK and PKC appeared as principal nodes in protein interactome maps (Fig. 3b and c).
Even though changes in their expression were not detected in our proteomic experiments, the alteration of some
of their targets may be compatible with a dysregulation of their functionality during AD progression at the level
of OB. Subsequent experiments were performed to monitor the activation state of p38 MAPK and PKC signaling
pathways across AD stages. MKK3 and MKK6 are two closely related dual-specificity protein kinases that activate
p38 MAPK. Western-blot analysis revealed a decrease in the activation status of upstream MKK3 and MKK6
in initial AD stages (Fig. 4a). This early down-regulation was accompanied by a fall in p38 MAPK levels and a
paralleled decrease in ATF2 and HSP27 phosphorylation (Fig. 4b), well-known downstream substrates of p38
MAPK. However, olfactory p38 MAPK activity tends to increase during AD progression, as demonstrated
by the increment in the phosphorylation status of HSP27 and ATF2 in advanced stages (Fig. 4b). PDK1 activity
depends on the autophosphorylation on Ser241 and activates PKC signal transduction by phosphorylation on the
activation loop. Although, a significant up-regulation in total PDK1 and PKC levels was evidenced in early
stages (Fig. 4c), PDK1 inactivation was accompanied by a decrease in the activation status of PKC isoforms in
intermediate stages, as revealed by Western-blot using a specific antibody against phosphorylated PKC isoforms
at a residue homologous to activated Thr514 of human PKC
γ
 (Fig. 4c). Accompanying the PKC inactivation,
Myristoylated alanine-rich C-kinase substrate (MARCKS), a substrate of PKC was also down-regulated in inter-
mediate stages (Supplementary Table 1). However, the accumulation of PKC isoforms maintained PKC active in
advanced stages, despite the PDK1 inactivation observed in these stages (Fig. 4c). Altogether, an early disruption
in upstream p38 MAPK pathway and a subsequent impairment of PDK1/PKC signaling axis occurs in the OB
from AD subjects. However, the tangled regulatory mechanisms that govern the PKC signaling needs further
exploration, to elucidate the specific role of each PKC isoform during the AD neurodegeneration that occurs in
the OB.

Dysregulation of AD-related protein interactomes in the OB during the neurodegenerative
process. We consider that the discovery of unexpected relationships between apparently unrelated proteins
and AD-causing neuropathological substrates is a powerful strategy for the characterization of novel AD caus-
itive/susceptibility proteins with a central role during the neurodegenerative process that occurs in olfactory
areas. We explored whether well-established AD-related proteins were indeed highly interconnected with the
stage-dependent differential olfactory proteomes. As shown in Fig. 5, differential functional interactors for
neuropathological substrates like APP and tau proteins were identified in the OB. With respect to OB controls,
the APP interactome was composed by 12, 24, and 32 differential targets in initial, intermediate, and advanced
AD stages respectively (Supplementary Fig. 3). In the case of tau protein, 9 differential targets were detected
in early AD stages, whereas 18 and 19 targets constituted the differential Tau interactome in intermediate and
advanced AD stages respectively (Fig. 5 and Supplementary Fig. 4). Interestingly, 5 differential targets were shared
between APP and Tau interactomes across AD grading. These proteins correspond to succinyl-CoA ligase [ADP/
GDP-forming] subunit alpha (SUCLG1), Stathmin 1 (STMN1), translationally-controlled tumor protein (TPT1),
protein S100-B (S100B), and syntaxin-binding protein 1 (STXBP1) (Supplementary Figs 3 and 4). Our analysis also revealed other functional interactomes that are modulated during AD progression in which the central nodes correspond to cAMP responsive element binding protein 1 (CREB1) and estrogen receptor 1 (ESR1) (Fig. 5 and Supplementary Figs 5 and 6).

Validation of differential olfactory proteins across AD grading: Focus on Prohibitin complex. Previous studies identified a down-regulation of olfactory XRCC5, and FABP5 in initial AD stages, together with the overexpression of CD166 antigen, V-type proton ATPase subunit H, and histone H4 in advanced AD stages. Our results confirm these previous observations (Supplementary Table 1), partially validating the label free-based liquid chromatography tandem mass spectrometry (LC-MS/MS) approach. With the aim to complement and validate quantitative proteome measurements, subsequent experiments were performed to check the steady-state levels of a subset of differential proteins using downstream assays. We consider the selection of assessing Vimentin (Vim) and Prohibitin-2 (Phb2) proteins for validation. The absence of the intermediate filament protein Vim exacerbates the amyloid plaque load and the increase in dystrophic neurites in APP/PS1 mouse model of AD and Phb2 deficiency leads to Tau hyperphosphorylation, and neurodegeneration in mice.

First, we performed immunohistochemical analysis to localize Vim and Phb subunits in the OB region during AD progression (Fig. 6). Vim tends to be expressed in the glomerular layer and preferentially distributed in the walls of the blood vessels (Fig. 6). Both Phb subunits were detected at dendritic connections in glomerular layer
and across the neuropil, being highly expressed in the cytoplasm of mitral cells and neurons of the anterior olfactory nucleus (AON) (Fig. 6). In order to evaluate the potential role of Vim and Phb subunits in the early-affected OB region in human AD phenotypes, protein expression levels were monitored by Western blotting across AD staging (Fig. 7). In accordance with proteomic data (Supplementary Table 1), immunoblotting analysis revealed a slight decrease in olfactory Vim protein levels in initial and advanced AD stages, and a down-regulation of

Figure 4. Signaling pathways disrupted in the OB across AD grading. Levels and residue-specific phosphorylation of MKK3/6 (a), p38 MAPK, ATF2, and HSP27 (b), PDK1, and PKC (c) in the OB across AD phenotypes. Equal loading of the gels was assessed by Ponceau staining and hybridization with a GAPDH specific antibody. Right panels show histograms of band densities. Data are presented as mean ± SEM from 5 independent OB samples per group. *P < 0.05 vs control group; **P < 0.01 vs control group. Representative Western blot gels (n = 3/experimental group) are shown. Full-length blots/gels are presented in Supplementary information.
Phb2 protein levels in intermediate and advanced AD stages with respect to controls (Fig. 7a). Mitochondrial prohibitin complex (constituted by Phb1 and Phb2) modulates mitochondrial dynamics, participates in the mitochondrial respiratory complex assembly, and exerts beneficial effects on neurons by reducing free radical production27, 28. Generally, repression of Phb2 is paralleled by a concomitant reduction of its assembly partner Phb1 and vice versa26, 29. In agreement with our mass spectrometry (MS) data (Supplementary Table 1), Phb1 levels were unchanged across AD stages (Fig. 7a), pointing out that Phb subunits are not functionally interdependent in the OB during AD neurodegeneration. However, phosphorylated Phb1 isoforms at Thr258 and Y259 were down-expressed in intermediate and advanced AD stages (Fig. 7b), suggesting potential fluctuations in the Phb1 interactome across AD stages.

OB protein expression of Prohibitin complex across Alzheimer-related co-pathologies. In contrast to the common separate investigation of neurological disorders, targeted cross-disease studies comparing shared molecular relationships may give new insights into possible olfactory perturbations common for all or some neurological backgrounds. To check the potential vulnerability of PHB complex across different Alzheimer-related co-pathologies at the level of OB, we have evaluated the OB protein expression of Phb2, Phb1, Thr258-, and Y259-phosphorylated Phb1 isoforms by Western-blot across AD-related diseases (n = 28 OB samples) (Supplementary Table 3). We have included pathologies with common smell impairment like FTLD 3, PSP where olfactory loss occurs to a lesser extent or is absent3, 30, and mixed dementia (Mix AD VD). Mixed dementia is a condition in which AD and vascular dementia occur at the same time, and both separate disorders often display olfactory dysfunction31. As shown in Fig. 8a, steady-state and phosphorylated levels of Phb1 remained unchanged in the OB from Mixed AD VD subjects respect to controls, while Phb2 protein expression is significantly increased. Moreover, a significant reduction in Phb1 expression (steady-state and phosphorylated levels) was detected in FTLD subjects, and no significant differences were observed with respect to Phb2 protein levels (Fig. 8b). On the other hand, both Phb subunits were statistically unchanged in PSP subjects, albeit 50% of PSP subjects presented a slight tendency to down-regulation in the case of Phb1 (Fig. 8c). Although equivalent olfactory deficits are observed between some AD-related co-pathologies, these data pointed out that the olfactory pattern of Phb subunits is proteinopathy-dependent, suggesting different mechanistic clues to the neurodegenerative process that occurs in the OB.

Discussion
During the last years, proteomics has emerged as a large-scale comprehensive approach to characterize and quantify specific olfactory-related proteomes in ageing and neurodegeneration11. Due to the early involvement of the olfactory dysfunction in AD4, we consider that the application of tissue proteomics in the OB is an ideal approach that allows zooming-in where pathophysiological changes are taking place. In the current work, we have used a discovery platform combining neuropathological diagnosis, label-free quantitative proteomics, physical and functional interaction data, and biochemical approaches in order to determine the chronologic regulation of the OB proteome during AD progression. Our group has previously identified common and distinct olfactory targets across tauopathies and synucleopathies using a labeling proteomic approach7. The use of labeling strategies usually results in the identification of more proteins, but label-free methods allow us to analyze and compare more samples individually, indicating that both workflows are complementary32. It is important to note that due to technical reasons, only the most abundant OB proteins were explored. Consequently, alterations other than those reported in this study might also participate in the AD neurodegeneration at the level of the OB. However, according to our integrative meta-analysis (Supplementary Table 4), most of the differential OB proteins reported in this study has not been previously reported in differential proteomic studies performed in human AD brains.

In agreement with previous studies performed in the APP/PS1 mouse model of AD14, our data revealed a stage-dependent synaptic proteostasis impairment in the OB during AD pathogenesis, where more than 50% of the differential proteome across AD phenotypes tends to localize to synaptic ending. Interestingly, 17% of the OB differential proteome (47 out of 278 proteins), is also deregulated in hippocampal tissue during AD progression12, suggesting a coordinated regulation of specific protein modules across AD-related brain structures.
that might turn out detrimental or potentially protective mechanisms during the disease evolution. The synaptic plasticity imbalance was accompanied by specific proteomic fingerprints that are dynamically modulated in a stage-dependent manner throughout the OB. Interestingly, 24 early-affected proteins involved in protein degradation, growth of neurites, long-term potentiation, and neuritogenesis were deregulated across all stages, highlighting their potential importance for targeting AD at an early stage. Assuming that causative and susceptibility proteins tend to be highly interconnected in AD, we have employed network-driven proteomics to yield novel insights into the signaling pathways that govern the evolution of AD at olfactory level. Our data point out that functional protein interactomes and specific pathways are dynamically modulated across AD staging in the OB, emphasizing the potential impact of stage-dependent analysis using high-throughput proteome screenings. It has been proposed that the aberrant regulation of a subset of kinases may represent the triggering events leading to the spread of an aberrant signaling in AD. In this context, the dysregulation of kinases regulating neuronal plasticity, learning, and memory have been proposed as the starting signal, which promotes neurotoxic outcomes. Analyzing the upstream signaling interactions of the differentially expressed proteomes in the OB, we determined upstream regulators (ERK1/2, p38 MAPK, and PKC) that were highly interconnected with downstream regulated proteins. Despite ERK1/2 is a well-defined pro-survival factor, neuronal ERK has been reported to be involved in the induction of cell death, APP processing, and Tau phosphorylation. Our data point out that olfactory ERK1/2 hyperactivation may be involved in the improper cytoskeletal coupling that occurs in olfactory neurons at early stages, probably driving the synaptic impairment. In contrast, we detected a specific upstream and downstream disruption in p38 MAPK pathway in early stages at the level of the OB, recovering normal levels in intermediate and advanced stages. This early deregulation was further validated by paralleled decrease in ATF2 and HSP27 phosphorylation, well-known downstream substrates of p38 MAPK. In spite of detrimental effects of p38 MAPK activation during AD pathogenesis, recent studies have dissected the role of specific p38 MAPK isoforms identifying different functions. It has been characterized that the inhibition of neuronal p38alpha, but not p38beta MAPK, provides neuroprotection in different neurotoxic environments. Interestingly, a depletion of neuronal p38alpha MAPK attenuates Aβ pathology in AD mouse and cell models. In line with these findings, the early down-regulation of p38 MAPK in olfactory neurons might be part of the neuroprotective mechanisms induced in initial stages against Aβ.

Figure 6. Immunohistochemical localization of OB Vim, Phb1 and Phb2 across AD grading. First line: Representative immunohistochemical staining pattern of Vim across AD grading. Positive staining in glomerular cell layer (GL) and wall of endothelial cells (ovals). Second line: Representative immunohistochemical staining pattern of Phb1 across AD grading. Positive staining in glomerular layer (GL), anterior olfactory nucleus (AON), and mitral cells (asterisks). Third line: Representative immunohistochemical staining pattern of Phb2 across AD grading. Positive staining in glomerular layer (GL), anterior olfactory nucleus (AON), and mitral cells (asterisks).
subsequent impairment of PDK1/PKC signaling axis occurs in the OB from AD subjects. An increment in PDK1 activity has been reported in the brain from AD patients, however, its inhibition or silencing points out a beneficial effect in AD-like pathology. 

*In vitro* studies have demonstrated that inhibition of PDK1 blocks neuronal cell death induced by Aβ51. Interestingly, quenching PDK1 activity in three APP-transgenic mouse models of AD rescued TACE-mediated neuroprotective cleavage of APP, and decreased Aβ deposition, counteracting memory and cognitive deficits. Thus, our data suggest that the significant reduction in PDK1 activation may be a signal to protect neuronal function in the OB. PDK1 is an upstream regulator of some PKC family members, critically involved in memory acquisition and maintenance. Deficits in neuronal PKC signal cascades are one of the earliest abnormalities in AD brains. Aβ can directly inhibit PKC isoforms, induces PKC degradation, and reduces PKC-mediated phosphorylation and membrane translocation. In line with these findings, the PKC inactivation observed in the OB could contribute to the Aβ-related toxicity across initial-intermediate phases of AD. Although PKC activation has been proposed for the treatment of dementias, the tangled regulatory mechanisms that govern the PKC signaling needs further exploration at olfactory level, in order to clarify the specific role of each PKC isoform during the neurodegenerative process.

Our proteomic screening further suggested an impairment of mitochondrial function in the OB, revealing dysregulation of 29 mitochondrial proteins across intermediate and advanced AD stages. This impairment is a common finding in human AD brains, and also in rodent, and cellular AD models, where intracellular Aβ accumulation leads to a decrease electron transfer efficiency, reduce ATP production, and increase ROS production. Interestingly, cross-validation by immunoblotting analysis revealed a down-regulation of Phb2 protein levels in intermediate and advanced stages. Phb complex (constituted by Phb1 and Phb2) is located in the mitochondrial inner membrane acting as a membrane-bound chaperone involved in the correct folding and assembly of some of the components of the mitochondrial respiratory chain. According to this hypothesis, a deficiency in Phb2

Figure 7. Olfactory expression of Vim and Phb subunits across AD stages. (a) Protein levels of Vim, Phb2, and Phb1 were monitored by Western-blotting. Equal loading of the gels was assessed by Ponceau staining and hybridization with a GAPDH specific antibody. Representative Western blot gels (n = 3/experimental group) are shown (left). Right panels show histograms of band densities. Data are presented as mean ± SEM from 3 independent OB samples per group. *P < 0.05 vs control group; ***P < 0.001 vs control group. (b) OB phosphorylation profiling of Phb1 across AD staging. Representative Western blot gels (n = 2/experimental group) are shown (left). Right panel shows histograms of band densities. Data are presented as mean ± SEM from 3 independent OB samples per group. *P < 0.05 vs control group; **P < 0.01 vs control group. Full-length blots/gels are presented in Supplementary information.
may impair the native and functional organization of respiratory proteins, compromising mitochondrial functionality. Interestingly, neuron-specific deletion of Phb2 induces an aberrant mitochondrial ultrastructure, and Tau hyperphosphorylation in mice, leading to behavioral impairments and cognitive deficiencies. Therefore, Phb2 deficiency might suggest a direct link between mitochondrial defects and tau pathology in olfactory neurons. We have observed a reversed Phb2 pattern between mixed dementia (Mix AD VD) respect to the protein profile observed in AD. Transcriptional and translational events may explain the difference observed in Phb2 protein levels. A possible explanation is that the vascular damage may induce a decrease in the Phb2 degradation rate at mRNA and/or protein levels at the level of the OB in mix AD VD. However, we should consider that the activation/inhibition of the transcription factor machinery that regulates the transcription of PHB2 gene may also be compromised (as a consequence of the vascular damage), leading to an increase in Phb2 mRNA and protein levels. However, additional validation studies should be conducted employing large cohorts to verify the protein expression changes observed in our sample set. Repression of Phb2 is usually paralleled by a concomitant reduction of its assembly partner Phb1 and vice versa. However, in agreement with previous studies performed in frontal cortex derived from AD subjects, steady-state levels of OB Phb1 was unchanged across AD grading, demonstrating that Phb subunits are not functionally interdependent in the OB during AD neurodegeneration. In addition, Phb1 and Phb2 are present in different subcellular localizations, presenting clear and distinctive

Figure 8. OB protein expression of Phb isoforms across AD-related proteinopathies. Olfactory expression of Phb2, Phb1, and Phb1 phosphorylated isoforms in Mix AD VD (a), FTLD (b), and PSP (c). Representative Western blot gels are shown for each Phb subunit. Histograms of band densities derived from 28 independent OB samples. Data are presented as mean ± SEM from: Controls (n = 4 cases), mixed dementia (mix AD VD) (n = 9 cases), FTLD (n = 6 cases), and PSP (n = 9 cases). *P < 0.05 vs control group; **P < 0.01 vs control group. Full-length blots/gels are presented in Supplementary Information.
functions. While some associations at the plasma membrane and in the mitochondria require both Phb1 and Phb2, both proteins function independently in the nucleus, influencing the activity of multiple transcription factors. However, based on our immunohistochemical analysis, most of Phb2 staining is not detected in the nucleus of olfactory neurons, suggesting that Phb2 may not be directly involved in transcriptional events. On the other hand, Phb subunits are post-translationally modified by O-linked N-acetylglucosamine, palmitoylation, transamidation, nitrrosylation, and phosphorylation. A decreased in phosphorylated Phb1 isoforms at T258 and Y259 was detected in the OB from AD subjects. Both residues are present in the C-terminal coiled-coil domain that is involved in protein-protein interactions, including the interaction between Phb1 and Phb2 as well as transcriptional regulation. Although the kinase that phosphorylates the Y259 remains unknown, it is well documented that Akt phosphorylates Phb1 at T258 in non-neuronal contexts. Specifically, Akt may phosphorylate this residue in the cytoplasm, promoting Phb1 mitochondrial translocation or in the lipid raft domain of the plasma membrane to activate the Ras-MAPK and PI3K/Akt pathways. However, previous work in our laboratory demonstrated that the activation state of Akt remains unchanged, and ERK is hyper-activated in the OB from AD subjects, suggesting that OB Phb1 dephosphorylation may be due to a phosphatase action or the inactivation of a specific kinase different than Akt. Although our results serve as a foundation for new areas of investigation into the role of olfactory signaling in human AD-related co-pathologies, further work is necessary to clarify the regulatory mechanisms involved in post-translational modifications of Phb subunits, in order to understand the final effect of olfactory Phb complex on cell survival and apoptosis across proteinopathies.

Methods

Materials. The following reagents and materials were used: anti-GAPDH (Calbiochem), anti-MKK3, anti-MKK6, anti-phospho MKK3 (Ser189)/MKK6 (Ser207), anti-p38 MAP kinase, anti-phospho p38 MAP kinase (Thr180/Tyr 182), anti-phospho HSP27 (Ser82), anti-phospho ATF2 (Thr71), anti-PDK1, anti-phospho PDK1 (Ser241), anti-PKC-Pan, anti-phospho PKC-pan (T514), anti-Prohibitin 1 (Phb1), anti-Prohibitin 2 (Phb2), (Cell signaling). Anti-vimentin antibody was purchased from Santa Cruz biotechnology. Anti-phospho Phb1 (T258), and anti-phospho Phb1 (Y259) were purchased from Signalway Antibody. Electrophoresis reagents were purchased from Biorad and trypsin from Promega.

Human samples. According to the Spanish Law 14/2007 of Biomedical Research, inform written consent forms of the Neurological Tissue Bank of Navarra Health Service, Brain Bank of IDIBELL, and Neurological Tissue Bank of IDIBAPS-Hospital Clinic (Barcelona, Spain) was obtained for research purposes from relatives of patients included in this study. The study was conducted in accordance with the Declaration of Helsinki and all assessments, post-mortem evaluations, and procedures were previously approved by the Clinical Ethics Committee of Navarra Health Service. For the proteomic phase, fourteen AD cases were distributed into different groups according to specific consensus diagnostic criteria: initial, intermediate, and advanced AD stages (n = 4–5/group). Three cases from elderly subjects with no history or histological findings of any neurological disease were used as a control group. All human brains considered in the proteomic study had a post-mortem interval (PMI) lower than 10 hours (Table 1). Brain processing and the neuropathological study for protein deposits aggregates Aβ and phospho-Tau were performed as previously described. For the discovery phase, neuropathological assessment was performed according to standardized neuropathological scoring/grading systems, including Bbath phases of Beta-amyloid deposition, Braak staging of neurofibrillary lesions. Consortium to Established a Registry for Alzheimer’s Disease, National Institute on Aging-Alzheimer’s Association (NIA-AA) guidelines, and primary age-related tauopathy (PART) criteria. For the validation phase, OB tissue from additional controls and AD subjects were included (n = 4–5/group) (Supplementary Table 3). This material was obtained from the Neurological Tissue Banks of IDIBELL and IDIBAPS-Hospital Clinic, Barcelona, Spain. For the specificity analysis, different NDs were considered: Progressive supranuclear palsy (PSP) (n = 9 cases; 4F/5M; median age: 74 years), frontotemporal lobar degeneration (FTLD) (n = 6; 3F/3M; median age: 81 years), mixed dementia (mix AD VD) (n = 9 cases; 4F/5M; median age: 85 years), and controls (n = 4; 1F/3M; median age: 79 years). In these cases, neuropathological assessment was performed according to standardized neuropathological guidelines: Mackenzie criteria for FTLD pathology, NINDS-AIREN criteria for vascular dementia, and NINDS criteria for PSP. 80% of the OB samples included in this phase had a PMI lower than 10 hours (Supplementary Table 3).

Sample preparation for proteomic analysis. OB specimens derived from control and AD cases were homogenized in lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT. The homogenates were spun down at 100.000 × g for 1 h at 15 °C. Prior to proteomic analysis, protein extracts were precipitated with methanol/choloroform, and pellets dissolved in 6M Urea, Tris 100mM pH 7.8. Protein quantitation was performed with the Bradford assay kit (Bio-Rad).

Label free LC-MS/MS. The protein extract for each sample was diluted in Laemmlli sample buffer and loaded into a 0.75 mm thick polyacrylamide gel with a 4% stacking gel casted over a 12.5% resolving gel. The run was stopped as soon as the front entered 3 mm into the resolving gel so that the whole proteome became concentrated in the stacking/resolving gel interface. Bands were stained with Coomasie Brilliant Blue and excised from the gel. Protein enzymatic cleavage (10 ug) was carried out with trypsin (Promega; 1:20, w/w) at 37 °C for 16 h as previously described. Purification and concentration of peptides was performed using C18 Zip Tip Solid Phase Extraction (Millipore). Peptides mixtures were separated by reverse phase chromatography using an Eksigent nanoLC ultra 2D pump fitted with a 75 μm ID column (Eksigent 0.075 × 250). Samples were first loaded for desalting and concentration into a 0.5 cm length 100 μm ID precolumn packed with the same chemistry as the separating column. Mobile phases were 100% water 0.1% formic acid (FA) (buffer A) and 100% Acetonitrile.

www.nature.com/scientificreports/
Peptide Identification and Quantification. MS/MS data acquisition was performed using Analyst 1.7.1 (AB Sciex) and spectra files were processed through Protein Pilot Software (v.5.0-ABSciex) using Paragon™ algorithm (v.4.0.0.0) for database search, Progroup™ for data grouping, and searched against the concatenated target-decoy UniProt proteome reference Human database (Proteome ID: UP000005640, 70902 proteins, December 2015). False discovery rate was performed using a non linear fitting method and displayed results were those reporting a 1% Global false discovery rate or better. The peptide quantification was performed using the Progenesis LC–MS software (ver. 2.0.5556.29015, Nonlinear Dynamics). Using the accurate mass measurements from full survey scans in the TOF detector and the observed retention times, runs were aligned to compensate for between-run variations in our nanoLC separation system. To this end, all runs were aligned to a reference run automatically chosen by the software, and a master list of features considering m/z values and retention times was generated. The quality of these alignments was manually supervised with the help of quality scores provided by the software. The peptide identifications were exported from Protein Pilot software and imported in Progenesis LC–MS software where they were matched to the respective features. Output data files were managed using R scripts for subsequent statistical analyses and representation. Proteins identified by site (identification based only on a modification), reverse proteins (identified by decoy database) and potential contaminants were filtered out. Proteins quantified with at least two unique peptides, an ANOVA p-value lower than 0.05, and an absolute fold change of <0.77 (down-regulation) or >1.3 (up-regulation) in linear scale were considered to be significantly differentially expressed. MS raw data and search results files have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifiers PXD005319.

Bioinformatics. The proteomic data were analyzed through the use of QIAGEN’s Ingenuity® Pathway Analysis (IPA) (QIAGEN Redwood City, www.qiagen.com/ingenuity), in order to detect and infer differentially activated/deactivated pathways as a result of AD phenotypes. This software comprises curated information from databases of experimental and predictive origin, enabling discovery of highly represented functions, pathways, and interactome networks.

Immunohistochemistry. For the immunohistochemical study, formalin fixed sections (3–5 mm-thick) were mounted on slides and deparaffinized. Tissue sections were labelled with the following primary antibodies: anti-vimentin (dilution 1/200), anti-Prohibitin-1 (Phb1) (dilution 1/120), and anti-Prohibitin-2 (Phb2) (dilution 1/50). The reaction product was visualized using an automated slide immunostainer (Leica Bond Max) with Bond Polymer Refine Detection (Leica Biosystems Newcastle Ltd).

Immunoblotting analysis. Equal amounts of protein (10 μg) were resolved in 12.5% SDS-PAGE gels. OB proteins derived from human samples were electrophoretically transferred onto nitrocellulose membranes using a Trans-blot Turbo transfer system (up to 25V, 7min) (Bio-rad). Equal loading of the gels was assessed by Ponceau staining. Membranes were probed with primary antibodies at 1:1000 dilution in 5% nonfat milk or BSA. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000), the immunoreactivity was visualized by enhanced chemiluminiscence (Perkin Elmer) and detected by a Chemidoc MP Imaging System (Bio-Rad). After densitometric analyses (Image Lab Software Version 5.2; Bio-Rad), optical density values were expressed as arbitrary units and normalized to GAPDH.

References

1. Qiu, C., De Ronchi, D. & Fratiglioni, L. The epidemiology of the dementias: an update. *Curr Opin Psychiatry* 20(4), 380 (2007).
2. Bettens, K., Sleegers, K. & Van Broeckhoven, C. Current status on Alzheimer disease molecular genetics: from past, to present, to future. *Hum Mol Genet* 19(R1), R4 (2010).
3. Attems, J., Walker, L. & Jellinger, K. A. Olfactory bulb involvement in neurodegenerative diseases. *Acta Neuropathol* 127(4), 459 (2014).
4. Roberts, R. O. et al. Association Between Olfactory Dysfunction and Amnestic Mild Cognitive Impairment and Alzheimer Disease Dementia. *JAMA Neurol* 73(1), 93 (2016).
5. Doty, R. L. The olfactory vector hypothesis of neurodegenerative disease: is it viable? *Ann Neurol* 63(1), 7 (2008).
6. Zelaya, M. V. et al. Olfactory bulb proteome dynamics during the progression of sporadic Alzheimer’s disease: identification of common and distinct olfactory targets across Alzheimer-related co-pathologies. *Oncotarget* 6(37), 39437 (2015).
7. Lachen-Montes, M. et al. An early dysregulation of FAK and MEK/ERK signaling pathways precedes the beta-amyloid deposition in the olfactory bulb of APP/PS1 mouse model of Alzheimer’s disease. *J Proteomics* 148, 149 (2016).
8. Fernandez-Irigoyen, J., Corrales, F. J. & Santamaria, E. Proteomic atlas of the human olfactory bulb. *J Proteomics* 75(13), 4005 (2012).
9. Nagayama, S., Homma, R. & Inamura, F. Neuronal organization of olfactory bulb circuits. *Front Neural Circuits* 8, 98 (2014).
10. Aebbersols, R. & Mann, M. Mass-spectrometric exploration of proteome structure and function. *Nature* 537(7620), 347 (2016).
11. Lachen-Montes, M., Fernandez-Irigoyen, J. and Santamaria, E., Deconstructing the molecular architecture of olfactory areas using proteomics. *Proteomics Clin Appl* (2016).
12. Hondius, D. C. et al. Profiling the human hippocampal proteome at all pathologic stages of Alzheimer’s disease. *Alzheimers Dement* 12(6), 654 (2016).
13. Krzyzanowska, A. et al. Expression of regulatory proteins in choroid plexus changes in early stages of Alzheimer disease. *J Neuropathol Exp Neurol* 74(4), 359 (2015).
60. Tillement, L., Lecanu, L. & Papadopoulos, V. Alzheimer’s disease: effects of beta-amyloid on mitochondria. *Nucleic Acids Res* **41** (Database issue), D843 (2013).

59. Sun, M. K. & Alkon, D. L. Activation of protein kinase C isozymes for the treatment of dementias. *Adv Pharmacol* **28**(6), 897 (2012).

58. Liang, W. S.

57. Mora, A., Komander, D., van Aalten, D. M. & Alessi, D. R. PDK1, the master regulator of AGC kinase signal transduction. *Semin Cell Dev Biol* **15**(2), 161 (2004).

56. Dehvari, N.

55. Desdouits, F.

54. Alkon, D. L., Sun, M. K. & Nelson, T. J. PKC signaling deficits: a mechanistic hypothesis for the origins of Alzheimer’s disease. *Neuropharmacology* **51**(9–10), e1003021 (2012).

53. Soler-Lopez, M.

52. Filiou, M. D.

51. Pietri, M.

50. Kraft, A. W.

49. Kempf, S. J. et al. An integrated proteomics approach shows synaptic plasticity changes in an APP/PS1 Alzheimer’s mouse model. *Mol Neurotroph* **7**(1), 33 (2006).

48. von Eichborn, J. et al. SynSysNet: integration of experimental data on synaptic protein-protein interactions with drug-target relations. *Nucleic Acids Res* **41** (Database issue), D834 (2013).

47. Hensley, K.

46. Pei, J. J.

45. Merkwirth, C.

44. Kempf, S. J.

43. Li, Y., Liu, L., Barger, S. W. & Griffin, W. S. Interleukin-1 mediates pathological effects of microglia on tau phosphorylation and on neurodegeneration. *PLoS Genet* **8**(11), e1003021 (2012).

42. Merkworth, C. et al. Loss of prohibitin membrane scaffolds impairs mitochondrial architecture and leads to tau hyperphosphorylation and neurodegeneration. *Neuropharmacology* **51**(9–10), e1003021 (2012).

41. Zigmond, J. et al. Targeting p38 MAPK pathway for the treatment of Alzheimer’s disease. *Neuropharmacology* **51**(9–10), e1003021 (2012).

40. Origlia, N., Arancio, O., Domenici, L. & Yan, S. S. MAPK, beta-amyloid and synaptic dysfunction: the role of RAGE. *Transl Psychiatry* **2**(2), e219 (2013).

39. Cheung, E. C. & Slack, R. S. Emerging role for ERK as a key regulator of neuronal apoptosis. *Curr Alzheimer Res* **9**(2), 127 (2012).

38. Hensley, K.

37. Desdouits, F. et al. Amyloid beta peptide formation in cell-free preparations. Regulation by protein kinase C, calmodulin and calcineurin. *J Biol Chem* **270**(4), 222 (2004).

36. Dehvari, N.

35. Hensley, K.

34. Soler-Lopez, M.

33. Soler-Lopez, M.

32. Filiou, M. D.

31. Pietri, M.

30. Doty, R. L. Olfactory dysfunction in Parkinson disease. *Neurology* **68**(12), 397 (2007).

29. Cheung, E. C. & Slack, R. S. Emerging role for ERK as a key regulator of neuronal apoptosis. *Science* **294**(5543), 944 (2001).

28. Artal-Sanz, M. & Tavernarakis, N. Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1: identification of five sites of phosphorylation in vivo. *Biochem J* **342**(Pt 2), 287 (1999).

27. Don, A., Bains, L., Alfares, M. A., Piner, C., Bu, B. S. & Fitch, S. The role of PKC in the regulation of synaptosome calcium channels. *J Pharmacol Exp Ther* **321**(2), 447 (2007).

26. Merkwirth, C.

25. Kraft, A. W.

24. Kempf, S. J.

23. Mora, A., Komander, D., van Aalten, D. M. & Alessi, D. R. PDK1, the master regulator of AGC kinase signal transduction. *Semin Cell Dev Biol* **15**(2), 161 (2004).

22. Casamayor, A., Morrice, N. A. & Alessi, D. R. Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. *Biochem* **354**(2), 1609 (1996).

21. Casamayor, A., Morrice, N. A. & Alessi, D. R. Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. *Biochem* **342**(Pt 2), 287 (1999).

20. Puig, B., Vinals, F. & Ferrer, I. Active stress kinase p38 enhances and perpetuates abnormal tau phosphorylation and deposition in Pick’s disease. *Acta Neuropathol* **107**(3), 185 (2004).

19. Song, C., Perides, G., Wang, D. & Liu, Y. F. beta-Amyloid peptide induces formation of actin stress fibers through p38 mitogen-activated protein kinase. *J Neurochem* **83**(4), 828 (2002).

18. Sun, M. K. & Alkon, D. L. Activation of protein kinase C isozymes for the treatment of dementias. *Adv Pharmacol* **28**(6), 897 (2012).

17. Lopez-Gonzalez, I. et al. Neuroinflammatory signals in Alzheimer disease and APP/PS1 transgenic mice: correlations with plaques, tangles and oligomeric species. *J Neuroimmunol Exp Neurol* **74**(4), 319 (2015).

16. von Eichborn, J. et al. SynSysNet: integration of experimental data on synaptic protein-protein interactions with drug-target relations. *Nucleic Acids Res* **41** (Database issue), D834 (2013).

15. Lopez-Gonzalez, I.

14. Kempf, S. J. et al. An integrated proteomics approach shows synaptic plasticity changes in an APP/PS1 Alzheimer’s mouse model. *Mol Neurotroph* **7**(1), 33 (2006).

13. Croning, M. D. et al. G2Cdb: the Genes to Cognition database. *Nucleic Acids Res* **37** (Database issue), D846 (2009).

12. Merkworth, C. et al. Loss of prohibitin membrane scaffolds impairs mitochondrial architecture and leads to tau hyperphosphorylation and neurodegeneration. *PLoS Genet* **8**(11), e1003021 (2012).

11. Zhou, P. et al. Prohibitin reduces mitochondrial free radical production and protects brain cells from different injury modalities. *J Neurosci* **32**(2), 583 (2012).

10. Pietri, M.

9. Alkon, D. L., Sun, M. K. & Nelson, T. J. PKC signaling deficits: a mechanistic hypothesis for the origins of Alzheimer’s disease. *Neuropharmacology* **51**(9–10), e1003021 (2012).

8. Liang, W. S.

7. Mora, A., Komander, D., van Aalten, D. M. & Alessi, D. R. PDK1, the master regulator of AGC kinase signal transduction. *Semin Cell Dev Biol* **15**(2), 161 (2004).

6. Kempf, S. J.

5. Hensley, K.

4. Kempf, S. J.

3. Don, A., Bains, L., Alfares, M. A., Piner, C., Bu, B. S. & Fitch, S. The role of PKC in the regulation of synaptosome calcium channels. *J Pharmacol Exp Ther* **321**(2), 447 (2007).

2. Casamayor, A., Morrice, N. A. & Alessi, D. R. Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. *Biochem* **342**(Pt 2), 287 (1999).

1. Von Eichborn, J. et al. SynSysNet: integration of experimental data on synaptic protein-protein interactions with drug-target relations. *Nucleic Acids Res* **41** (Database issue), D834 (2013).
Acknowledgements

We are very grateful to the patients and relatives that generously donor the brain tissue for research purposes. The authors thank the Neurological Tissue Bank of Navarra Health Service, for providing us the OB specimens used in the proteomic and specificity phases. We are indebted to the Neurological Tissue Banks of IDIBELL and IDIBAPS-Hospital Clinic (Barcelona, Spain), for sample and data procurement. We appreciate the help from Federico Garcia-Bragado from Pathology Department of Navarra Hospital Complex (Pamplona, Spain). Authors thank all PRIDE Team for helping with the mass spectrometric data deposit in ProteomeXchange (PRIDE). This work was funded by grants from the Spanish Ministry of Economy and Competitiveness (MINECO) (Ref. SAF2014-59340-R), Department of Economic Development from Government of Navarra (Ref. PC025), and Obra Social la Caixa to ES. AGM and KA are supported by PEJ-2014-A-61949 and PEJ-2014-A-72151 (MINECO). EPV is supported by PTA-2013/8711/I (MINECO). MLM is supported by a predoctoral fellowship from the Public University of Navarra (UPNA). The Proteomics Unit of Navarrabiomed is a member of Proteored, PRBB-ISCIII, and is supported by grant PT13/0001, of the PE I + D + I 2013-2016 funded by ISCIII and FEDER. This project is part of the HUPO Brain Proteome Project and these results are lined up with the Spanish Initiative on the Human Proteome Project (SpHPP).

Author Contributions

M.L.M. in collaboration with A.G.M. performed sample preparation and proteomic analysis. M.V.Z. and I.F. performed the selection of human samples and immunohistochemistry results. M.L.M., A.G.M., E.P., and K.A. performed validation and specificity analysis. J.F.I. and E.S. performed liquid chromatography mass spectrometry analysis and data interpretation. Finally, J.F.I. and E.S. designed and supervised the complete study.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-09481-x

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

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/. © The Author(s) 2017