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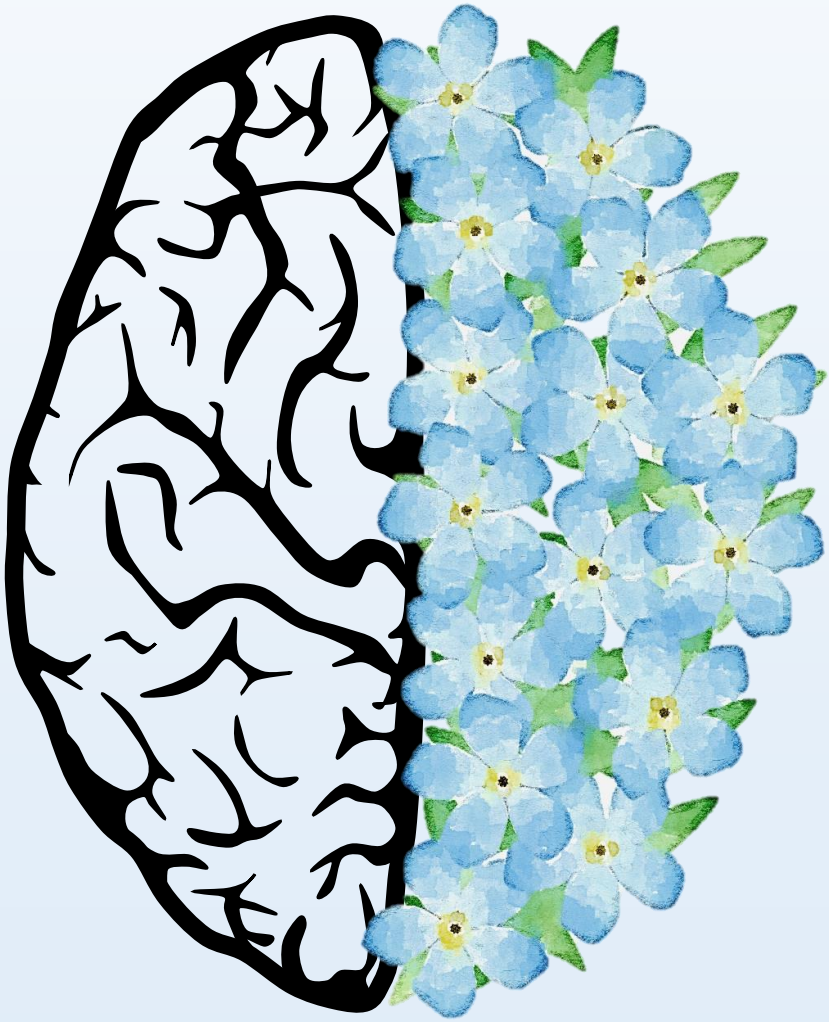

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FACULTAT DE BIOLOGIA

DEPARTAMENT DE BIOLOGIA CEL·LULAR, FISIOLOGIA I IMMUNOLOGIA

PARC CIENTÍFIC DE BARCELONA

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Memòria presentada per Eva Dávila Bouziguet, graduada en Ciències Biomèdiques, per optar al grau de Doctora per la Universitat de Barcelona.

Els estudis de tercer cicle s'han emmarcat en el programa de Doctorat en Biomedicina de la Universitat de Barcelona. El projecte de Tesi Doctoral està adscrit al Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Facultat de Biologia de la Universitat de Barcelona. El treball experimental i la redacció de la memòria han estat dirigits per la Dra. Marta Pascual Sánchez, Professora Titular de la Universitat de Barcelona, i pel Dr. Eduardo Soriano García, Catedràtic de la Universitat de Barcelona, i tutoritzats per la Dra. Marta Pascual Sánchez.

Barcelona, març de 2022.

Vist i plau dels directors de Tesi: La candidata a Doctora:

Dra. Marta Pascual Sánchez
Directora i Tutora

Dr. Eduardo Soriano García
Director

Eva Dávila Bouziguet

A mis padres.

A Elvira.

Sentía yo entonces vivísima curiosidad—algo novelesca—por la enigmática organización del órgano del alma. … Conocer el cerebro—nos decíamos en nuestros entusiasmos idealistas—equivale a averiguar el cauce material del pensamiento y de la voluntad. … El jardín de la neurología brinda al investigador espectáculos cautivadores y emociones artísticas incomparables. … ¡Como el entomólogo a la caza de mariposas de vistosos matices, mi atención perseguía, en el vergel de la substancia gris, células de formas delicadas y elegantes, las misteriosas mariposas del alma, cuyo batir de alas quién sabe si esclarecerá algún día el secreto de la vida mental! … Porque, aun desde el punto de vista plástico, encierra el tejido nervioso incomparables bellezas. ¿Hay en nuestros parques algún árbol más elegante y frondoso que el corpúsculo de Purkinje del cerebelo o la célula psíquica, es decir, la famosa pirámide cerebral?

Santiago Ramón y Cajal

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Ja s'apropa el final d'aquesta etapa. Quan tiro la vista enrere veig com, després de prop de 7 anys al lab, han passat per la meva vida moltes persones que em fan sentir agraïda.

Per descomptat he de començar per tu, Marta. Tu em vas donar la oportunitat de començar a fer ciència. M'has ensenyat infinitat de coses. M'has ajudat a créixer com a científica, i en molts altres aspectes. M'has donat moltes eines i punts de vista que no tenia. I soc la científica que soc gràcies a tu. Gràcies per tot.

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Alba… Albes? Si vas estar al lab l'any 2021, hi ha bastants probabilitats de que et diguessis Alba. I un terç de que fossis tu l'Alba a la que cridaven.

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Aquesta tesi és meva i també vostra. Gràcies a totes i tots.

ABSTRACT

The neuropathological hallmarks of Alzheimer's disease are senile plaques, extracellular deposits of amyloid-β peptide (Aβ), and neurofibrillary tangles, intracellular aggregates of hyperphosphorylated Tau protein (P-Tau). At the functional level, Alzheimer's disease involves synaptic dysfunction, aberrant network activity, and cognitive impairment, eventually resulting in dementia. Remarkably, individuals presenting Aβ and P-Tau without cognitive impairment have been reported. This cognitive resilience to Alzheimer's disease is believed to involve synaptic preservation.

In Alzheimer's disease, Aβ and P-Tau exert toxicity either independently or synergistically. However, Tau is pivotal in mediating the synaptotoxicity induced by Aβ, neurodegeneration, and cognitive decline. Tau is a canonical microtubule-binding protein. Nevertheless, its physiological roles are currently recognized to extend far beyond microtubule stabilization, including participation in synaptic plasticity and DNA protection. Although the regulation of Tau function depends on phosphorylation, the association of P-Tau with Alzheimer's disease and other tauopathies has established the notion that it is invariably pathological.

Aberrant network activity, hyperexcitability, and altered oscillatory activity, which indicate an imbalance between excitation and inhibition in neural circuits, have been described in Alzheimer's disease. Hence, dysfunction of the GABAergic system has been suggested to underlie network abnormalities. Studies from our group have revealed synaptic alterations in the GABAergic septohippocampal pathway and its implication in the emergence of abnormal hippocampal oscillatory activity in J20 and VLW animals, Alzheimer's disease mouse models that accumulate Aβ and P-Tau, respectively. GABAergic neurons in the medial septum control GABAergic hippocampal interneurons, which govern principal cell activity, hence modulating hippocampal oscillations.

Given the myriad of physiological roles of Tau and the impact of phosphorylation on its activity, we have studied its phosphorylation pattern in physiological and pathological conditions in mice and human subjects. Our data show that control mice and human subjects present Tau phosphorylated at Thr205 and Ser262 in the soma of hippocampal interneurons, pointing to a physiological role of these P-Tau species in this neuron population. Moreover, we have observed these P-Tau species in the soma of hippocampal interneurons in Alzheimer's disease patients and in J20 and VLW mice. The presence of Tau phosphorylated at Thr205 correlates with the Aβ plaque burden in J20 animals, suggesting an inductive effect of Aβ on Tau phosphorylation at this site. Conversely, the accumulation of Tau phosphorylated at Ser262 is unchanged in pathological conditions, implying a physiological role of this P-Tau. In addition, our data indicate that

the presence of mutant human Tau in pyramidal neurons in VLW mice induces the phosphorylation of endogenous murine Tau at Thr231 in hippocampal interneurons.

Furthermore, to recapitulate the complete spectrum of Alzheimer's disease pathology, we have crossed J20 and VLW mice. In the resulting J20/VLW animals, which simultaneously present Aβ and P-Tau, our findings reveal no differences in Aβ burden or P-Tau levels compared to single transgenic mice. However, J20/VLW animals present a distinct Tau phosphorylation pattern in hippocampal interneurons. Our data show that GABAergic septohippocampal innervation is dramatically altered in J20 and VLW animals, whereas it is preserved in J20/VLW mice. Moreover, we have found that hippocampal oscillations are partially conserved in J20/VLW animals, in contrast to single transgenic mice. Finally, J20/VLW mice display preserved cognitive function, contrary to J20 and VLW animals.

Taken together, our findings point to a physiological role of Tau phosphorylation in the somatodendritic compartment of hippocampal interneurons. Moreover, they suggest that a particular Tau phosphorylation signature in this neuron population protects against the loss of GABAergic septohippocampal innervation, thereby avoiding alterations in hippocampal oscillatory activity and, thus, preventing cognitive impairment in J20/VLW mice. Lastly, these data highlight J20/VLW mice as a suitable animal model to explore the cognitive resilience to Alzheimer's disease.

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Aβ	amyloid-β peptide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	amyloid precursor protein
CA	Cornu Ammonis
CB	calbindin
Cdk5	cyclin-dependent kinase 5
CR	calretinin
CRAD	cognitive resilience to Alzheimer's disease
FTDP-17	frontotemporal dementia with Parkinsonism linked to chromosome 17
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GSK3β	glycogen synthase kinase 3β
HCN	hyperpolarization-activated cyclic nucleotide-gated channel
IPSP	inhibitory postsynaptic potential
LFP	local field potential
LTD	long-term depression
LTP	long-term potentiation
MSDB	medial septum and diagonal band of Broca complex
NDAN	non-demented with Alzheimer's disease neuropathology
NMDA	N-methyl-D-aspartic acid
P-Tau	hyperphosphorylated Tau protein
PSD-95	postsynaptic density protein 95
pSer262 Tau	Tau phosphorylated at serine 262
pThr205 Tau	Tau phosphorylated at threonine 205
pThr231 Tau	Tau phosphorylated at threonine 231
PV	parvalbumin
REM	rapid eye movement
Thy1	thymocyte differentiation antigen 1
WT	wild-type

INTRODUCTION

1. The septohippocampal pathway

1.1 Components of the septohippocampal pathway

1.1.1 Septal region

The septum is a cerebral structure that forms the medial interventricular wall in the human brain (Risold, 2004). It is a telencephalic subcortical structure and part of the basal forebrain. The basal forebrain is a complex brain region composed of heterogeneous structures and neuron types, which present different morphologies, neurotransmitter contents, and projection patterns. It is involved in cortical activation, attention, motivation, and memory (Zaborszky et al., 2012).

1.1.1.1 Septal region organization

In rodents, the septum is bound anteriorly and dorsally by the corpus callosum and laterally by the lateral ventricles. Four main groups of nuclei can be defined in the septum: medial, lateral, ventral, and posterior. The medial group contains the medial septal nucleus and the nucleus of the diagonal band of Broca. The lateral group consists of the lateral septal, septofimbrial, and septohippocampal nuclei. The ventral group is made of the bed nucleus of the stria terminalis. Lastly, the posterior group is formed by the triangular nucleus, the bed nucleus of the anterior commissure, and the bed nucleus of the stria medullaris (Risold, 2004). This thesis deals with the septohippocampal pathway, which originates in the medial septum. Therefore, not much detail on other septal regions will be given.

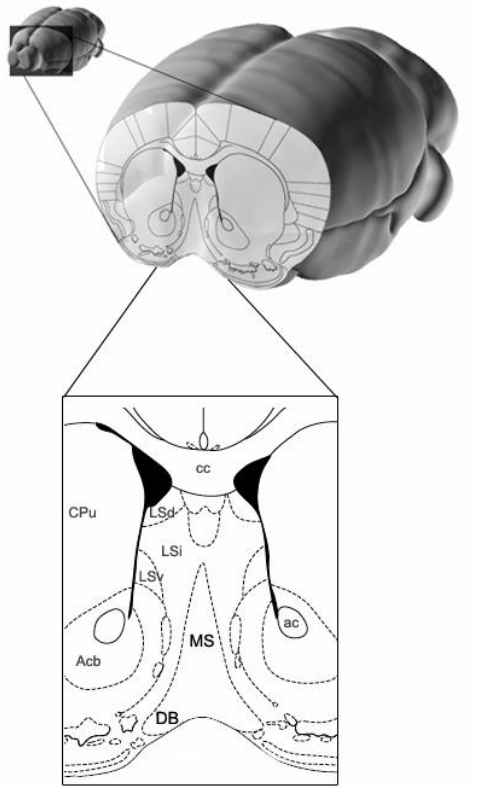

Figure 1. Anatomy of the mouse medial septum. Location of the medial septal nucleus (MS) and the nucleus of the diagonal band of Broca (DB) in a coronal section of the mouse brain corresponding to the midpoint of the septal area on the anteroposterior axis (Bregma: 0.98*). Courtesy of S.E. Rubio.*

As mentioned above, the medial septum consists of the medial septal nucleus and the nucleus of the diagonal band of Broca, which constitute the medial septal nucleus and diagonal band of Broca (MSDB) complex. The medial septal nucleus is arranged in a vertical band around the medial line of the septum. The nucleus of the diagonal band of Broca comprises a vertical and a horizontal limb,

located in the most medial and lateral parts of the ventral septum, respectively (Paxinos & Franklin, 2001). Together, the medial septal nucleus and the nucleus of the diagonal band of Broca form an inverted Y-shaped structure in coronal sections (Figure 1).

In humans, the septum consists of two distinct parts: the septum pellucidum and the septum verum. The septum pellucidum is a double membrane of connective tissue situated in the medial line, separating the anterior horns of the lateral ventricles. The septum verum or septal area is a region below the anterior end of the corpus callosum on the medial aspect of the frontal lobe and includes the septal nuclei and the septal cortex. The septal nuclei lay deep below the corpus callosum, and in humans, contrary to rodents, they are not located within the septum pellucidum (Hendelman, 2005). However, the human correlate of the rodent medial septum equally consists of the medial septal nucleus and the nucleus of the diagonal band of Broca (Ashwell & Mai, 2012). Closely related to the human MSDB complex is another basal forebrain structure, the nucleus basalis of Meynert (Ashwell & Mai, 2012).

1.1.1.2 Septohippocampal neurons

The septohippocampal pathway comprises projection neurons located in the MSDB complex that innervate all hippocampal regions (Figure 2) (Meibach & Siegel, 1977; Mosko et al., 1973; Swanson & Cowan, 1979). Septohippocampal axons project to the hippocampal formation mainly via the fimbria and dorsal fornix, while some travel through the supracallosal stria to innervate more posterior hippocampal regions and others take a ventral route through and around the amygdaloid complex and terminate in the ventral subiculum (Dutar et al., 1995). Although the septohippocampal pathway is primarily an ipsilateral projection, some axons reach the contralateral hippocampus (Gaykema et al., 1990).

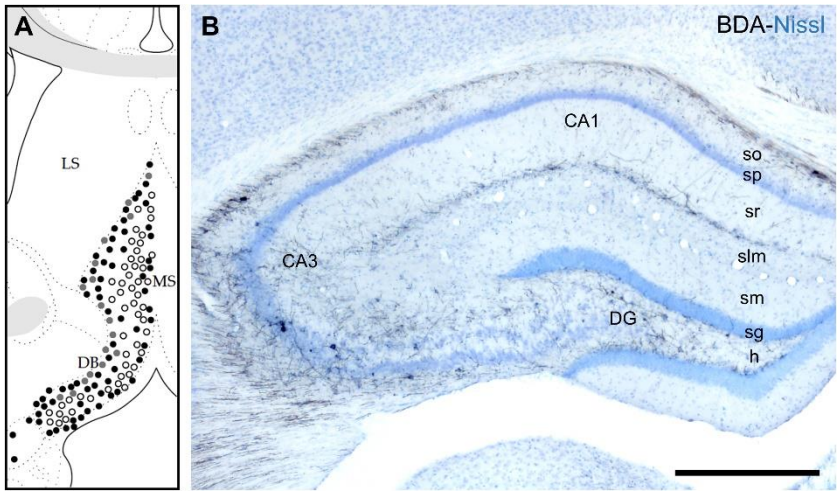

Figure 2. Distribution of the somas and axons of septohippocampal neurons. (A) Location of the somas of GABAergic (open dots), cholinergic (black dots), and glutamatergic (gray dots) neurons in the MSDB complex of the rat. (B) Septohippocampal axons (BDA, black) labelled through tract-tracing methods profusely innervate the hippocampal formation, especially the CA3 region, the *stratum oriens* and *stratum lacunosum-moleculare* of the CA1 region, and the hilus of the dentate gyrus. Abbreviations: BDA, biotinylated dextran-amine; DB, nucleus of the diagonal band of Broca; DG, dentate gyrus; h, hilus; LS, lateral septal nucleus; MS, medial septal nucleus; sg, granule cell layer; slm, *stratum lacunosum-moleculare*; sm, *stratum moleculare*; so, *stratum oriens*; sp, pyramidal cell layer; sr, *stratum radiatum*. Scale bar: 500 μm. *Adapted from Risold, 2004 (A).*

The medial septal nucleus projects to the dorsal hippocampal area from its most anteromedial portion,

whereas its most posterolateral part innervates ventral hippocampal levels, denoting a topographic arrangement of the septohippocampal projection. On the other hand, both the vertical and the horizontal limbs of the diagonal band of Broca project to all hippocampal levels (Gaykema et al., 1990; Yoshida & Oka, 1995).

Three main septohippocampal neuron types have been described, based on neurotransmitter content: GABAergic, cholinergic, and glutamatergic.

1.1.1.2.1 GABAergic neurons

GABAergic neurons employ the inhibitory neurotransmitter γ-aminobutyric acid (GABA). They are located throughout the MSDB complex and represent 45–65% of septohippocampal neurons (Amaral & Kurz, 1985; Wainer et al., 1985).

In the septum, GABAergic neurons are a diverse neuron population that encompasses local circuit interneurons and projection neurons. Specifically, the subpopulation in the MSDB complex that projects to the hippocampal formation can be identified by the expression of the calcium-binding protein parvalbumin (PV) (Freund, 1989). These PV-positive GABAergic neurons have a high degree of contact specificity in the hippocampus at both the cellular and subcellular levels, as they exclusively innervate the somatodendritic compartment of GABAergic interneurons (Figure 3 and Figure 4). GABAergic septohippocampal neurons possess thick myelinated axons and establish numerous, large synaptic boutons arranged in basket-shaped structures around the soma and proximal dendrites of their targets, thereby characterizing them as basket cells (Figure 3). Their GABAergic interneuron targets are located in all layers and regions of the hippocampal formation, although mainly in the *stratum oriens* and *stratum radiatum* of the *Cornu Ammonis* (CA) 3 region and in the hilus and granule cell layer of the dentate gyrus (Freund & Antal, 1988; Gulyás et al., 1991).

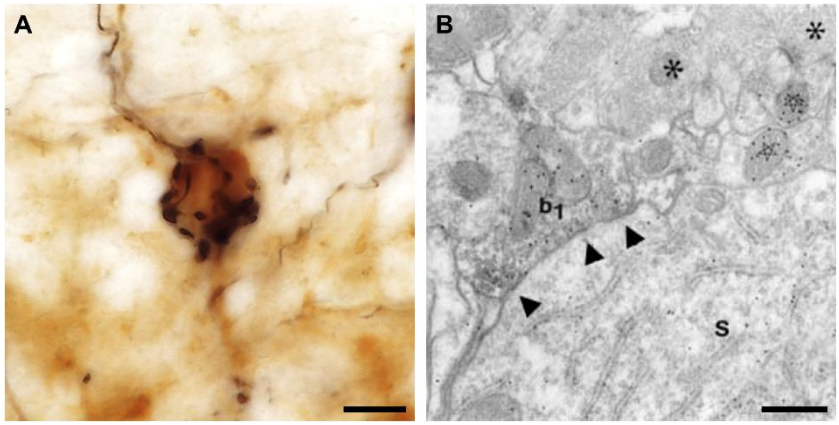

Figure 3. GABAergic septohippocampal synaptic contacts.
(A) The basket-shaped contacts established by GABAergic septohippocampal axons can comprise tens of synaptic boutons (black) located around the soma and proximal dendrites of GABAergic hippocampal interneurons, in this case a PV-positive interneuron (brown). (B) GABAergic synapses (arrow heads) established by GABAergic septohippocampal boutons (b1) on the soma of GABAergic hippocampal interneurons (S) visualized through electron microscopy, where gold particles (black) indicate the presence of the neurotransmitter GABA in both the presynaptic (b1) and postsynaptic (S) compartments. Scale bar: 10 μm (A), 500 nm (B). *Adapted from Freund & Antal, 1988 (B).*

Remarkable features of septohippocampal neurons are their electrophysiological properties. GABAergic septohippocampal neurons are fast- and burst-firing, meaning they can fire action potentials at high frequencies, higher than the rest of septal neurons, and are able to generate rhythmic bursts of action potentials, respectively. This attributes are often presented by PV-positive GABAergic neurons, hence, termed fast-spiking cells (Kawaguchi et al., 1987; Simon et al., 2006; Sotty et al., 2003).

1.1.1.2.2 Cholinergic neurons

Cholinergic neurons utilize the excitatory neurotransmitter acetylcholine. They are positioned all over the MSDB complex and make up 35–45% of septohippocampal neurons (Amaral & Kurz, 1985; Wainer et al., 1985).

Cholinergic neurons have historically been studied using acetylcholinesterase enzyme activity as a marker. More recently, they have been identified by the expression of choline acetyltransferase, another enzyme essential for acetylcholine metabolism. Cholinergic septohippocampal neurons predominantly innervate the dendritic shafts of pyramidal and granule neurons, as well as of interneurons, thus contacting all hippocampal neuron types (Figure 4). These neurons have thin unmyelinated axons and form small synaptic contacts on their targets, primarily within or in close proximity to the pyramidal and granule cell layers (Frotscher & Léránth, 1985, 1986; P. R. Lewis & Shute, 1967).

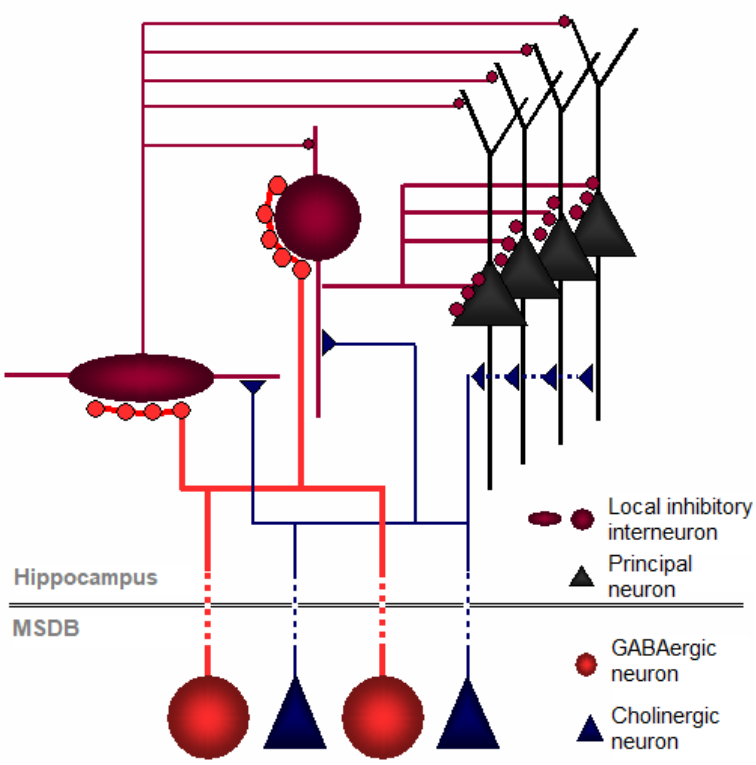

Figure 4. Target selection of the septohippocampal pathway components. Cholinergic septohippocampal axons establish synaptic contacts with all hippocampal neuron types. Conversely, GABAergic septohippocampal axons specifically contact GABAergic hippocampal interneurons, which in turn innervate hippocampal principal neurons. Dashed lines indicate the path of the axons from the MSDB complex to the hippocampus and are not to scale. *Courtesy of M. Pascual.*

Cholinergic septohippocampal neurons are slow-firing, and they fire at much lower frequencies than their GABAergic counterparts (Simon et al., 2006; Sotty et al., 2003).

1.1.1.2.3 Glutamatergic neurons

Glutamatergic neurons use the excitatory neurotransmitter glutamate. They are located within the MSDB complex, mainly at the border between the lateral and the medial septum, and represent 4–23% of septohippocampal neurons (Colom et al., 2005; Hajszan et al., 2004; Henderson et al., 2010).

Compared to cholinergic and GABAergic neurons, glutamatergic neurons were a late addition to the known populations of septohippocampal projection neurons, and they can be identified by the expression of vesicular glutamate transporter 2. Glutamatergic septohippocampal projections in the hippocampus are sparse and innervate both pyramidal neurons and interneurons in or near the *stratum oriens*. However, data indicate strong connections of these neurons within the septum, but scarce functional connections with the hippocampus (Huh et al., 2010; J. Robinson et al., 2016; Sun et al., 2014).

Glutamatergic septohippocampal neurons display heterogeneous action potential firing patterns, as they can be slow-, fast-, burst-, or cluster-firing (Huh et al., 2010; Sotty et al., 2003).

1.1.2 Hippocampal formation

The hippocampal formation is a telencephalic allocortical structure present in both cerebral hemispheres. In rodents, it appears as an elongated, C-shaped structure, with its long axis extending from the septal nuclei of the basal forebrain anterodorsally, over and behind the diencephalon, into the posteroventral portion of the hemisphere (Cappaert et al., 2015; Witter, 2012). This long axis is referred to as the dorsoventral axis in mice and the septotemporal axis in rats, and orthogonal to it is the transverse axis. In humans, the hippocampal formation is located deep within the medial aspect of the temporal lobe, and its anteroposterior extent partially occupies the cavity of the temporal horn of the lateral ventricle (Hendelman, 2005; Insausti & Amaral, 2012).

The hippocampal formation has a laminar organization and encompasses the hippocampus proper, the dentate gyrus, and the subiculum. Closely related to the hippocampal formation is another telencephalic cortical structure: the parahippocampal region, which includes the presubiculum, the parasubiculum, the entorhinal cortex, and the perirhinal and postrhinal cortices (Cappaert et al., 2015). The parahippocampal region composes the most posterior and ventral portion of the cerebral cortex and wraps around the posteroventral part of the hippocampal formation (Witter, 2012).

1.1.2.1 Hippocampal cytoarchitecture

The hippocampus proper can be divided into three major regions: CA1, CA2, and CA3. Frequently, the CA2 and CA3 are grouped into a single region. The distinction of the CA2 has been a subject of debate, as it resembles a terminal portion of the CA3. There are differences in pyramidal neuron size between the CA1 and CA3, but the main differences between regions are the intrinsic and extrinsic connectivity patterns. The laminar organization is similar for all hippocampal regions, with their different layers stacked in parallel to the surface of the lateral ventricle (Figure 5) (Cappaert et al., 2015; Witter, 2012).

From the surface of the ventricle to the hippocampal fissure, the CA regions of the hippocampus proper contain the following layers:

- **Alveus**. It is located above the *stratum oriens* and contains afferent and efferent axons.

- ***Stratum oriens***. It is a narrow layer located above the pyramidal cell layer. It encompasses the basal dendrites of pyramidal neurons as well as GABAergic neurons, among which are both interneurons and neurons that project to the septum.

- **Pyramidal cell layer** (***stratum pyramidale***). It is the principal cell layer. It contains the somas of glutamatergic pyramidal neurons and some GABAergic interneurons.

- ***Stratum lucidum***. It is a narrow layer located adjacent to the pyramidal cell layer, exclusively in the CA3. It is occupied by granule neuron axons, referred to as mossy fibers, originating from the dentate gyrus, and some GABAergic interneurons.

- ***Stratum radiatum***. It is below the pyramidal cell layer in the CA1 and adjacent to the *stratum lucidum* in the CA3. It encompasses the apical dendrites of pyramidal neurons and some GABAergic interneurons. It can be defined as the suprapyramidal region, where CA3 to CA3 associative connections and CA3 to CA1 Schaffer collateral connections are located.

- ***Stratum lacunosum-moleculare***. It is adjacent to the *stratum radiatum* and contains the most distal part of the apical dendrites of pyramidal neurons, alongside GABAergic interneurons and Cajal-Retzius cells. It is where perforant pathway fibers from the entorhinal cortex travel and terminate.

The dentate gyrus is composed of three layers:

- **Molecular layer** (***stratum moleculare***). It is the most superficial layer of the dentate gyrus and borders the hippocampal fissure and the pia. Although it has relatively sparse cells, it comprises several GABAergic interneuron types and Cajal-Retzius cells. It

contains the dendrites of granule neurons and receives axons originating from the entorhinal cortex and the hilus.

- **Granule cell layer** (***stratum granulosum***). It lies deep to the molecular layer. It comprises tightly packed glutamatergic granule neurons and some GABAergic interneurons.

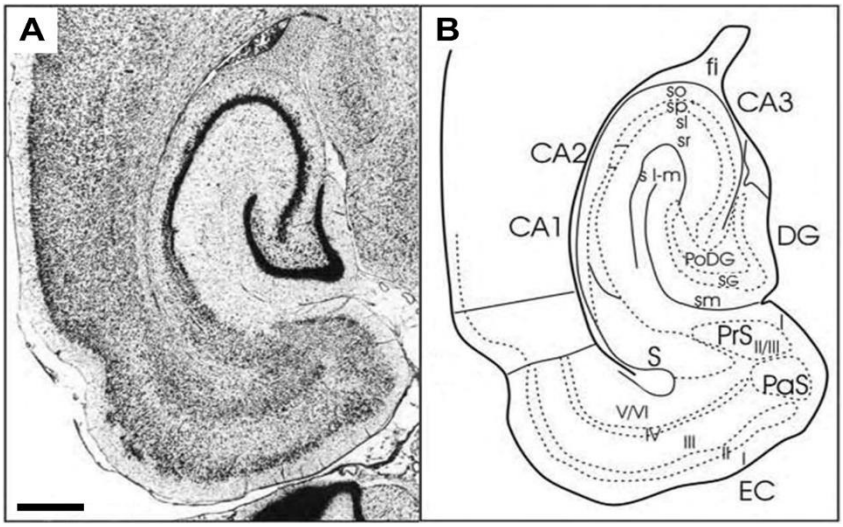

Figure 5. Cytoarchitecture of the hippocampal formation.
(A) Laminar organization of the hippocampus proper and dentate gyrus visualized through Nissl staining. (B) Main regions and layers of the hippocampal formation and the parahippocampal region. Abbreviations: DG, dentate gyrus; EC, entorhinal cortex; fi, fimbria; PaS, parasubiculum; PoDG, polymorphic layer of the dentate gyrus; PrS, presubiculum; sg, granule cell layer; sl, *stratum lucidum*; slm, *stratum lacunosum-moleculare*; sm, *stratum moleculare*; so, *stratum oriens*; sp, pyramidal cell layer; sr, *stratum radiatum* S, subiculum. Scale bar: 500 μm (A). *Adapted from Witter & Amaral, 2004 (A and B).*

Together, the granule cell and molecular layers form a V- or U-shaped structure enclosing the third layer of the dentate gyrus:

- **Hilus or polymorphic layer**. It is the inner layer of the dentate gyrus, surrounded by the granule cell layer. It contains the axons of granule neurons that project to the CA3, or mossy fibers, as well as glutamatergic mossy cells and GABAergic interneurons (Cappaert et al., 2015; Witter, 2012).

1.1.2.2 Hippocampal circuits

The connectivity of the hippocampal formation and parahippocampal region is highly complex and intricate, regarding both the intrinsic connectivity within its regions and the extrinsic connectivity between them and with other brain areas (Cappaert et al., 2015). An outstanding hippocampal network is the trisynaptic circuit (Figure 6), which involves three unidirectional excitatory connections that allow the hippocampal formation to process information coming from neocortical regions through the entorhinal cortex and send it back to the neocortex (Andersen et al., 1966). The trisynaptic circuit consists of the following connections:

- **Perforant pathway**. Axons of pyramidal, stellate, and multipolar neurons located in layer II of the entorhinal cortex project to the molecular layer of the dentate gyrus, where they contact the dendrites of granule neurons.

- **Mossy fiber pathway**. Axons of granule neurons, or mossy fibers, located in the dentate gyrus project to the *stratum lucidum* of the CA3, where they contact the dendrites of pyramidal neurons.

- **Schaffer collateral**. Axons of pyramidal neurons located in the CA3 project to the *stratum radiatum* and *stratum oriens* of the CA1, where they contact the dendrites of pyramidal neurons.

The trisynaptic circuit is closed when the information that reaches the CA1 is projected back to the entorhinal cortex. This information flow occurs directly, as axons of pyramidal neurons in the CA1 project to layer V of the entorhinal cortex, and indirectly, through the subiculum.

It should be noted that this is a rather simplified conception of hippocampal circuitry, given that many extrinsic and intrinsic networks overlap with the trisynaptic circuit. However, it highlights the fact that the hippocampal formation is organized very differently from the rest of cortical regions. For instance, unidirectional connectivity is a peculiarity of the hippocampal formation. Furthermore, it is one of few brain regions receiving highly processed multimodal sensory information from various neocortical areas. At the same time, it possesses highly associative intrinsic networks (Andersen et al., 2007; Cappaert et al., 2015; Witter, 2012).

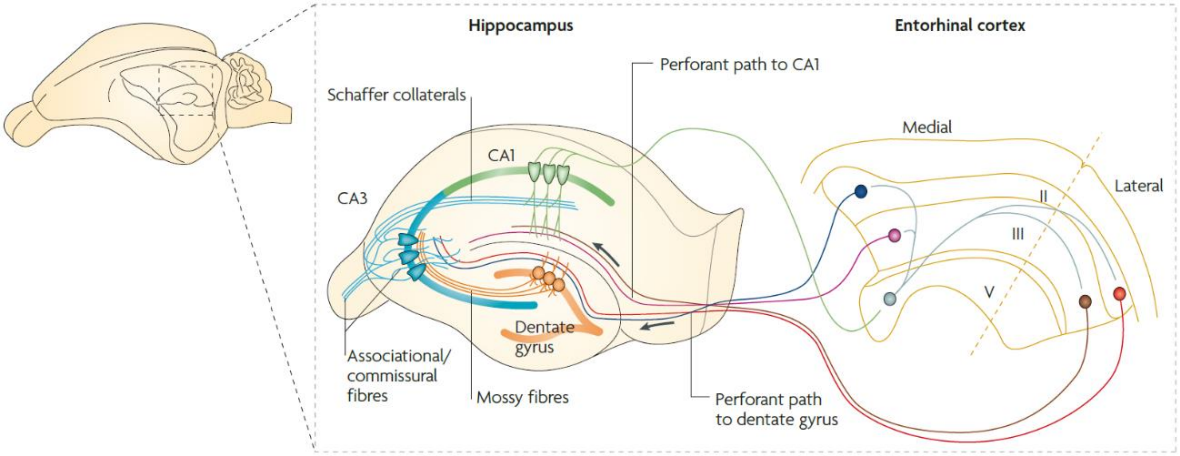

Figure 6. Trisynaptic circuit of the hippocampus.
Connections that compose the trisynaptic circuit, encompassing the perforant pathway, the mossy fiber pathway, and the Schaffer collateral, as well as additional entorhinal cortex inputs and associational and commissural connections that contribute to the complex hippocampal circuitry. *Adapted from Neves et al., 2008.*

Further remarkable circuits of the hippocampal formation are the recurrent autoassociative connections established by CA3 pyramidal neurons, which, in addition to the Schaffer collateral projecting to the CA1, emit numerous local axon collaterals that innervate other pyramidal neurons located in the CA3. Moreover, CA3 pyramidal neurons

also establish commissural projections to the contralateral hippocampal formation that innervate CA3 and CA1 pyramidal neurons, as well as some interneurons (Cappaert et al., 2015; Witter, 2012).

In the context of this thesis, it is worth mentioning the complex network established by inhibitory interneurons within the hippocampal formation. Hippocampal inhibitory interneurons form vast local circuits through which they modulate the activity of principal neurons and other interneurons. In this way, local circuit inhibitory interneurons are able to control both the excitability of single cells and the temporal window for principal neuron population activity, which is crucial for the coordination and integration of hippocampal functions (Freund & Buzsáki, 1996).

As a result of the intricate, singular organization of its connectivity, the hippocampal formation is endowed with the ability to integrate information from all sensory modalities, making it a unique region for possessing this quality (Andersen et al., 2007).

1.1.2.3 Hippocampal interneurons

Interneurons are present across all brain regions, where they carry out essential functions within neural circuits. This section will delve into the field of hippocampal interneurons.

Hippocampal interneurons are mainly inhibitory local circuit neurons. However, some cells included in this class display features typical of principal projection neurons, such as establishing commissural axon collaterals or projecting to extrahippocampal areas. By definition, inhibitory interneurons utilize the neurotransmitter GABA. Therefore, they express the GABAergic cell marker glutamic acid decarboxylase (GAD), the synthesizing enzyme of GABA (Andersen et al., 2007; Freund & Buzsáki, 1996; Pelkey et al., 2017).

GABAergic local circuit inhibitory interneurons are scattered throughout all hippocampal subfields and layers but are far less abundant than principal neurons, accounting for 10–15% of the total hippocampal neuron population. Despite this, an extraordinary diversity of interneuron classes has been described, and this diverse population is a major regulator of cortical circuit function (Andersen et al., 2007; Freund & Buzsáki, 1996; K. D. Harris et al., 2018; Pelkey et al., 2017).

Classification of hippocampal interneurons into subpopulations has been attempted based on their anatomical, neurochemical, and physiological properties, location, or connectivity (Freund & Buzsáki, 1996; Mátyás et al., 2004). However, due to their exceptional diversity and heterogeneity, finding a representative, non-overlapping classification scheme is not straightforward. The present thesis defines three non-overlapping

subpopulations of hippocampal interneurons based on their calcium-binding protein content (Figure 7): PV, calretinin (CR), and calbindin (CB).

1.1.2.3.1 Parvalbumin-positive interneurons

PV-positive interneurons represent 20–24% of the total GABAergic hippocampal neuron population. Their somas are heterogeneous and large compared to other interneurons, and they are mainly located in the pyramidal cell layer and *stratum oriens* of the hippocampus and the granule cell layer and hilus of the dentate gyrus. According to their connectivity, PV-positive interneurons can be basket, axo-axonic, or bistratified cells (Freund & Buzsáki, 1996; Mátyás et al., 2004; Pelkey et al., 2017).

PV-positive basket cells establish numerous, large synaptic boutons arranged in basket-shaped structures around the soma and proximal dendrites of principal neurons. They account for 60% of the total PV-positive cell population, and a single one of these cells contacts up to 2500 pyramidal neurons with an average of seven synaptic boutons onto each one. PV-positive axo-axonic, or chandelier, cells establish approximately seven synaptic boutons organized in a row on the axon initial segment of each principal neuron they innervate. They represent 16% of the total PV-positive cell population, and an individual cell of this type contacts up to 1200 pyramidal neurons. Finally, bistratified cells simultaneously contact both the apical and basal dendrites of principal neurons, with 20% of these synapses made onto dendritic spines. They account for 24% of the total PV-positive cell population, and a single bistratified cell contacts approximately 1600 pyramidal neurons with an average of six synaptic boutons onto each (Bezaire & Soltész, 2013; Freund & Buzsáki, 1996; Pelkey et al., 2017).

Several features presented by PV-positive GABAergic neurons endow them with the ability to exert a strong influence on their targets and modulate hippocampal rhythmic activity patterns. Because each PV-positive interneuron innervates a large number of principal neurons, they facilitate the synchronization of principal neuron population activity (Cobb et al., 1995; Klausberger & Somogyi, 2008). In addition, their electrophysiological properties play a crucial role in the induction of synchrony. Like PV-expressing GABAergic septohippocampal neurons, PV-positive interneurons are fast-spiking cells, meaning they can generate action potentials at high frequencies (Kawaguchi et al., 1987; Simon et al., 2006; Sotty et al., 2003). Moreover, PV-positive neurons present gap junctions, electrical synapses that allow fast communication within their population, thereby forming complex functional interneuron networks (Fukuda & Kosaka, 2000; Katsumaru et al., 1988; Venance et al., 2000). This fast electrical coupling leads to the synchronization of action potentials in the PV-positive neuron population, contributing to

their role in promoting the cooperative activity of principal neurons (Buzsáki & Chrobak, 1995; Cobb et al., 1995; Klausberger & Somogyi, 2008).

Because of their relevance in the topics addressed in this thesis, PV-positive neurons are covered in extensive detail in this and later sections.

1.1.2.3.2 Calretinin-positive interneurons

CR-positive interneurons account for 13% of the total GABAergic hippocampal neuron population. Their somas are mostly multipolar, and they can be found throughout all hippocampal layers, although more abundantly in the pyramidal cell layer, the *stratum radiatum,* and the *stratum oriens*. Regarding their connectivity, CR-positive interneurons selectively or preferentially innervate other interneurons. In particular, these cells belong to the type 1 interneuron-selective interneuron class and establish synaptic contacts in a climbing fiber-like manner on the soma and dendrites of their targets. Moreover, CR-positive interneurons that coexpress vasoactive intestinal peptide belong to the type 3 interneuron-selective interneuron class and contact the dendrites of their targets (Bezaire & Soltész, 2013; Freund & Buzsáki, 1996; Gulyás et al., 1996; Mátyás et al., 2004; Pelkey et al., 2017).

It should be considered that not all hippocampal neurons expressing CR are GABAergic interneurons. For instance, Cajal-Retzius cells, located close to the hippocampal fissure in the *stratum lacunosum-moleculare* of the hippocampus and the molecular layer of the dentate gyrus, also contain CR (Soriano et al., 1994). This neuron population is abundant during embryonic development, but only a small fraction remains in the adult brain due to its transitory nature. However, Cajal-Retzius cells are glutamatergic and do not belong to the inhibitory interneuron category. Similarly, mossy cells located in the hilus of the dentate gyrus also express CR in some species, including mice, hamsters, and rhesus monkeys, yet they are glutamatergic neurons and not inhibitory interneurons (Y. Liu et al., 1996; Mátyás et al., 2004; Seress, 2007).

1.1.2.3.3 Calbindin-positive interneurons

CB-positive interneurons account for approximately 11% of the total GABAergic hippocampal neuron population. Their somas are generally multipolar, and they are mainly located in the *stratum radiatum* and are abundant near the border of the *stratum lacunosum-moleculare* of the hippocampus. As for their connectivity, CB-positive interneurons are dendrite-targeting interneurons and innervate pyramidal neurons (Freund & Buzsáki, 1996; Mátyás et al., 2004; Pelkey et al., 2017; Tóth & Freund, 1992).

Analogous to CR, CB is expressed by other hippocampal neurons that are not inhibitory interneurons but glutamatergic neurons, namely granule neurons of the dentate gyrus and superficial pyramidal neurons of the CA1 region (Baimbridge & Miller, 1982; Mátyás et al., 2004).

Notably, there is a subpopulation of GABAergic CB-positive neurons located in the *stratum oriens* that projects to the MSDB complex, where they mainly innervate GABAergic neurons, some of which are GABAergic septohippocampal neurons that project back to the hippocampus (Tóth et al., 1993; Tóth & Freund, 1992). Hence, this hippocamposeptal pathway closes a septo-hippocampo-septal loop, as will be described in a later section (see section 1.2.2.2).

The GABAergic septohippocampal pathway exerts a robust control over the network of GABAergic local circuit interneurons, heavily influencing principal neuron activity. Thus, it has a pivotal role in determining hippocampal function, as will be detailed in subsequent sections (see section 1.3).

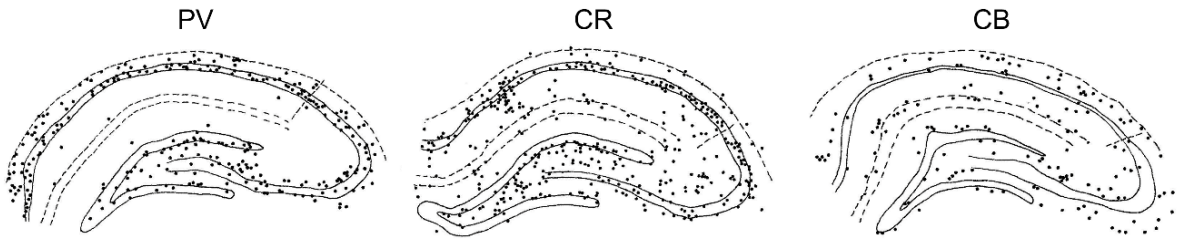

Figure 7. Distribution of hippocampal interneuron subpopulations based on their calcium-binding protein content. Camera lucida drawings of the distribution of interneurons immunoreactive for PV, CR, and CB in coronal sections of the rat hippocampus. Each dot represents a cell. *Adapted from Freund & Buzsáki, 1996.*

1.2 Afferent connections of septohippocampal neurons

As a subcortical structure, the septum links the cerebral cortex and effector regions of the hypothalamus and brainstem. It integrates information from a descending system, responsible for neuroendocrine and autonomic functions involving homeostatic control and social behavior, and an ascending system, comprising cognitive processes such as learning and memory (L. Medina & Abellán, 2012).

In particular, septohippocampal neurons receive afferents from local circuit neurons located in the MSDB complex and from projection neurons found in other brain regions.

1.2.1 Local circuits

Within the MSDB complex, cholinergic septohippocampal neurons contact GABAergic septohippocampal neurons via axon collaterals (Alreja et al., 2000; M. Wu et al., 2000). Likewise, glutamatergic local circuit neurons innervate cholinergic and GABAergic septohippocampal neurons (Hajszan et al., 2004; M. Wu et al., 2004). As a result of the actions of these neurons through the local network within the MSDB complex, GABAergic septohippocampal neurons are activated, which is crucial for the generation and modulation of hippocampal rhythmic activity patterns (J. Robinson et al., 2016), as will be described in later sections (see section 1.3).

1.2.2 External afferent connections

The main external afferent connections of septohippocampal neurons are described below.

1.2.2.1 Lateral septum

The lateral septum sends projections to the MSDB complex, in part originating from GABAergic neurons (Leranth & Frotscher, 1989; Raisman, 1966; Risold & Swanson, 1997; Swanson & Cowan, 1979). At the time of the description of this connection, the available anatomical and electrophysiological data led to theorizing that the lateral to medial septum projection was in a position to influence septohippocampal neurons. Therefore, it had to be one of the principal components of the septo-hippocampo-septal loop. Nevertheless, tract-tracing studies have proved that this connection is sparse and thus cannot account for relaying the feedback from the hippocampal formation to the MSDB complex, while some studies have reported the absence of this projection in species other than rodents (Gulyás et al., 1991; Leranth et al., 1992; Staiger & Nürnberger, 1991; Witter et al., 1992).

1.2.2.2 Hippocampal formation

The septum and the hippocampal formation are reciprocally connected (Raisman, 1966). Hence, in addition to the septohippocampal pathway, an hippocamposeptal connection also exists. The hippocamposeptal pathway is one of the most influential afferent connections of septohippocampal neurons.

Hippocampal pyramidal neurons project to the lateral septum, where they primarily contact GABAergic neurons (A. Alonso & Köhler, 1982; Leranth & Frotscher, 1989; Raisman et al., 1966; Swanson & Cowan, 1977). Notably, a subpopulation of GABAergic neurons located in the *stratum oriens* of the hippocampus project to the medial septum (A. Alonso & Köhler, 1982; Gaykema, van der Kuil, et al., 1991; Tóth et al., 1993; Tóth & Freund, 1992). Most of these neurons express CB, and a large proportion contains the neuropeptide somatostatin (Gulyás et al., 2003; Tóth & Freund, 1992). In some cases, more than half of hippocampal CB-positive neurons have been found to project to the medial septum (Tóth & Freund, 1992). The main targets of GABAergic hippocamposeptal neurons are GABAergic septohippocampal neurons located in the MSDB complex (Tóth et al., 1993). Moreover, direct reciprocity within the septo-hippocampo-septal loop has been demonstrated, as GABAergic septohippocampal neurons innervate GABAergic hippocamposeptal neurons from which they receive projections (Takács et al., 2008; Tóth et al., 1993).

On the one hand, the projection from pyramidal neurons to the lateral septum constitutes an indirect input to GABAergic septohippocampal neurons. Given the existing lateral to medial septum connection, lateral septal GABAergic neurons can inhibit GABAergic septohippocampal neurons. As a result, increased activity of hippocampal pyramidal neurons triggers the inhibition of GABAergic septohippocampal neurons in order to downregulate the input from the MSDB complex to the hippocampus.

On the other hand, the connection from GABAergic neurons to the medial septum represents a direct input to GABAergic septohippocampal neurons. The firing of GABAergic hippocamposeptal neurons is synchronized to principal neuron population activity, as they are mostly driven by local axon collaterals of pyramidal neurons. Thus, their input to GABAergic septohippocampal neurons enforces the same activity pattern in this neuron population (Blasco-Ibáñez & Freund, 1995; Freund & Buzsáki, 1996; Tóth et al., 1993). In this way, hippocampal rhythmic activity patterns can be propagated to the MSDB complex. Although this projection is inhibitory, it results in the rhythmic activation of GABAergic septohippocampal neurons and hence upregulates the input from the MSDB complex to the hippocampus. This is because the regular inhibitory input leads to rhythmic hyperpolarization of GABAergic septohippocampal neurons, which evokes their rebound

activation, meaning that they fire immediately after the rebound from inhibition (Manseau et al., 2008).

GABAergic hippocamposeptal neurons also contact a small number of cholinergic septohippocampal neurons in the MSDB complex, with a minor, net inhibitory effect (Manseau et al., 2008; Tóth et al., 1993).

Altogether, the septo-hippocampo-septal loop is completed by these direct and indirect connections from the hippocampal formation to the medial septum, ensuring thorough communication and coordination between both structures by regulating the activity of septal projection neurons according to hippocampal rhythmicity.

1.2.2.3 Other afferent connections

The medial hypothalamus projects to the MSDB complex from both the posterior hypothalamic nucleus and the supramammillary nucleus, contacting GABAergic and cholinergic septohippocampal neurons (Borhegyi et al., 1998; Cullinan & Zaborszky, 1991; Vertes, 1992; Vertes et al., 1995). This hypothalamic connection is part of a synchronizing ascending system that has a role in influencing hippocampal rhythmic activity (Hernández-Pérez et al., 2015; Oddie et al., 1994; Pan & McNaughton, 2004).

The median raphe nucleus innervates GABAergic septohippocampal neurons in the MSDB complex (Alreja, 1996; Conrad et al., 1974; Leranth & Vertes, 1999). Moreover, the ventral tegmental area, the locus coeruleus, and the reticular formation project to the MSDB complex (Aransay et al., 2015; Berridge & Foote, 1996; Fallon & Moore, 1978; Gaykema & Zaborszky, 1996; Jones & Yang, 1985; Segal, 1976; Zaborszky et al., 1991).

Finally, the entorhinal cortex and the prefrontal cortex innervate GABAergic neurons in the MSDB complex (Gaykema, Van Weeghel, et al., 1991; Leranth et al., 1999; Sesack et al., 1989; Zaborszky et al., 1997).

Together, these afferents enable the integration of information coming from cortical and subcortical regions in the septum, either in relation to cognitive processes or to neuroendocrine and autonomic functions (L. Medina & Abellán, 2012). Furthermore, some of them are reciprocal connections, such as those with the hypothalamus, the brainstem, and certain cortical regions, further contributing to a comprehensive septal function (L. Medina & Abellán, 2012; Risold, 2004).

1.3 Role of the septohippocampal pathway

The septum functions as an interface between the cerebral cortex and numerous subcortical regions. It is involved in homeostatic control and social behavior owing to its descending connections with the hypothalamus, brainstem, and amygdala, among others. Moreover, the medial septum, and especially the septohippocampal pathway, is involved in learning and memory due to its ascending connections with cortical areas like the hippocampus (L. Medina & Abellán, 2012).

1.3.1 Disinhibitory circuit of the GABAergic septohippocampal pathway

The septohippocampal pathway controls hippocampal activity through a complex connectivity pattern, which enables it to regulate large populations of hippocampal neurons.

Cholinergic septohippocampal neurons tonically increase the excitability of hippocampal principal neurons and interneurons (Gulyás et al., 1990; Krnjević & Ropert, 1982). Conversely, GABAergic septohippocampal neurons specifically innervate GABAergic hippocampal interneurons (Freund & Antal, 1988; Gulyás et al., 1990). At the same time, GABAergic hippocampal interneurons mainly contact principal neurons (Freund & Buzsáki, 1996). Therefore, GABAergic neurons in the medial septum inhibit GABAergic interneurons in the hippocampus, which in turn inhibit a large number of principal neurons. In this context, it was proposed that the GABAergic septohippocampal pathway participates in a disinhibition process: activation of GABAergic septohippocampal neurons, through inhibition of GABAergic hippocampal interneurons, abolishes the inhibition exerted on hippocampal principal neurons, which are thus indirectly activated (Figure 8) (Freund & Antal, 1988). This hypothesis was subsequently confirmed by means of *in vitro* electrophysiological studies in brain slices (Tóth et al., 1997).

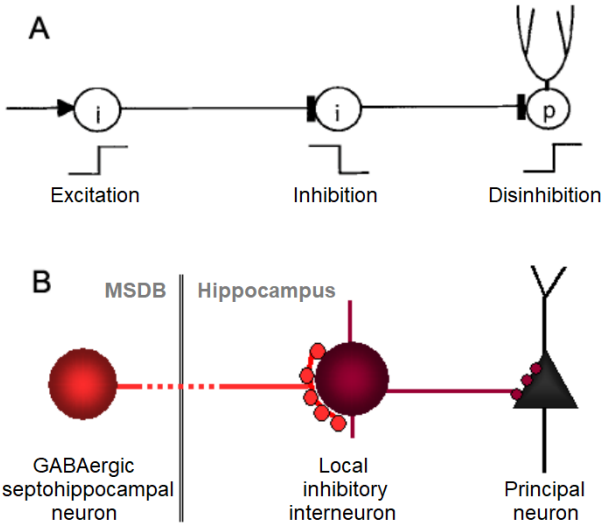

Figure 8. Disinhibition process mediated by the GABAergic septohippocampal pathway.
(A) Series circuit of two inhibitory interneurons (i) and a principal neuron (p). Activation of the first interneuron causes inhibition of the second interneuron, abolishing the inhibition that fell upon the principal neuron which is, thus, disinhibited.
(B) Elements that compose the disinhibitory circuit in the septohippocampal system. Activation of the GABAergic septohippocampal neuron inhibits the local inhibitory interneuron, leading to disinhibition of the principal neuron.
Adapted from Freund & Buzsáki, 1996 (A) and M. Pascual (B).

It is worth noting that the basket-shaped synaptic contacts established by GABAergic septohippocampal neurons are highly efficient at inhibiting their targets. This is due to the numerous synaptic boutons composing each contact and to their location on the soma and proximal dendrites of their targets (Freund & Antal, 1988). In this way, the inhibitory input falls on cellular domains that are key for synaptic integration, owing to their proximity to the action potential initiation site in the axon initial segment (Buzsáki, 2006; Takács et al., 2008). Moreover, each GABAergic septohippocampal neuron contacts multiple GABAergic hippocampal interneurons simultaneously, each of which in turn innervates from hundreds to thousands of principal neurons, many of them through strongly inhibitory basket-shaped synaptic contacts as well (Freund & Buzsáki, 1996; Takács et al., 2015). Thereby, the GABAergic septohippocampal pathway is endowed with the ability to effectively control the activity of large neuron populations, not only by regulating single-cell excitability but also by providing a temporal window for activity to take place via its inhibitory input (Freund & Buzsáki, 1996; Freund & Gulyás, 1997).

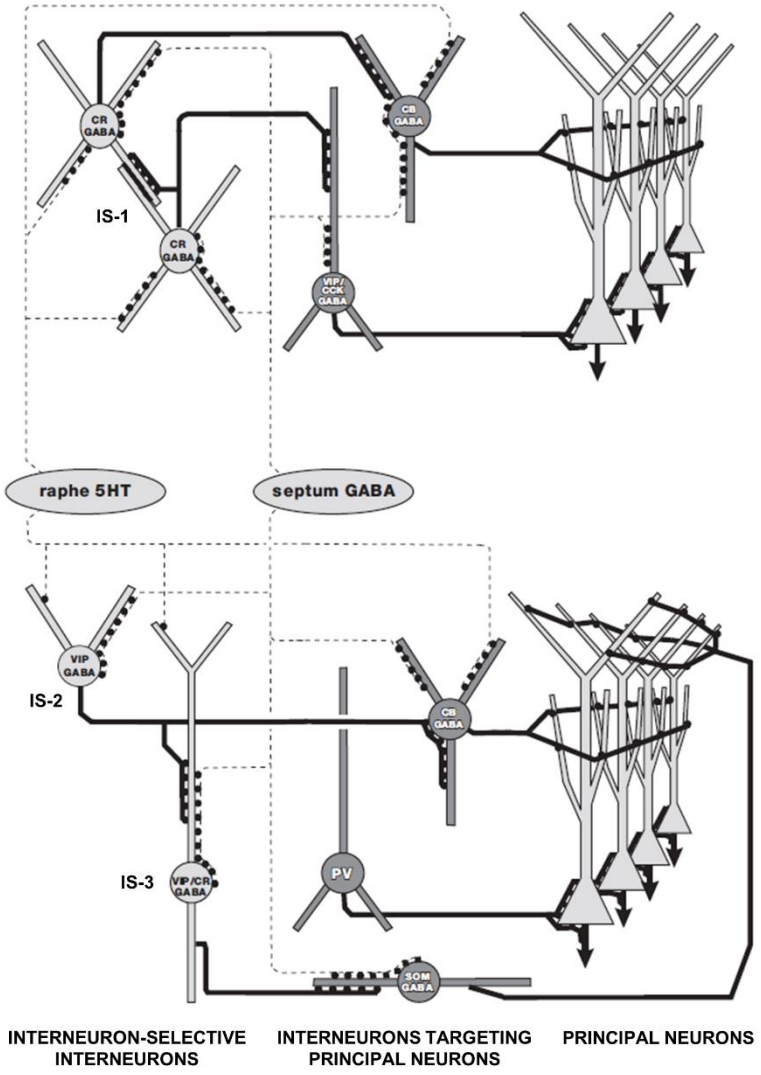

Figure 9. Control of hippocampal circuits by local and subcortical inhibitory afferents.
Septal GABAergic afferents innervate the somatodendritic compartment of all hippocampal interneuron types. IS interneurons (light gray) contact several interneuron types (dark gray) that target different compartments of principal neurons. Hence, GABAergic septohippocampal neurons are able to profoundly influence the activity of hippocampal inhibitory local circuits and, thus, of principal neurons. Median raphe nucleus serotonergic afferents innervate CR-, CB-, and VIP-positive interneurons. IS-1 interneurons contain CR, IS-2 interneurons contain VIP, and IS-3 interneurons contain both CR and VIP. Abbreviations: 5HT, serotonin; CCK, cholecystokinin; IS, interneuron-selective; SOM, somatostatin; VIP, vasoactive intestinal peptide. *Adapted from Freund & Gulyás, 1997.*

Notably, the robust disinhibitory action of GABAergic septohippocampal axons occurs at the three stages of hippocampal information processing, that is, the trisynaptic circuit: the dentate gyrus, the CA3 region, and the CA1 region (Freund, 1989). Likewise, considering the profuse connectivity between local circuit inhibitory interneurons in the hippocampus, GABAergic septohippocampal neurons influence a wide range of inhibitory local circuits by targeting most GABAergic hippocampal interneurons (Freund & Antal, 1988; Gulyás et al., 1990). On the whole, the GABAergic septohippocampal pathway is capable of integrating inputs from the medial septum and other subcortical regions connected to it, together with the existing information in the hippocampus and, thus, is in a position to influence the activity of hippocampal circuits (Figure 9) (Freund & Gulyás, 1997).

Opposite to its cholinergic counterpart, the GABAergic septohippocampal pathway has received very little attention in human studies. However, its existence has been demonstrated in rhesus monkeys. As in rodents, it terminates specifically on GABAergic hippocampal interneurons. Moreover, the synaptic organization of the GABAergic septohippocampal projection in rhesus monkeys is consistent with the disinhibitory function identified in rodents (Gulyás et al., 1991).

Altogether, the GABAergic septohippocampal pathway is able to exert robust control over the hippocampus, which is manifested as a series of oscillations generated by the rhythmic activity patterns of hippocampal neuron populations (Freund & Buzsáki, 1996). These oscillatory activities will be described in the next section.

1.3.2 Hippocampal oscillatory activity

Neuronal activity elicits extracellular voltage changes due to transmembrane currents, giving rise to different electrical fields known as electroencephalographic activity or local field potential (LFP). These terms are synonymous, but for historical reasons, electroencephalographic activity refers to the mean-field potential recorded with scalp electrodes that reflects the average behavior of a large number of neurons located in superficial layers of the cortex (Buzsáki, 2006). In contrast, LFPs are recorded with deep electrodes and arise from the temporal summation of currents of neurons in a particular location, primarily reflecting the synaptic activity of those that have been synchronously activated. However, they are also influenced by intrinsic neuronal properties such as subthreshold oscillations, Ca^{2+} spikes, and spike afterhyperpolarizations (Buzsáki, 2006; Buzsáki et al., 2012; Llinás, 1988). Overall, these electrical activity patterns reflect the activity of a large number of neurons and are particularly robust when these neurons are firing synchronously (Andersen et al., 2007).

Hippocampal activity is shaped by the interactions between the excitatory and inhibitory elements of its circuits. The functional complexity of networks arises from these interactions, especially from the inhibitory interneuron system. Inhibitory circuits modify the spread of activity, and the outcome depends on the details of individual connections. Complex feedback and feedforward circuits, established mainly by local circuit inhibitory interneurons, act complementarily to bring stability and synchrony to the hippocampal network (Buzsáki, 2006).

On the one hand, in feedback inhibition, axon collaterals from local principal neurons activate local interneurons, which in turn inhibit said principal neurons. Feedback inhibition has a stabilizing effect that involves the emergence of oscillations, since it provides periods of marked inhibition of principal neuron activity shortly after the firing of these cells (Andersen et al., 2007; Buzsáki, 2006).

On the other hand, in feedforward inhibition, axon collaterals from excitatory afferent connections activate local interneurons, which in turn inhibit local principal neurons. Feedforward inhibition enforces a temporal framework for activity on its targets and filters the effect of afferent excitation, as the excitatory input falls upon both interneurons and principal neurons. Doing so can determine the timing of principal neuron activation, hence increasing the temporal precision of firing and, therefore, driving synchrony (Andersen et al., 2007; Buzsáki, 2006). The reason for this is that feedforward inhibition imposes a narrow temporal window for the summation of excitatory and inhibitory inputs, which results in sub-millisecond order spike timing precision (Pouille & Scanziani, 2001).

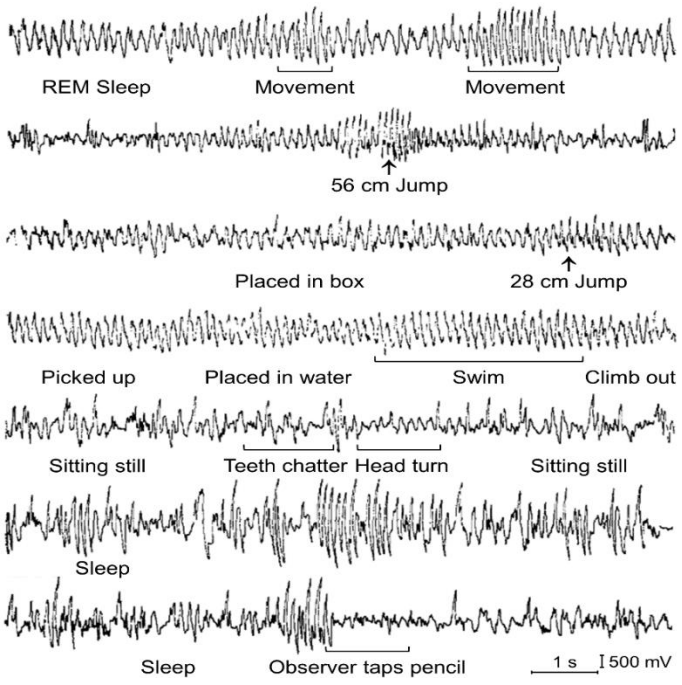

Figure 10. Hippocampal electroencephalographic activity during different behaviors in the rat.
During exploratory behaviors (movement, jumping, swimming, head turn) and REM sleep, the characteristic rhythmic activity pattern of theta oscillations is present. During other behaviors (sitting still, teeth chatter, sleep), small irregular amplitude activity and large irregular amplitude activity are present. Hippocampal electroencephalographic activity was recorded through an electrode placed in the CA1 region of the hippocampus of a rat. *Adapted from Whishaw & Vanderwolf, 1973.*

In concert with the hippocampal feedback and feedforward circuits, afferent connections from the septum and other brain regions, like the

hypothalamus and brainstem, provide the modulatory input needed to guarantee adequate hippocampal oscillatory activity (Andersen et al., 2007; Cappaert et al., 2015).

Several electrical activity patterns can be recorded from the hippocampus (Figure 10). These hippocampal LFPs are associated with particular behavioral or psychological states, such as alertness, anatomical location, movement, and sensory stimulation (Andersen et al., 2007; Whishaw & Vanderwolf, 1973). Each type of LFP is generated by distinct mechanisms, is related to specific neuronal firing properties, and carries out different functions (Buzsáki, 2006; Colgin, 2016).

Broadly, hippocampal electrical activity can be divided into rhythmic and non-rhythmic. According to their frequency range, six rhythmic activity patterns exist: delta (1–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), beta (12–26 Hz), gamma (26–100 Hz), and ripple (100–200 Hz) oscillations (Andersen et al., 2007; Buzsáki, 2006; Colgin, 2016). Regarding non-rhythmic activity, it can be classified in small irregular amplitude activity and large irregular amplitude activity (Andersen et al., 2007; Vanderwolf, 1969). Some of these electrical activity patterns co-occur, such as theta and gamma oscillations, whereas others are mutually exclusive, like theta and ripple oscillations (Andersen et al., 2007). In the framework of this thesis, only theta and gamma oscillations will be covered.

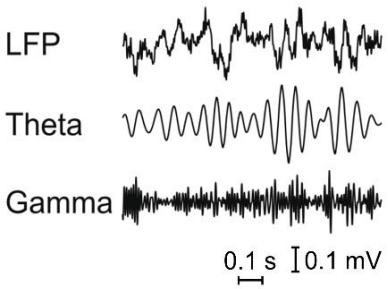

Figure 11. Theta and gamma oscillations of hippocampal LFPs.
Raw LFP recording and filtered signals for the theta (4-12 Hz) and gamma (26-100 Hz) frequency bands. Hippocampal LFPs were recorded through an electrode placed in the hippocampus of a mouse. *Adapted from A. Ittner et al., 2014.*

1.3.2.1 Theta oscillations

Hippocampal oscillatory activity in the theta frequency band (Figure 11)—4–12 Hz in rodents, where theta oscillations extend into the frequency band of alpha oscillations as the latter are not present in the 8–12 Hz band (Derdikman & Knierim, 2014)—depends on ongoing behavior and has a distinct set of behavioral correlates (Andersen et al., 2007; Vanderwolf, 1969; Whishaw & Vanderwolf, 1973). Broadly, theta oscillations are present during exploratory motor activity, meaning "voluntary" or "attentional" movement, and rapid eye movement (REM) sleep (Vanderwolf, 1969; Whishaw & Vanderwolf, 1973). Moreover, theta oscillations are associated with mnemonic functions, particularly spatial and episodic learning and memory (Buzsáki, 2005; Colgin, 2013, 2016). Theta states are necessary for binding events and spatial locations together in time so that the neuronal ensembles encoding each item are correctly connected regarding the spatiotemporal framework (Buzsáki, 2005; Colgin, 2016). A broad term to encompass the diverse roles of theta oscillations during wakefulness could be navigation, since theta oscillations can be

viewed as a temporal organizer for navigating both the physical space, during movement, and the neuronal space, during memory processes (Buzsáki, 2006).

Theta oscillations arise mainly from the summation of excitatory postsynaptic potentials and inhibitory postsynaptic potentials (IPSPs) in principal neurons of the hippocampal CA1 and CA3 regions and the dentate gyrus, with additional contribution from those of the subiculum, entorhinal cortex, and perirhinal cortex (Buzsáki, 2002; Kamondi et al., 1998; Pernía-Andrade & Jonas, 2014; Soltész & Deschenes, 1993). These cells are the principal current generators of the LFP in the theta frequency band, that is, theta oscillations (Buzsáki, 2002).

At this point, it is worth noting the distinction between the concepts of "current generator" and "rhythm generator". On the one hand, "current generator" denotes the transmembrane currents contributing to the LFP, thus, the cells whose electric activity directly influences the recorded oscillation. On the other hand, "rhythm generator" refers to the mechanisms that control the emergence and regulation of oscillatory activity, these being at the cellular or network level (Buzsáki, 2002).

Regarding theta oscillations, several intra- and extra-hippocampal rhythm generators have been described, involving both principal neurons and interneurons. It has long been recognized that the MSDB complex and the septohippocampal pathway are required for the emergence of hippocampal theta oscillations since they are abolished by lesions of the MSDB complex or the fimbria and dorsal fornix, through which septohippocampal axons travel (Green & Arduini, 1954; M'Harzi & Monmaur, 1985; Petsche et al., 1962). The mechanism by which the septohippocampal pathway is believed to drive theta oscillations is through the rhythmic disinhibition of principal neurons, which depends on the GABAergic component of the projection (see section 1.3.1).

An increase in the excitatory input to GABAergic septohippocampal neurons, both from external afferent connections, such as the hypothalamus and brainstem, and from local MSDB complex cholinergic and glutamatergic neurons, leads to the sudden activation of GABAergic septohippocampal neurons (Hangya et al., 2009; Manseau et al., 2005; Vertes & Kocsis, 1997; M. Wu et al., 2000). This activation triggers pacemaker mechanisms possessed by GABAergic septohippocampal neurons and precipitates a fast spread of synchronous activity by coupling hippocampal oscillatory activity to that of septal neurons. In this way, synchrony spreads and oscillations at the population level emerge through the recruitment and entrainment of an increasing number of neurons from the network. The hippocampal interneuron population, the target of GABAergic septohippocampal neurons, entrains their target principal neurons so that they oscillate synchronously. This entrainment of principal neurons, the main current generators of the LFP in the theta frequency band, causes an

increase in theta oscillatory activity within the septohippocampal system (Hangya et al., 2009). In turn, the hippocamposeptal projection enhances the synchrony of its target GABAergic septohippocampal neurons (Takács et al., 2008; Tóth et al., 1993). However, hippocamposeptal feedback is not only necessary for strengthening the synchrony. It is essential for the generation, coordination, and maintenance of theta oscillations (Hangya et al., 2009). Therefore, this reciprocal inhibitory loop is at the center of the network generating theta oscillations (Figure 12).

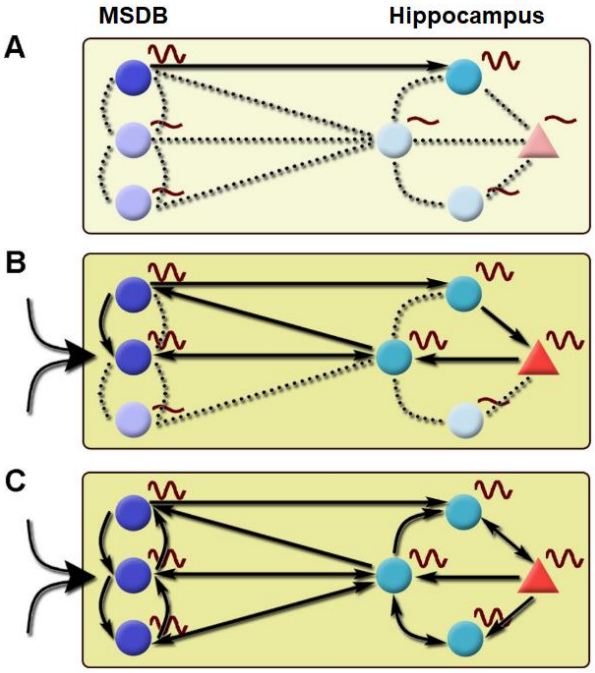

Figure 12. Mechanism of theta oscillations generation by the GABAergic septohippocampal pathway.
(A) In non-theta states, only constitutively firing neurons (in dark color) present oscillatory activity in the theta frequency band, but independently from one another. (B) GABAergic septohippocampal neurons are abruptly activated by afferent excitation from both external connections (arrows in the left) and local cholinergic and glutamatergic inputs (intensity of yellow color background). (C) Theta oscillations in septal GABAergic neurons spread across the MSDB complex and the hippocampus, entraining different neuron types. Reciprocal hippocamposeptal connections contribute to the maintenance of theta oscillations. Circles symbolize GABAergic neurons; triangles represent pyramidal neurons. *Adapted from Hangya et al., 2009 (A–C).*

Evidence supporting the role of the GABAergic septohippocampal neurons of the MSDB complex in pacing theta oscillations is the expression of pacemaker channels by these cells (Sotty et al., 2003; Varga et al., 2008; Xu et al., 2004). Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels underlie the I_h current, which is responsible for the pacemaker designation given that it acts as a pacemaker current to initiate rhythmic firing (He et al., 2014). In fact, the presence of the I_h current has been associated with the ability of neurons to be entrained by oscillations in the theta frequency band (He et al., 2014; H. Hu et al., 2002; Xu et al., 2004). Furthermore, the participation of HCN channels in the generation of spontaneous subthreshold oscillations and rhythmic action potentials has been demonstrated (B. D. Bennett et al., 2000; C. T. Dickson et al., 2000; He et al., 2014). Therefore, GABAergic septohippocampal neurons, expressing HCN channels and exhibiting theta rhythmic firing, are pointed to as essential components of the network generating hippocampal theta oscillations (Varga et al., 2008; Xu et al., 2004). Nevertheless, mechanisms independent of GABAergic septohippocampal neurons are also involved in driving theta oscillations in the hippocampus.

On the one hand, excitatory inputs from external afferent connections are crucial during theta oscillations. In particular, the entorhinal cortex provides an excitatory input necessary for theta oscillations during active behaviors, and its removal or inactivation

strongly reduces theta activity (Kamondi et al., 1998; Pernía-Andrade & Jonas, 2014; Ylinen et al., 1995). Cholinergic input, presumably from cholinergic septohippocampal neurons, also provides excitation to the hippocampus and is required for theta oscillations during inactive states, like in REM sleep or in the absence of active behaviors (Kramis et al., 1975; Nakajima et al., 1986).

On the other hand, the contribution of intra-hippocampal rhythm generators to theta oscillations has also been established, specifically of the CA3 recurrent collateral connections. Independent theta oscillations have been proven to emerge from the CA3 recurrent collateral connections, and they persist after removal of the entorhinal cortex as long as CA3 recurrent collaterals remain intact (Bragin et al., 1995; Kocsis et al., 1999; Montgomery et al., 2009). Moreover, specific modulation of these connections with optogenetic tools has confirmed their role as theta rhythm generators (López-Madrona et al., 2020). Furthermore, the intrinsic properties of neurons and circuits of the hippocampus play a key role in theta oscillations. Precisely, the electrophysiological properties of pyramidal neurons facilitate rhythmic firing in the theta frequency band. Pyramidal neurons present theta resonance, meaning they respond more effectively to inputs in the theta frequency band (H. Hu et al., 2002; Pike et al., 2000). In relation to this, they also express pacemaker HCN channels (C. T. Dickson et al., 2000; H. Hu et al., 2002). It has been demonstrated that theta rhythmic activity in PV-positive interneurons induces theta oscillations in pyramidal neurons, and blocking HCN channels in pyramidal neurons reduces them (Stark et al., 2013).

Taken together, accumulating data indicate that hippocampal theta oscillations arise from multiple rhythm and current generators but are primarily orchestrated by complex interactions between the MSDB complex and the hippocampus, owing to the properties of their circuits and the intrinsic properties of their neurons (Buzsáki, 2002; Colgin, 2016; Nuñez & Buño, 2021).

1.3.2.2 Gamma oscillations

Oscillatory activity of the hippocampus in the gamma frequency band (Figure 11)—26–100 Hz—is much less understood than theta oscillations in terms of its complete spectrum of behavioral correlates, but it is known to be associated with olfactory behavior in response to a wide range of olfactory stimuli (Vanderwolf, 2001). Due to their fast nature, gamma oscillations are endowed with the ability to prompt rapid selection of relevant inputs, association of neurons into neuronal ensembles, and coordination of these ensembles (Colgin & Moser, 2010). Moreover, the synchronization of gamma oscillations across various regions of the neocortex could contribute to binding together the individual elements of compound representations (Andersen et al., 2007; Colgin & Moser, 2010). Therefore, it has

been suggested that gamma oscillations play a role in complex processes. Remarkably, it is believed that gamma oscillations support memory encoding and retrieval (Bieri et al., 2014; Colgin, 2016; Colgin & Moser, 2010; Zheng et al., 2016).

Gamma oscillations reflect IPSPs in principal neurons and mainly occur in the hippocampal CA1 and CA3 regions, the dentate gyrus, and the entorhinal cortex (Bragin et al., 1995; Buzsáki & Wang, 2012; Csicsvari et al., 2003; Pernía-Andrade & Jonas, 2014; Soltész & Deschenes, 1993). Therefore, these cells are the primary current generators of gamma frequency LFPs (Colgin & Moser, 2010). Contrary to theta frequency LFPs, gamma oscillations are not produced by the generalized activity of all principal neurons but by the activation of particular neuronal ensembles at certain times (Colgin & Moser, 2010).

Regarding the origin of gamma oscillations, there are two independent gamma rhythm generators. There is one rhythm generator in the CA3 region whose activity spreads to the CA1, while another rhythm generator is in the entorhinal cortex and propagates its oscillations to the dentate gyrus and the hippocampal CA1 and CA3 regions (Bragin et al., 1995; Colgin et al., 2009; Csicsvari et al., 2003). This fact was first noted with the observation that gamma activity is heavily reduced in the dentate gyrus after removing the entorhinal cortex, whereas it is increased in the CA1 region (Bragin et al., 1995). In relation to this, the gamma frequency band encompasses two different oscillatory activities, namely slow gamma (26–55 Hz) and fast gamma (56–100 Hz) oscillations, which depend upon different rhythm generators. While slow gamma is entrained by the CA3 region input, fast gamma is driven by the entorhinal cortex (Belluscio et al., 2012; Colgin et al., 2009; Kemere et al., 2013; Schomburg et al., 2014).

The intrinsic properties of hippocampal neurons, especially the synaptic, electrophysiological, and connectional characteristics of GABAergic interneurons, play a crucial role in the generation of gamma oscillations. Of particular relevance are the perisomatic-targeting PV-positive interneurons, that is, basket and axo-axonic cells (Bartos et al., 2007; Hájos & Paulsen, 2009; H. Hu et al., 2014; Kann, 2016; Korotkova et al., 2010).

Several facts account for the relevance of PV-positive interneurons for gamma oscillations. First, PV-positive interneurons are fast-spiking cells. This means they can fire at high frequencies for prolonged periods of time, with weak or no accommodation, allowing PV-positive interneurons to sustain action potential firing during each cycle of gamma oscillations (Jonas et al., 2004; Kawaguchi et al., 1987; Simon et al., 2006; Sotty et al., 2003).

Second, PV-positive interneurons form complex functional interneuron networks well suited to synchronize spiking within this neuron population. PV-positive interneurons

have gap junctions and thus can communicate rapidly via these electrical synapses (Fukuda & Kosaka, 2000; Katsumaru et al., 1988; Venance et al., 2000). The presence of gap junctions between GABAergic interneurons is necessary for the generation of gamma oscillations and can enhance synchrony in this frequency band (Hormuzdi et al., 2001; Traub et al., 2000, 2001). Aside from electrical synapses, GABAergic interneurons are also reciprocally connected by chemical synapses, an essential factor underlying oscillations in the gamma frequency band (Whittington et al., 1995). Overall, both types of fast synapses are suited for synchronizing activity with high temporal fidelity (Bartos et al., 2007).

Third, since an individual PV-positive interneuron innervates a large number of principal neurons by targeting their soma and proximal dendrites, it can pace the firing in multiple principal neurons and synchronize the activity of the principal neuron population (Bezaire & Soltész, 2013; Cobb et al., 1995; Freund & Buzsáki, 1996; Klausberger & Somogyi, 2008). In this sense, perisomatic fast IPSPs have a strong synchronizing effect during gamma oscillations, both between basket cells and between basket cells and principal neurons (Bartos et al., 2002; Whittington et al., 1995). Moreover, feedback inhibition, in which axon collaterals from principal neurons activate PV-positive interneurons, entails an even more efficient synchronization and the generation of oscillations (Andersen et al., 2007; Buzsáki, 2006) (see section 1.3.2.1). Hence, through their influence on principal neurons, PV-positive interneurons are endowed with the ability to control the output of the network (Hájos & Paulsen, 2009; Kann, 2016; Klausberger & Somogyi, 2008).

Another very significant factor is that fast-spiking PV-positive interneurons present gamma resonance, meaning they respond strongly and with the highest temporal precision to inputs in the gamma frequency band (Pike et al., 2000). Notably, for resonance to contribute significantly to the LFP, it has to occur synchronously in adjacent neurons, which is likely to happen in inhibitory interneurons (Buzsáki et al., 2012).

The GABAergic septohippocampal pathway may have a role in the modulation of gamma oscillations since it profusely innervates GABAergic hippocampal interneurons, the most abundant subtype of which are PV-positive interneurons. Moreover, PV-positive GABAergic projection neurons from basal forebrain regions other than the septum control cortical gamma oscillations through the innervation of cortical PV-positive interneurons (Kim et al., 2015).

Frequently, gamma oscillations are concurrent with theta oscillations, but both oscillatory activities are generated independently (Leung, 1992; Stumpf, 1965). Nevertheless, when they co-occur, the properties of gamma oscillations are modulated depending on, or collectively with, theta oscillations (Chrobak & Buzsáki, 1998). This mechanism is known as cross-frequency coupling (Figure 13), and both phase-amplitude

coupling, or nesting, and phase-phase coupling, or phase synchronization, occur between theta and gamma oscillations (Belluscio et al., 2012; Bragin et al., 1995; Colgin et al., 2009; Csicsvari et al., 2003; Soltész & Deschenes, 1993; Zheng et al., 2016; Zheng & Zhang, 2013).

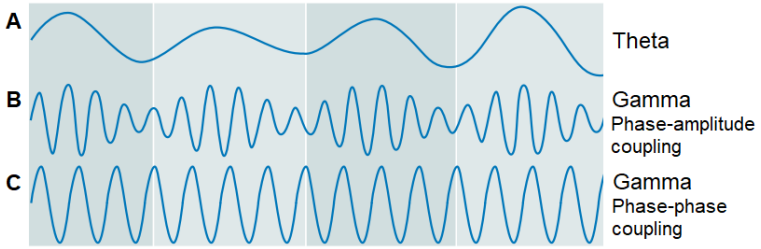

Figure 13. Theta-gamma cross-frequency phase-amplitude and phase-phase coupling.
(A) The phase of a lower-frequency oscillation (theta) can modulate the oscillatory activity of a higher-frequency one. (B) Amplitudes of the higher-frequency oscillation (gamma) are maximal during the positive phase of the lower-frequency oscillation and minimal during its negative phase. (C) In each cycle of the lower-frequency oscillation, several cycles of the higher-frequency oscillation take place and their phase relationship remains fixed (phase-locked), meaning that peaks of the higher-frequency oscillation always coincide with the same phase values of the lower-frequency one. Dark and light boxes indicate consecutive cycles of the lower-frequency oscillation. *Adapted from Fell & Axmacher, 2011 (A–C).*

These two phenomenon imply that neuronal activity is precisely coordinated at multiple time scales, which could be advantageous for information transfer and plasticity (Belluscio et al., 2012; Bergmann & Born, 2018; Buzsáki & Wang, 2012; Fell & Axmacher, 2011). Phase-amplitude and phase-phase coupling may occur independently or represent two different facets of the same mechanism. This common mechanism leading to the temporal coordination of both oscillations could be perisomatic inhibition by fast-spiking PV-positive interneurons (Belluscio et al., 2012; Bragin et al., 1995; Buzsáki & Wang, 2012).

Altogether, fast-spiking PV-positive interneurons and the complex inhibitory interneuron networks they establish are endowed with the synaptic, electrophysiological, and connectional features needed to enable the emergence of hippocampal gamma oscillations. Hence, gamma frequency spiking of PV-positive interneurons, through strong rhythmic perisomatic inhibition of the principal neuron population, produces gamma oscillations (Bartos et al., 2007; Kann, 2016; Klausberger & Somogyi, 2008; Pernía-Andrade & Jonas, 2014).

2. Alzheimer's disease continuum

In the following sections, a general outlook of the Alzheimer's disease continuum will be given. Rather than being a thorough review of Alzheimer's disease, this section pretends to provide an overview of the neuropathological, pathophysiological, and clinical features that are most relevant in the framework of this thesis.

For a long time, Alzheimer's disease was definitively diagnosed at autopsy, while in life it was diagnosed as possible or probable Alzheimer's disease (Khachaturian, 1985; McKhann et al., 1984). Gradually, the separation of neuropathological alterations from clinical manifestations became vague. As a result, the term Alzheimer's disease became ambiguously employed to designate two distinct entities: a clinical condition without confirmed neuropathological changes and the neuropathological changes characteristic of Alzheimer's disease (Beach et al., 2012; Jack et al., 2018; Knopman et al., 2019).

Because the definition of Alzheimer's disease in living people has been based on signs and symptoms, it is a syndromic construct rather than a biological paradigm. Conversely, what defines Alzheimer's disease as a distinct neurodegenerative disease among those causing cognitive impairment is a definite set of neuropathological features. Therefore, it is believed that defining Alzheimer's disease as a biological construct based on postmortem studies or *in vivo* biomarker analyses will facilitate the understanding of the mechanisms leading to the cognitive decline associated with it and the intervention to target the pathways involved (Dubois et al., 2007, 2010, 2014; Jack et al., 2011, 2018).

Another matter that is key to understanding the processes underlying clinical manifestations and developing disease-modifying treatments is considering Alzheimer's disease as a continuum, rather than several separate clinically defined entities (Dubois et al., 2014; Jack et al., 2018). The reason for this is the notion that cognitive impairment and biomarker measurements progress continuously over a long period of time (Bateman et al., 2012; Fagan et al., 2014; Resnick et al., 2010; Villemagne et al., 2013). Thus, the separate diagnostic guidelines for the different stages of Alzheimer's disease have been unified, and now the Alzheimer's disease continuum encompasses all stages across the disease spectrum, regardless of the age of onset or the presence of clinical symptoms (Albert et al., 2011; Jack et al., 2018; McKhann et al., 2011; Sperling et al., 2011). In fact, the diagnosis of Alzheimer's disease includes asymptomatic individuals presenting neuropathological alterations or biomarker evidence of pathology, irrespective of the absence of cognitive impairment (Dubois et al., 2016; Hyman et al., 2012; Jack et al., 2018; Montine et al., 2012).

In subsequent sections, the classical manifestations of Alzheimer's disease and a related but distinct clinical entity will be addressed.

2.1 Alzheimer's disease

Alzheimer's disease is a neurodegenerative disease first described by German psychiatrist and neuropathologist Aloysius *Alois* Alzheimer at the beginning of the twentieth century and later published in a case report titled *About a peculiar disease of the cerebral cortex* (Alzheimer, 1907).

The neuropathological hallmarks of Alzheimer's disease are senile plaques and neurofibrillary tangles present in particular brain regions. Along with the aberrant processes leading to these deposits, synaptic loss and selective neuronal degeneration are fundamental aspects of the pathophysiological process (Knopman et al., 2021; Long & Holtzman, 2019).

At the clinical level, Alzheimer's disease is characterized by progressive cognitive impairment, categorized as dementia upon becoming severe enough to interfere with normal functioning. Its typical manifestation is predominantly an amnesic clinical presentation consisting of impairment in learning and memory, accompanied by alterations in other cognitive domains affecting attention, language, visuospatial function, and behavior (Knopman et al., 2021; McKhann et al., 2011). Nevertheless, non-amnesic clinical presentations also exist, mainly based on language, visuospatial, or executive dysfunctions (Crutch et al., 2017; Gorno-Tempini et al., 2008; Ossenkoppele et al., 2015). Moreover, neuropsychiatric symptoms often co-occur, such as depression and anxiety in intermediate stages, or delusions, hallucinations, and aggressiveness in advanced stages (D'Onofrio et al., 2012; Knopman et al., 2021).

Within the Alzheimer's disease continuum, a clinical stage preceding overt dementia is mild cognitive impairment, in which clinical symptoms are present but do not entail the loss of independence. Mild cognitive impairment does not invariably evolve into dementia, but it does in a substantial proportion of cases. As previously introduced, when considering Alzheimer's disease as a continuum, its spectrum includes the preclinical stage of normal cognition and the clinical stages of mild cognitive impairment and dementia (Dubois et al., 2016; Hyman et al., 2012; Jack et al., 2018; Montine et al., 2012).

Alzheimer's disease is the most common type of dementia and may account for 60–70% of all cases (World Health Organization, 2017). There were over 55 million people worldwide living with dementia in 2020, and it is estimated that this number will increase to 78 million by 2030 and 139 million by 2050 (World Health Organization, 2021).

The majority of Alzheimer's disease cases are diagnosed in persons older than 65 years, constituting late-onset Alzheimer's disease. Diagnoses before 65 years are rare,

representing 5% of all cases, and correspond to early-onset Alzheimer's disease (Long & Holtzman, 2019; World Health Organization, 2021). Approximately 1% of Alzheimer's disease cases are inherited in an autosomal dominant-manner, thus belonging to early-onset Alzheimer's disease. In most instances, these cases are due to mutations in the *APP*, *PSEN1*, or *PSEN2* genes, which encode proteins that are involved in the disease's pathogenesis, as will be described in the next section (Kang et al., 1987; Levy-Lahad et al., 1995; Sherrington et al., 1995). Hence, it is termed familial Alzheimer's disease, in contrast to the prevalent sporadic Alzheimer's disease. Besides the genes responsible for familial early-onset Alzheimer's disease, hundreds of genes have been investigated as susceptibility factors for late-onset Alzheimer's disease. Among these genes, the ε4 allele of *APOE*, encoding apolipoprotein E, is the most influential genetic risk factor, although variants of many other genes collectively contribute significantly to Alzheimer's disease risk (Corder et al., 1993; I. E. Jansen et al., 2019; Strittmatter et al., 1993).

Despite immense efforts to develop therapeutic strategies for Alzheimer's disease, no effective disease-modifying treatments are available yet. Currently, no treatments prevent, cure, or significantly slow disease progression. The existing pharmacological approaches are palliative, limited, and their effects are rather modest, at most delaying symptom progression by 6 months (Fink et al., 2018). Nevertheless, because of the social and economic burden of Alzheimer's disease, the efforts of the scientific community to discover the underlying causes, involved pathophysiological mechanisms, and possible therapeutic approaches for the treatment of Alzheimer's disease are not decreasing. One of the most exploited approaches to achieve this has been the generation and analysis of animal models of Alzheimer's disease, especially transgenic mouse lines bearing mutations in genes related to familial Alzheimer's disease, as will be described in a later section (see section 2.2).

2.1.1 Neuropathological features

The definitive diagnosis of Alzheimer's disease has historically been determined at autopsy by the presence of its neuropathological hallmarks: senile plaques and neurofibrillary tangles (Figure 14). Currently, a definitive diagnosis can be established in life through biomarker measures of these features. Thus, what biologically defines Alzheimer's disease is these neuropathological alterations and the proteins they involve (Hyman et al., 2012; Jack et al., 2018; Montine et al., 2012). The following sections will delve into these hallmarks.

2.1.1.1 Amyloid-β peptide and senile plaques

Senile plaques are extracellular deposits containing amyloid-β (Aβ) peptide and, hence, can be termed amyloid plaques or Aβ plaques (Glenner & Wong, 1984; Masters et al.,

1985). The amyloid terminology is not exclusive of Aβ deposits and applies to several other proteins. Amyloid can be defined as insoluble, fibrous, mostly extracellular deposits composed of monomers of proteins or peptides misfolded into β-strands that are usually organized in cross-β-sheet structures. Like other amyloids, Aβ plaques can be histologically visualized through staining with aromatic compounds such as Thioflavin S and T or Congo red, or more specifically by means of immunodetection of its components (Figure 14A and B) (Haass & Selkoe, 2007; Salahuddin et al., 2021).

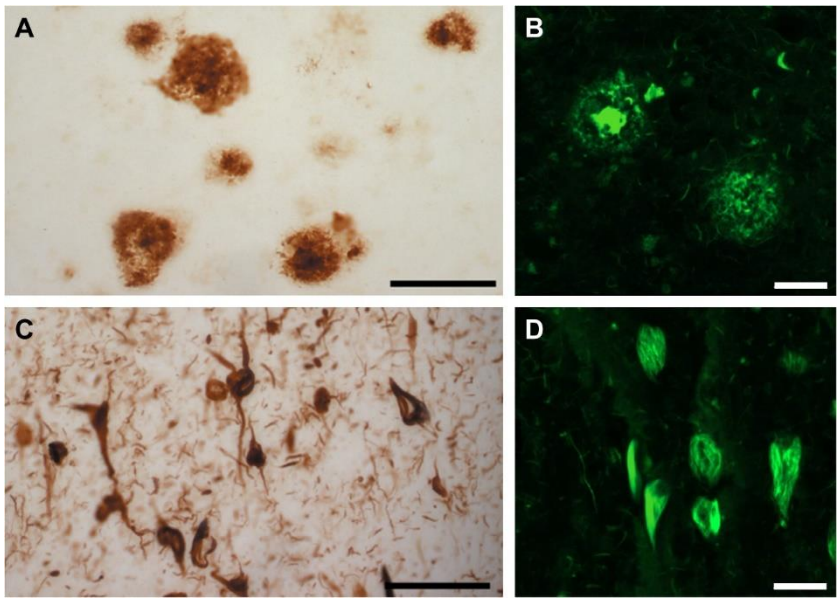

Figure 14. Senile plaques and neurofibrillary tangles in the brain of Alzheimer's disease patients.
(A and B) Visualization of senile plaques through immunodetection of $A\beta_{1-42}$ (A) or Thioflavin S staining (B). (C and D) Identification of neurofibrillary tangles by immunodetection of paired helical filament-1 Tau (C) or Thioflavin S staining (D). Scale bar: 125 μm (A), 20 μm (B and D), 62.5 μm (C). *Adapted from LaFerla & Oddo, 2005 (A and C) and Petersen et al., 2019 (B and D).*

Aβ peptide is produced by proteolytic processing of amyloid precursor protein (APP), a type I transmembrane protein encoded by the *APP* gene located on chromosome 21 and expressed ubiquitously in neuronal and non-neuronal cells, in a physiological pathway (Haass et al., 1992; Kang et al., 1987; Slunt et al., 1994). In neurons, APP is located in the somatodendritic and axonal compartments and is enriched at the presynaptic active zones of axons (Haass et al., 2012; Kins et al., 2006; Lassek et al., 2013). However, the majority of APP localizes to the secretory pathway, where it undergoes several post-translational modifications before only a fraction of the total APP reaches the plasma membrane (Haass et al., 2012; Jiang et al., 2014; Kins et al., 2006). Its physiological functions are not completely understood, but it is involved in the development of the nervous system and in synapse formation, function, and plasticity (Müller et al., 2017).

The processing of APP mainly takes place via two canonical and mutually exclusive pathways: the amyloidogenic pathway, which leads to Aβ production (Figure 15A), and the non-amyloidogenic pathway, which prevents it (Figure 15B). In the amyloidogenic pathway, 36–43 amino acid-long Aβ peptides are generated through sequential cleavage of APP by β-secretase and the γ-secretase complex and then released to the extracellular medium as monomers (Haass et al., 2012; Kang et al., 1987; Tomita, 2014; Vassar et al., 2014).

Production and release of Aβ are regulated by synaptic activity at both the presynaptic and postsynaptic compartments (Cirrito et al., 2005, 2008; Kamenetz et al., 2003; Verges et al., 2011; Wei et al., 2010). The catalytic subunits of the γ-secretase complex are the proteins presenilin-1 and presenilin-2, encoded by *PSEN1* and *PSEN2* genes, respectively. For this reason, mutations in the *APP*, *PSEN1*, or *PSEN2* genes that favor the amyloidogenic pathway are linked to familial Alzheimer's disease (Kang et al., 1987; Levy-Lahad et al., 1995; Sherrington et al., 1995).

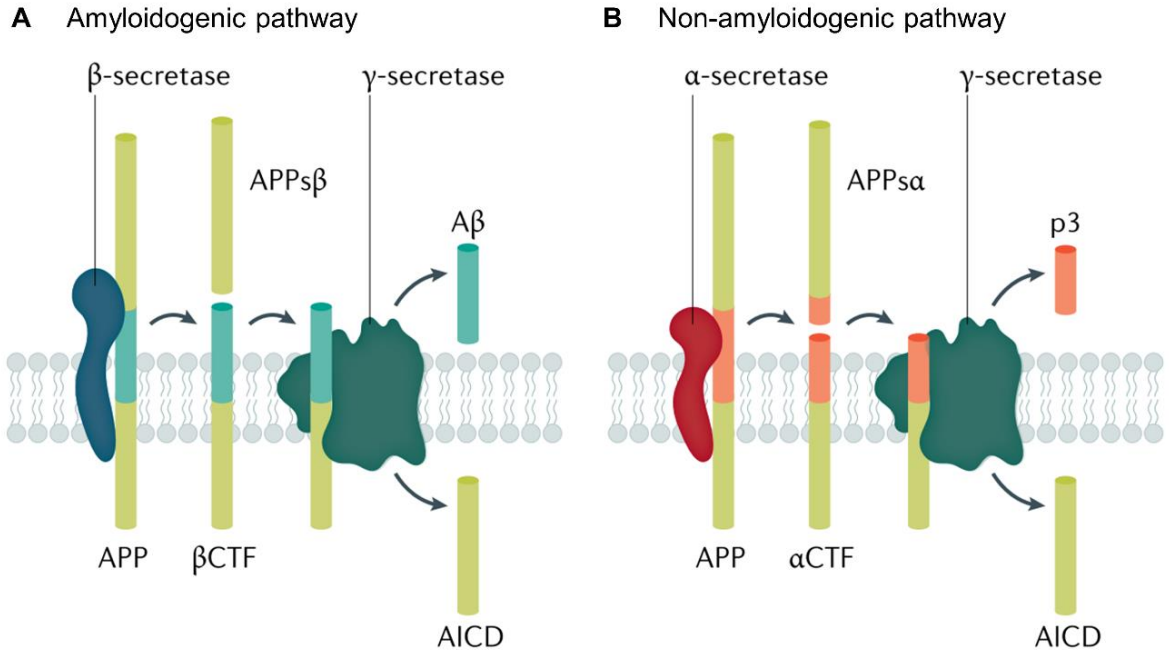

Figure 15. Proteolytic processing of APP and Aβ production.
(A) In the amyloidogenic pathway, β-secretase processes the APP, and subsequent proteolysis mediated by the γ-secretase complex produces Aβ. (B) In the non-amyloidogenic pathway, α-secretase proteolyzes the domain of APP that contains Aβ, preventing the generation of Aβ by the γ-secretase complex. Abbreviations: AICD, amyloid precursor protein intracellular domain; APPsα, amyloid precursor protein secreted ectodomain α; APPsβ, amyloid precursor protein secreted ectodomain β; αCTF, α-carboxy-terminal fragment; βCTF, β-carboxy-terminal fragment; p3, amino-terminally truncated Aβ. *Adapted from Knopman et al., 2021 (A and B).*

Due to its sequence, Aβ peptide has the intrinsic propensity to convert from a largely α-helical conformation within APP to a β-sheet conformation once cleaved. However, different Aβ species differ in their tendency to aggregate due to having distinct biochemical properties and post-translational modifications that influence this propensity (Kummer & Heneka, 2014; Watson et al., 2005). One relevant factor is the peptide's carboxy-terminal length and, thus, the proteolytic cleavage site of APP that generates it. For instance, among the two principal components of Aβ plaques, $A\beta_{1\text{-}40}$ is the most abundantly produced Aβ species, but $A\beta_{1\text{-}42}$ is the most prone to aggregation owing to the increased hydrophobicity of its extended carboxy-terminal end (Haass & Selkoe, 2007; Masters & Selkoe, 2012; Watson et al., 2005).

Once they are released, Aβ peptides undergo successive stages of aggregation: from monomers to oligomers of increasing orders, to protofibrils, and ultimately to highly insoluble fibrils, which are rich in β-sheet structures organized in cross-β assemblies and accumulate in extracellular deposits (Figure 16A) (Glenner & Wong, 1984; Haass & Selkoe, 2007; W. L. Klein et al., 2001; Masters et al., 1985; Masters & Selkoe, 2012). In particular, Aβ fibrils comprise two twisted protofibrils that possess helical symmetry and, in turn, are composed of Aβ monomers stacked in a parallel, in-register cross-β structure. Every subunit, corresponding to a single Aβ monomer, forms an LS-shaped structure (Figure 16A) with an L-shaped amino-terminal domain and an S-shaped carboxy-terminal domain (Gremer et al., 2017).

Aβ deposits are morphologically heterogeneous, but two types stand out: neuritic plaques, which are what senile plaques classically refer to, and diffuse plaques, which are presumed to be the initial stage of neuritic plaques (D. W. Dickson, 1997; Haass & Selkoe, 2007; Serrano-Pozo et al., 2011). Neuritic plaques are formed by a dense core of Aβ fibrils and a halo of dystrophic neurites and other degenerating proteins and cellular components as well as Aβ oligomers, surrounded by the processes of activated microglia and reactive astrocytes. Conversely, diffuse plaques are amorphous deposits of Aβ with no dense core of Aβ fibrils nor associated dystrophic neurites or glial response (Haass & Selkoe, 2007; Masters & Selkoe, 2012; Serrano-Pozo et al., 2011).

Classically, Aβ fibrils of neuritic plaques were considered the toxic factor that initiates the neurodegenerative process of Alzheimer's disease. However, countless data have demonstrated that soluble Aβ species in the form of oligomers are the most toxic forms (Cleary et al., 2005; Jin et al., 2011; Koffie et al., 2009; Lesné et al., 2013; Shankar et al., 2007, 2008; Spires-Jones et al., 2007; Spires et al., 2005; Walsh et al., 2002; Wei et al., 2010). Currently, a range of soluble Aβ oligomers, mainly small and diffusible oligomeric species, are believed to be the bioactive elements inducing neurotoxicity and synaptotoxicity, especially during the early stages of Alzheimer's disease (Haass & Selkoe, 2007; W. L. Klein et al., 2001; S. Li & Selkoe, 2020; Salahuddin et al., 2021). Moreover, Aβ oligomers are a better quantitative correlate of the degree of cognitive impairment than Aβ plaques in the late stages of Alzheimer's disease (Mc Donald et al., 2010; McLean et al., 1999; Tomic et al., 2009).

Brain regions become affected by the neuropathological changes of Alzheimer's disease in a hierarchical order. In a prion-like manner, Aβ species that have already acquired a β-sheet conformation can induce, or seed, the aggregation of further Aβ molecules and the formation of Aβ fibrils and larger aggregates (Figure 16A) (Eisele et al., 2009, 2010; Ye et al., 2015). In the case of senile plaques, Aβ deposition starts in the neocortex and continues in the allocortex, including the entorhinal cortex and the hippocampal formation (Figure 16B).

Subsequently, it proceeds in the diencephalon and basal ganglia, next in the brainstem, and finally in the cerebellum. Senile plaques are present in normal aging as well but are restricted to the neocortex, allocortex, diencephalon, and basal ganglia (Thal et al., 2002; van der Kant et al., 2020).

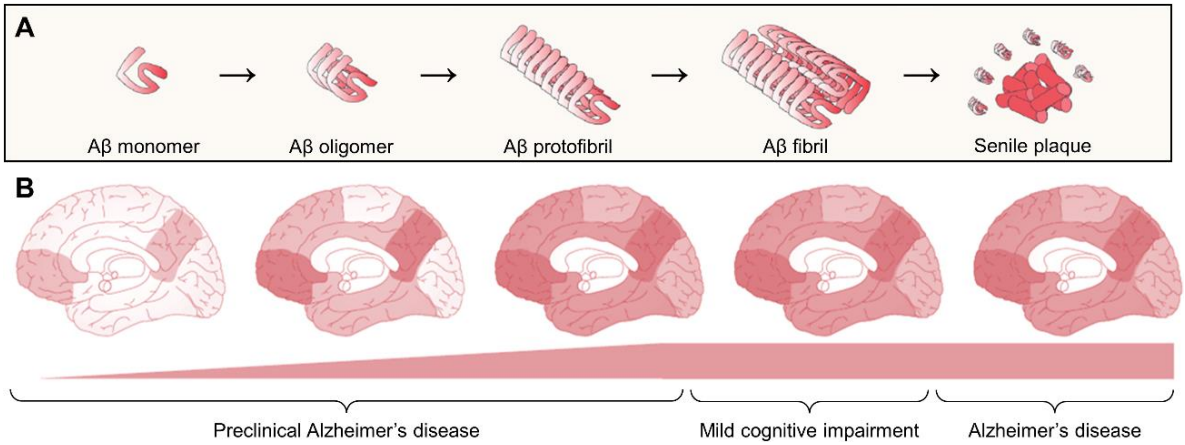

Figure 16. Progression of Aβ pathology in Alzheimer's disease.
(A) Aβ monomers assemble oligomers, which aggregate into protofibrils and eventually into fibrils that accumulate in senile plaques in the extracellular medium. Senile plaques are composed of a dense core of Aβ fibrils and are surrounded by Aβ oligomers. (B) Aβ pathology starts in the neocortex and spreads to the allocortex in the early stages of Alzheimer's disease. Subsequently, Aβ pathology spreads to the diencephalon, basal ganglia, brainstem, and cerebellum in mild cognitive impairment and Alzheimer's disease patients. *Adapted from Long & Holtzman, 2019 (A) and van der Kant et al., 2020 (B).*

2.1.1.2 Tau protein and neurofibrillary tangles

Neurofibrillary tangles are intracellular deposits consisting of post-translationally modified Tau protein, mostly hyperphosphorylated Tau (P-Tau) (Grundke-Iqbal, Iqbal, Tung, et al., 1986; Kosik et al., 1986). These inclusions typically have a flame-shaped appearance and occupy the soma and proximal dendrites of neurons, principally of pyramidal neurons of the hippocampus and certain cortical regions (H. Braak & Braak, 1991). Neurofibrillary tangles can be histologically visualized through silver stainings like the Gallyas silver stain, by Thioflavin S and T staining, or through specific immunodetection methods (Figure 14C and D) (Congdon & Sigurdsson, 2018; Ferrari & Sorbi, 2021).

Neurofibrillary tangles and Tau pathology as a whole are not exclusive of Alzheimer's disease. On the contrary, Tau pathology is characteristic of several dementias generally termed tauopathies, such as Alzheimer's disease itself or a subset of frontotemporal dementias, which are a common cause of early-onset dementia (Cairns et al., 2007; Congdon & Sigurdsson, 2018; Goedert, 2018).

Tau is a microtubule-associated protein encoded by the *MAPT* gene located on chromosome 17 and mainly expressed in neurons, although it is expressed in astrocytes and oligodendrocytes as well (Binder et al., 1985; LoPresti et al., 1995; Papasozomenos & Binder, 1987; Shin et al., 1991). In mature neurons, Tau is primarily located in axons, but it is also

present in dendrites at lower levels and can be in both the presynaptic and postsynaptic compartments (Binder et al., 1985; Hoover et al., 2010; L. M. Ittner et al., 2010; Mondragón-Rodríguez et al., 2012). Moreover, Tau can localize to the soma and the nucleus, and it can be associated with the plasma membrane (Brandt et al., 1995; C. Liu & Götz, 2013; Loomis et al., 1990; Papasozomenos & Binder, 1987; Sultan et al., 2011; Violet et al., 2014).

Four main domains can be distinguished in Tau: the amino-terminal domain, the proline-rich domain, the microtubule-binding domain, and the carboxy-terminal domain (Figure 17) (Guo et al., 2017; Mandelkow & Mandelkow, 2012). In the adult central nervous system, alternative splicing of exons 2, 3, and 10 generates six main isoforms of Tau that can be differentiated by the presence of none (0N), one (1N), or two (2N) inserts within the amino-terminal domain, and three (3R) or four (4R) repeats within the microtubule-binding domain (Figure 17) (Andreadis et al., 1992; Goedert et al., 1989). Tau expression is developmentally regulated, and in the fetal brain only the shortest isoform, 0N3R Tau, is expressed (Goedert & Jakes, 1990; Kosik et al., 1989). In the adult human brain, 3R and 4R Tau isoforms are present in approximately equal amounts (Goedert & Jakes, 1990; M. Hong et al., 1998). Conversely, in the adult mouse brain, 4R Tau isoforms are almost exclusively expressed, while 3R Tau isoforms are only expressed in the fetal and newborn brain (Brion et al., 1993; Spillantini & Goedert, 1998).

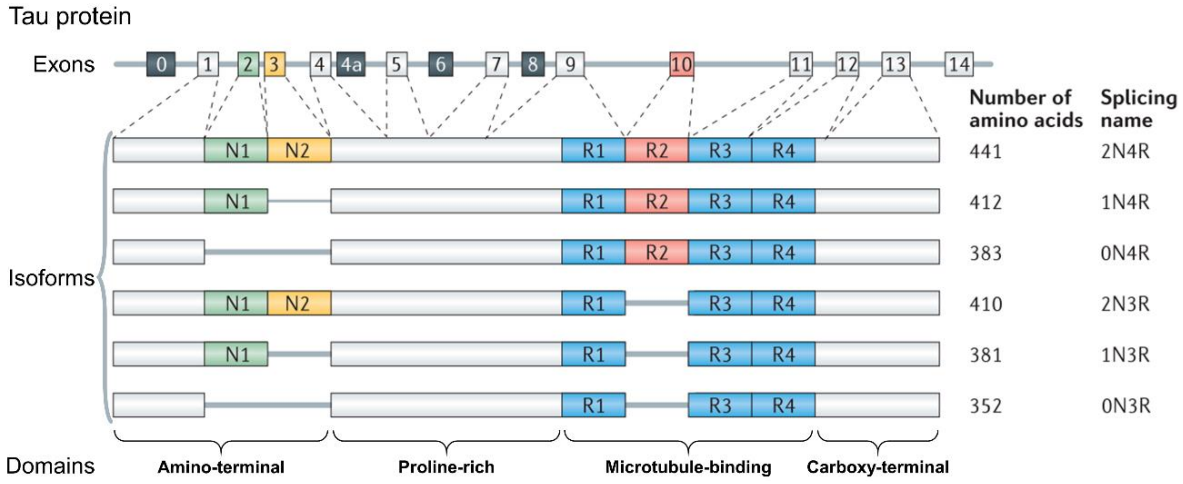

Figure 17. Exons, isoforms, and domains of Tau protein.
Alternative splicing of exons 2, 3, and 10 of the *MAPT* gene generates six Tau isoforms in the adult human brain, which contain 0, 1, or 2 amino-terminal inserts (0N, 1N, or 2N, respectively) and 3 or 4 microtubule-binding repeats (3R or 4R, respectively). Exons 0 and 14 are transcribed but not translated, while exons 4a, 6, and 8 are transcribed only in peripheral tissue. The regions corresponding to the amino-terminal, proline-rich, microtubule-binding, and carboxy-terminal domains are indicated. *Adapted from Y. Wang & Mandelkow, 2016.*

In terms of structure, Tau has the peculiarity of being an intrinsically disordered protein, meaning its polypeptide chain is very flexible, has a low content of secondary structures, and these are transient (Ávila et al., 2016; Cleveland et al., 1977; Lee et al., 1988;

Mukrasch et al., 2007; Schweers et al., 1994). The natively unfolded quality of Tau, which stems from the high hydrophilicity of its primary structure and the related lack of stable secondary structure, renders it a highly dynamic molecule. Despite being intrinsically disordered, Tau possesses a certain degree of global folding, namely tertiary structure, which arises from intramolecular interactions between the amino-terminal and carboxy-terminal domains and results in a paper-clip conformation (Ávila et al., 2016; Jeganathan et al., 2006; Mukrasch et al., 2009; Schwalbe et al., 2014).

Tau protein is subject to a broad variety of post-translational modifications, encompassing phosphorylation, acetylation, methylation, glycosylation, glycation, ubiquitination, SUMOylation, protonation, oxidation, nitration, prolyl-isomerization, and proteolytic cleavage (Alquezar et al., 2021; Guo et al., 2017; Tapia-Rojas et al., 2019). Post-translational modifications are pivotal in the regulation of Tau function. Phosphorylation is the most studied post-translational modification of Tau and has a profound impact on its activity, as Tau contains 85 putative phosphorylation sites (Figure 18), 53 of which have been demonstrated to be phosphorylated in human tissue (Alquezar et al., 2021; Ercan et al., 2017; Hanger et al., 2007; Morishima-Kawashima et al., 1995). Commonly, post-translational

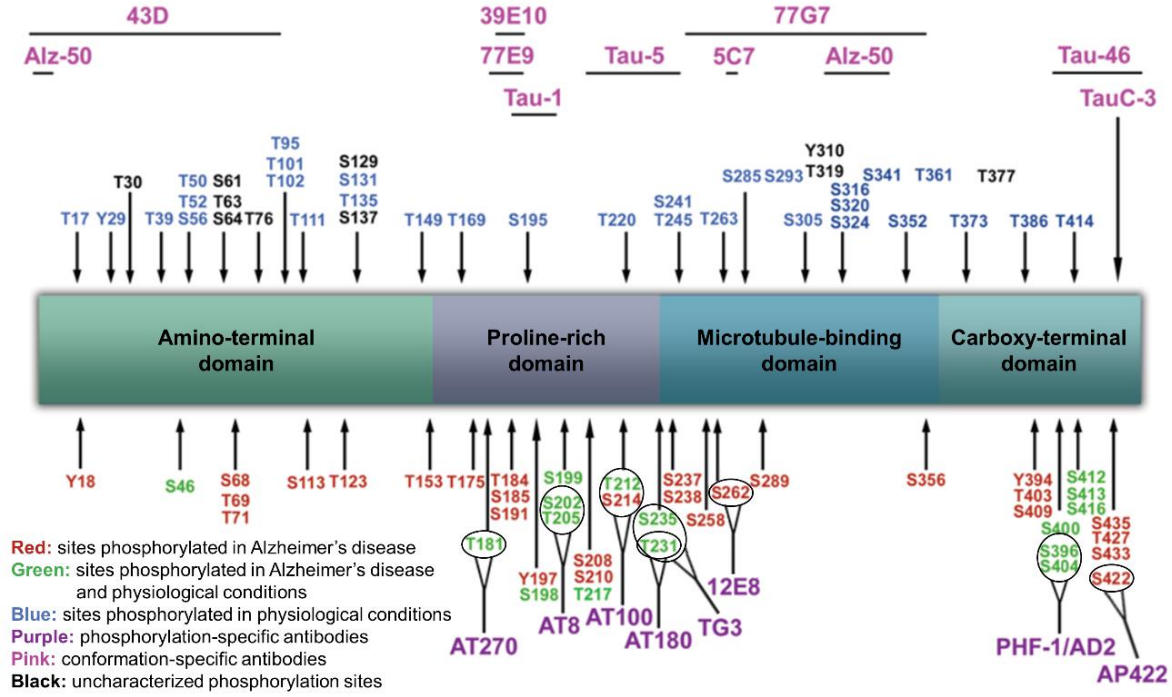

Figure 18. Tau phosphorylation sites and major Tau antibodies.
Putative phosphorylation sites on Tau protein and epitopes corresponding to major Tau antibodies. Red color indicates residues phosphorylated in the brain in Alzheimer's disease, green color denotes residues phosphorylated in both Alzheimer's disease and physiological conditions, blue color represents residues phosphorylated in physiological conditions, and black color designates phosphorylation sites that have not been fully characterized yet. Purple color illustrates antibodies detecting specific phosphorylated Tau epitopes and pink color shows antibodies detecting specific Tau conformations. Alz-50 (amino acids 2–10 and 312–342), 43D (amino acids 1–100), 77E9 (amino acids 185–195), 39E10 (amino acids 189–195), Tau-5 (amino acids 210–230), 5C7 (amino acids 267–278), Tau-1 (amino acids 195, 198, 199, and 202), 77G7 (amino acids 270–375), Tau-46 (amino acids 404–441), TauC-3 (tau cleaved on amino acid 421). *Adapted from Šimić et al., 2016.*

modifications regulate the ability of Tau to bind to microtubules (Mi & Johnson, 2006; S. Park et al., 2018; Tapia-Rojas et al., 2019). In general, Tau phosphorylation decreases its affinity for microtubules and often results in its detachment, but the number and location of phosphorylation sites determine the specific effects on Tau function (Kiris et al., 2011; F. Liu et al., 2007; Mi & Johnson, 2006).

The full range of physiological functions of Tau has not been completely unveiled yet. However, there is a general consensus that it is a multifunctional protein participating in microtubule-related processes in axons as well as in non-microtubule-related activities in several subcellular compartments. Because of its association with tubulin and ability to promote microtubule assembly under experimental conditions, it has long been claimed that the main function of Tau is to stabilize microtubules in the axon in a phosphorylation-dependent manner (Drubin & Kirschner, 1986; Weingarten et al., 1975).

Besides its participation in the modulation of microtubule stability, other prominent roles of Tau in the axon include the regulation of axonal transport, specifically of motor-driven anterograde fast axonal transport of membrane-bound organelles, the contribution to microtubule-actin interactions, and the control of the flexural rigidity of microtubules (Biswas & Kalil, 2018; Cabrales Fontela et al., 2017; Dixit et al., 2008; Felgner et al., 1997; Fulga et al., 2007; Kanaan et al., 2011, 2012; Seitz et al., 2002).

In dendrites, Tau regulates the functionality of the postsynaptic compartment in glutamatergic synapses. Specifically, it controls synaptic plasticity, including both long-term potentiation (LTP) and long-term depression (LTD), which underlie learning and memory (Bin Ibrahim et al., 2021; Dringenberg, 2020). Tau exerts this function through the modulation of Fyn kinase localization to the postsynaptic density, the regulation of N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and the modification of the cytoskeletal structure of dendrites and dendritic spines (Ahmed et al., 2014; Frandemiche et al., 2014; Fulga et al., 2007; L. M. Ittner et al., 2010; Kimura et al., 2014; Mondragón-Rodríguez et al., 2012; Regan et al., 2015; Reynolds et al., 2008; Usardi et al., 2011). Postsynaptic targeting of Fyn kinase by Tau leads to the formation of Tau/Fyn/postsynaptic density protein 95 (PSD-95)/NMDA receptor complexes and, thus, to upregulation of NMDA receptor activity at the postsynaptic density (L. M. Ittner et al., 2010). Tau may also have a role in GABAergic synapses, although data regarding Tau function in GABAergic neurons are scarce (Nykänen et al., 2012)

The role of Tau within the nucleus is unclear. However, several functions related to the regulation of nuclear processes have been proposed, such as the control of heterochromatin stability, the maintenance of the integrity of genomic and ribosomal DNA and cytoplasmic and nuclear RNA, and the regulation of ribosomal DNA transcription (Bou

Samra et al., 2017; Camero et al., 2014; Loomis et al., 1990; Mansuroglu et al., 2016; Sultan et al., 2011; Violet et al., 2014).

In association with the plasma membrane, Tau contributes to the modulation of signaling pathways triggered by insulin and neurotrophic factor receptors linked to synaptic plasticity and survival, like pathways involving mitogen-activated protein kinases (Leugers et al., 2013; Marciniak et al., 2017). Moreover, Tau has a role in neurite outgrowth and is especially necessary for growth cone behaviors during axonogenesis (Biernat et al., 2002; Biswas & Kalil, 2018; Gauthier-Kemper et al., 2011; Leugers & Lee, 2010; C. A. Liu et al., 1999; Sayas et al., 2015). Furthermore, in oligodendrocytes, Tau participates in mechanisms that contribute to myelination (C. Klein et al., 2002; Lee et al., 1998; Seiberlich et al., 2015).

Although Tau is natively unfolded, several factors can promote its aggregation, including post-translational modifications (Guo et al., 2017; Tapia-Rojas et al., 2019). Specific post-translational modifications or patterns of post-translational modifications of Tau can affect its oligomerization, seeding potential, and aggregation propensity, which determine its influence in the pathological process and are associated with the rate of clinical progression of Alzheimer's disease (Clavaguera et al., 2013; Dujardin et al., 2020; Sanders et al., 2014). In the context of this thesis, two factors are particularly relevant concerning the induction of Tau aggregation: mutations and hyperphosphorylation (A. del C. Alonso et al., 2001, 2004; Hutton et al., 1998; Kopke et al., 1993).

No mutations in the Tau-encoding *MAPT* gene are associated with Alzheimer's disease. However, mutations in the *MAPT* gene are related to several tauopathies, such as frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), a familial tauopathy also known as frontotemporal lobar degeneration (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). Tau mutations can be divided into missense mutations, which change its sequence, and splicing mutations, which alter the proportions of different Tau isoforms. Most missense mutations are located in or near the microtubule-binding domain, and some of them cause Tau to have reduced affinity for microtubules and to be more prone to hyperphosphorylation and aggregation (A. del C. Alonso et al., 2004; Barghorn et al., 2000; M. Hong et al., 1998; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998; von Bergen et al., 2001).

Phosphorylation of Tau is developmentally regulated, and it is high in the fetal brain but considerably lower in the adult brain (Brion et al., 1993, 1994; Yu et al., 2009). In Alzheimer's disease, Tau is hyperphosphorylated, containing three- to four-fold more phosphates per Tau molecule than in the brain of healthy adult subjects (Kopke et al., 1993; Ksiezak-Reding et al., 1992). In Alzheimer's disease and other tauopathies, Tau

hyperphosphorylation is one of the earliest events in the disease development and generally precedes and induces aggregation (A. del C. Alonso et al., 2001; E. Braak et al., 1994).

Phosphorylation at specific residues occurs early in the disease's pathological process and is associated with pre-tangles, that is, non-fibrillar structures comprising oligomers of different orders (Augustinack et al., 2002; Luna-Muñoz et al., 2007). Oligomers are intermediate Tau species formed during the process of aggregation into neurofibrillary tangles, with partial β-sheet structure (Lasagna-Reeves, Castillo-Carranza, Sengupta, Sarmiento, et al., 2012; Maeda et al., 2007; Takashima, 2013). Hence, hyperphosphorylation commonly precedes the formation of Tau oligomers (Guo et al., 2017; Iqbal et al., 2013). However, whenever mutations in the *MAPT* gene are present, the formation of oligomers and fibrillar aggregates occasionally precede hyperphosphorylation (Dujardin et al., 2018; Holmes et al., 2014).

A subset of Tau phosphorylation sites has received a greater interest as they are present in Alzheimer's disease and are part of epitopes recognized by antibodies used as diagnostic markers (Figure 18) (Biernat et al., 1992; Goedert et al., 1994; Hasegawa et al., 1992; Mandelkow & Mandelkow, 2012; Paudel et al., 1993; Šimić et al., 2016). For instance, residue serine 262 (Ser262) is one of the few sites located in the microtubule-binding domain repeats and its phosphorylation reduces the affinity of Tau for microtubules, alters the stability of its binding, and promotes its detachment (Biernat et al., 1993; Drewes et al., 1995; A. Schneider et al., 1999; Sengupta et al., 1998). However, phosphorylation at this site also protects Tau from aggregating into paired helical filaments (A. Schneider et al., 1999). Similarly, residue threonine 231 (Thr231) is located in the proline-rich domain and its phosphorylation decreases the ability of Tau to polymerize microtubules, regulates microtubule binding, and detaches Tau from microtubules (Amniai et al., 2009; Cho & Johnson, 2004; Goedert et al., 1994; Schwalbe et al., 2015; Sengupta et al., 1998). Moreover, residue threonine 205 (Thr205) is also located in the proline-rich domain and its phosphorylation can contribute to the dendritic localization of Tau (Jin et al., 2011; Zempel et al., 2010). Remarkably, phosphorylation at this site may confer neuroprotection (A. Ittner et al., 2016).

Similar to Aβ peptide, various Tau species are present in Alzheimer's disease: monomers, oligomers of different orders, paired helical filaments, and straight filaments (Crowther, 1991; Grundke-Iqbal, Iqbal, Quinlan, et al., 1986; Kidd, 1963; Lasagna-Reeves, Castillo-Carranza, Sengupta, Sarmiento, et al., 2012; Maeda et al., 2007; Terry, 1963). Furthermore, neurofibrillary tangles contain both paired helical and straight filaments, which originate from identical protofilaments but differ in the way these are packed. However, the predominant components of neurofibrillary tangles in Alzheimer's disease

are paired helical filaments (Crowther, 1991; Fitzpatrick et al., 2017; Mandelkow & Mandelkow, 2012; Oakley et al., 2020). Within paired helical filaments, Tau is also abnormally hyperphosphorylated (Grundke-Iqbal, Iqbal, Quinlan, et al., 1986; Grundke-Iqbal, Iqbal, Tung, et al., 1986).

Paired helical filaments display an overall cross-β structure. They comprise a core consisting of the microtubule-binding domain repeats and a fuzzy coat made of the amino- and carboxy-terminal domains (Berriman et al., 2003; Crowther, 1991; Wischik et al., 1988). The core of paired helical filaments consists of two protofilaments arranged as a double-helical stack of C-shaped subunits. Every C-shaped subunit corresponds to a single Tau molecule and contains eight β-strands (Fitzpatrick et al., 2017). This C-shaped conformation has been termed the Alzheimer fold (Berriman et al., 2003; Fitzpatrick et al., 2017; von Bergen et al., 2000). The nature of Tau as a natively unfolded protein has complicated the detection of its quality as an amyloid-forming protein. However, it has been demonstrated that it stems from hexapeptide motifs located at the beginning of the second and third microtubule-binding domain repeats with a high β-sheet propensity (Mandelkow & Mandelkow, 2012; von Bergen et al., 2000).

Within Tau-related neuropathological alterations, the Tau isoform and species composition, such as the type of filament present and the most common sites that are hyperphosphorylated, depend on each particular disease (Goedert, 2018; Oakley et al., 2020). In Alzheimer's disease, neurofibrillary tangles contain 3R and 4R Tau isoforms in similar proportions and both straight and paired helical filaments (Crowther, 1991; Goedert et al., 1989, 1995). However, in other tauopathies, there is a predominance of either 3R or 4R Tau isoforms. In the case of FTDP-17, the isoform predominance depends on the exact mutation present in the *MAPT* gene, but both straight and paired helical filaments are present as well (Crowther & Goedert, 2000; R. de Silva et al., 2006; Heutink, 2000).

Other than neurofibrillary tangles, Tau pathology in Alzheimer's disease manifests histologically in the form of dystrophic neurites and neuropil threads. Dystrophic neurites are abnormal neuronal processes containing Tau filaments and found surrounding senile plaques, while neuropil threads are dystrophic neurites found in the neuropil independently from senile plaques. Furthermore, after the degeneration and death of neurons containing neurofibrillary tangles, these become extracellular structures known as ghost tangles (E. Braak et al., 1994; H. Braak & Braak, 1991; Knopman et al., 2021; C. Li & Götz, 2017b).

Regarding the toxicity of Tau species, it has been established that soluble Tau oligomers, but not neurofibrillary tangles or Tau fibrils, are the most toxic species (Flach et al., 2012; Mufson et al., 2013; SantaCruz et al., 2005; Tian et al., 2013). Soluble Tau oligomers

of different orders are believed to be responsible for neurotoxicity and synaptotoxicity (Fá et al., 2016; Kaniyappan et al., 2017; Lasagna-Reeves et al., 2011; Tepper et al., 2014; Tian et al., 2013; Yoshiyama et al., 2007). Furthermore, prefibrillar Tau oligomers are a better correlate of cognitive status than neurofibrillary tangles in the early stages of Alzheimer's disease (Lasagna-Reeves, Castillo-Carranza, Sengupta, Sarmiento, et al., 2012; Maeda et al., 2007; Mufson et al., 2013).

Analogous to senile plaques, the progression of neurofibrillary tangles follows a hierarchical sequence. Tau pathology spreads in a prion-like manner. Pathological Tau is transmitted trans-synaptically and further propagates Tau pathology by seeding, or inducing, the alteration of other Tau molecules (Figure 19A) (Brettschneider et al., 2015; Clavaguera et al., 2009, 2013; De Calignon et al., 2012; DeVos et al., 2018; Frost et al., 2009; L. Liu et al., 2012). Several mechanisms have been proposed for Tau transmission, among which stands out its release regulated by synaptic activity (Pooler et al., 2013; J. W. Wu et al., 2016; Yamada et al., 2014). Thereby, Tau pathology spreads intercellularly through the brain in a stereotyped fashion across anatomically connected networks, in such a way that its distribution pattern can be correlated with the clinical stages of Alzheimer's disease through Braak staging (Figure 19B). In stages I and II—transentorhinal stages—neurofibrillary tangles develop in the transentorhinal cortex. In stages III and IV—limbic stages—they propagate to the entorhinal cortex and the hippocampal formation. Finally, in stages V and VI—neocortical stages—they spread throughout the entire neocortex. In normal aging, neurofibrillary tangles are also present but are limited to the entorhinal and limbic areas (E. Braak et al., 1991; Hyman et al., 1984; van der Kant et al., 2020).

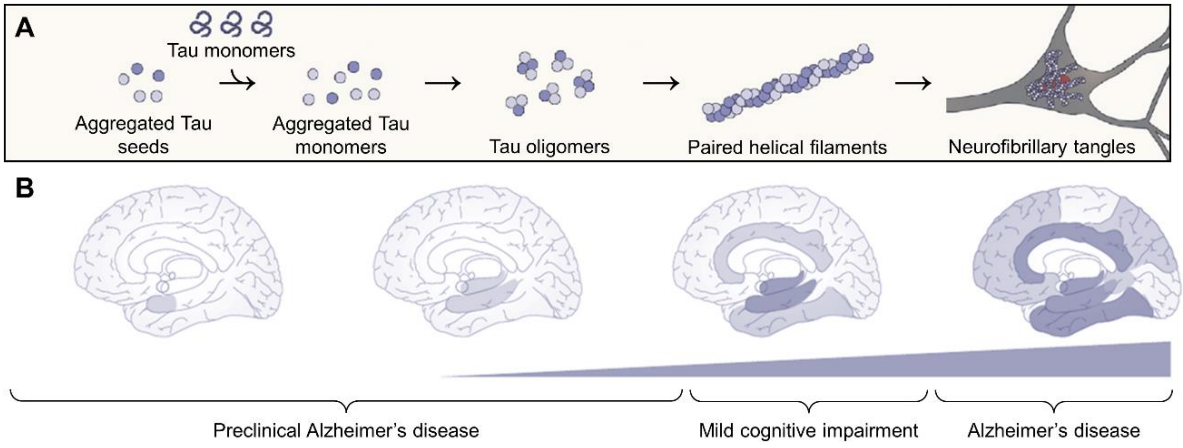

Figure 19. Progression of Tau pathology in Alzheimer's disease.
(A) Tau monomers aggregate as a result of several causes, including induction by aggregated Tau seeds. Aggregated Tau monomers assemble oligomers, which form paired helical filaments and these accumulate in neurofibrillary tangles in the somatodendritic compartment of neurons. (B) Tau pathology starts in the transentorhinal cortex in the early stages of Alzheimer's disease and spreads to the entorhinal cortex and the hippocampal formation in mild cognitive impairment patients. Finally, Tau pathology propagates to the neocortex in Alzheimer's disease patients. *Adapted from Long & Holtzman, 2019 (A) and van der Kant et al., 2020 (B).*

2.1.2 Pathophysiological features

In the last decade of the twentieth century, the amyloid cascade hypothesis was proposed to describe the pathophysiological process underlying Alzheimer's disease. Although it has undergone modifications over time, it has been the predominant model of Alzheimer's disease pathogenesis. The amyloid cascade hypothesis postulates that Aβ accumulation in the brain is the initiating factor of Alzheimer's disease pathogenesis and leads to Tau pathology, neuroinflammation, synaptic dysfunction, neuronal loss, and eventually cognitive impairment (Hardy & Higgins, 1992; Hardy & Selkoe, 2002; Selkoe & Hardy, 2016).

However, this linear model is not consistent with clinical observations, as there are discrepancies between Aβ burden and clinical symptoms, and therapeutic approaches that aim to reduce Aβ levels fail to stop disease progression (Golde et al., 2018; Karran et al., 2011; Mesulam, 1999; Nelson et al., 2012). Hence, the further development of the model has

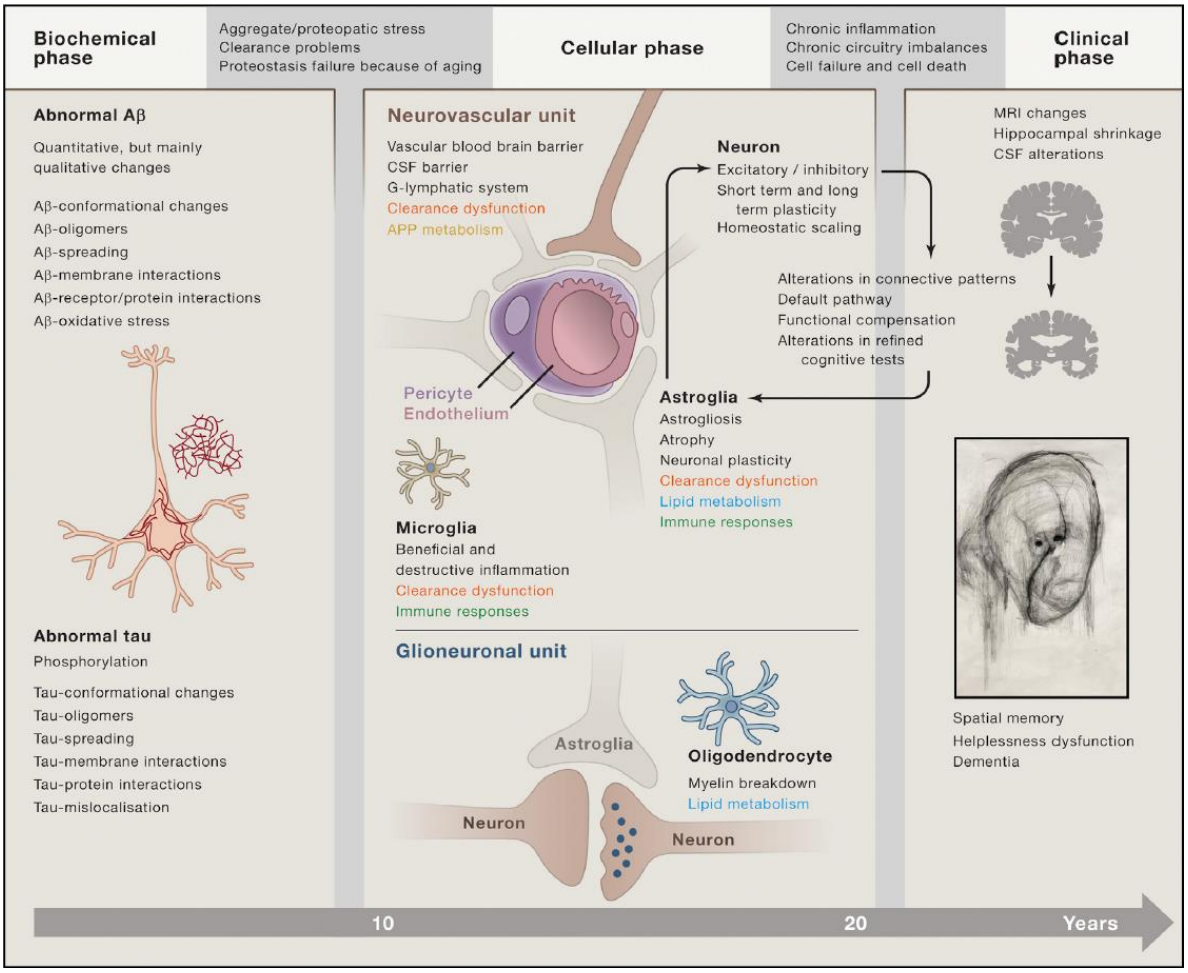

Figure 20. Biochemical, cellular, and clinical phases of Alzheimer's disease.
Progressive accumulation of abnormal Aβ and Tau over a decade goes unnoticed during the biochemical phase. Over another decade, the cellular phase gradually progresses from reversible physiological responses to irreversible pathological compensatory mechanisms. Upon severe disturbance of the normal brain homeostasis, the clinical phase ensues. *Adapted from De Strooper & Karran, 2016.*

brought about the cellular phase hypothesis (Figure 20). The cellular phase hypothesis posits that the accumulation of Aβ and Tau pathologies is progressive and well-tolerated by neurons and glia in the early stages of Alzheimer's disease: the biochemical phase. However, at some point, the compensatory mechanisms countering the proteopathy and cellular stress transform into chronic, irreversible, pathological processes: the cellular phase. The cellular phase is characterized by defective clearance mechanisms that contribute to the proteopathy, dysfunction of the neurovascular unit, aberrant network activity, and altered microglial and astrocytic functions. The pathological processes that occur in the cellular phase lead to the failure of the cellular homeostatic mechanisms. When the normal brain homeostasis has been disrupted, the clinical phase of Alzheimer's disease begins, culminating in widespread neurodegeneration and overt dementia (De Strooper & Karran, 2016; Herrup, 2015; Long & Holtzman, 2019).

Thus, Aβ and Tau pathologies, together with neuroinflammation and alterations in synapses, neurons, microglia, astrocytes, the vasculature, the blood-brain barrier, and the glymphatic and other clearance systems drive disease progression covertly over the course of the years prior to the manifestation of cognitive impairment (Da Mesquita et al., 2018; De Strooper & Karran, 2016; Labzin et al., 2018; Plog & Nedergaard, 2018; Sweeney et al., 2018).

2.1.2.1 Synaptic dysfunction

Alzheimer's disease can be viewed as a synaptic dysfunction disorder comprising alterations at the molecular, cellular, and circuit levels. Synaptic pathophysiology is a unifying subject for understanding the relationship between the toxicity induced by Aβ and Tau and cognitive impairment through data from genetics, cell biology, neuropathology, and clinical manifestations (Knopman et al., 2021; Palop & Mucke, 2016; Spires-Jones & Hyman, 2014).

The final outcome of chronic synaptic dysfunction, synaptic loss, is a robust correlate of cognitive impairment in Alzheimer's disease (DeKosky & Scheff, 1990; Terry et al., 1991). From the early stages of disease, synaptic loss in the hippocampus and dentate gyrus correlate better with cognitive status than neuropathological changes, including neuronal loss and Aβ and Tau accumulation (Masliah et al., 1994; Scheff et al., 1996, 2007). In agreement with this, synaptic plasticity—comprising LTP and LTD—is the cellular correlate underlying learning and memory, especially in the hippocampus, and is altered in Alzheimer's disease (Bin Ibrahim et al., 2021; Cuestas Torres & Cardenas, 2020; Dringenberg, 2020; Styr & Slutsky, 2018).

Both Aβ and Tau, particularly their oligomeric forms, are able to exert synaptotoxicity independently or synergistically (Busche & Hyman, 2020; Long &

Holtzman, 2019). For years, several lines of evidence have indicated that Aβ exerts a part of its toxic effects through Tau as a mediator. On this subject, multiple data indicate that Tau is required for Aβ-induced toxicity, such as excitotoxicity at glutamatergic synapses (Figure 21A), both *in vitro* and *in vivo* (Frandemiche et al., 2014; L. M. Ittner et al., 2010; Jin et al., 2011; Rapoport et al., 2002; Roberson et al., 2007, 2011; Zempel et al., 2013). For instance, this is supported by the fact that removal of endogenous Tau prevents Aβ-associated synaptic (Figure 21C), network, and cognitive deficits (Roberson et al., 2007, 2011).

Likewise, the spread of Tau pathology and the subsequent neurodegeneration triggered by Tau-induced toxicity requires the co-occurrence of Aβ pathology, and the production rate of Tau—comprising its synthesis and release into the cerebrospinal fluid—positively correlates with the Aβ burden (Pontecorvo et al., 2019; Price & Morris, 1999; Sato et al., 2018; L. Wang et al., 2016). Nevertheless, it appears that neurodegeneration and cognitive decline are driven by Tau pathology, and thus Tau is a better predictor of cognitive deficits than Aβ. In particular, cognitive decline manifests when Tau pathology spreads from the entorhinal cortex into the neocortex (Price et al., 2009; Price & Morris, 1999). More importantly, accumulating data indicate that Tau accumulation is a predictor of cognitive impairment, while Aβ accumulation is a predictor of Tau accumulation and severity of Tau-associated cognitive impairment (Aschenbrenner et al., 2018; D. A. Bennett et al., 2004; Brier et al., 2016; Hanseeuw et al., 2019).

On the one hand, soluble Aβ oligomers are present at synapses, at both the presynaptic and postsynaptic compartments, and synaptic loss in the vicinity of senile plaques correlates with the burden of oligomeric Aβ species (Koffie et al., 2009, 2012; Spires et al., 2005). Notably, Aβ oligomers can inhibit LTP, enhance LTD, reduce dendritic spine density, and impair cognitive function (Arbel-Ornath et al., 2017; Cleary et al., 2005; Shankar et al., 2007, 2008; Spires-Jones et al., 2007; Spires et al., 2005; Walsh et al., 2002; Wei et al., 2010). Among the molecular mechanisms leading to synaptic dysfunction and loss, Aβ-induced increases in Ca^{2+} levels, namely excitotoxicity, play a crucial role and are associated with impaired LTP and LTD, internalization of AMPA and NMDA receptors, and loss of dendritic spines (Demuro et al., 2005; Hsieh et al., 2006; Lambert et al., 1998; S. Li et al., 2009; Mattson et al., 1992; Shankar et al., 2008; Snyder et al., 2005; Walsh et al., 2002; Zempel et al., 2010).

On the other hand, soluble Tau oligomers—typically composed of P-Tau—are also present at synapses, again at both the presynaptic and postsynaptic compartments, cause loss of dendritic spines by mediating Aβ-induced toxicity, and increased levels of P-Tau at synapses correlate with cognitive impairment (DeVos et al., 2018; Hoover et al., 2010; Perez-Nievas et al., 2013; Tai et al., 2012; Zempel et al., 2010; Zhou et al., 2017). The effects of Tau

pathology stem from both the gain of toxic function and the loss of physiological function. In Alzheimer's disease, axonal Tau is missorted to the somatodendritic compartment, compromising the integrity of axonal microtubules and disrupting axonal transport (Ebneth et al., 1998; L. M. Ittner et al., 2009; Kanaan et al., 2011; Kopeikina et al., 2012). In addition, since Tau is missorted to synapses, including dendritic spines, it can impair both LTP and LTD through several mechanisms, leading to synaptic dysfunction and loss (Decker et al., 2015; Fá et al., 2016; Hoover et al., 2010; L. M. Ittner et al., 2010; Tracy et al., 2016; Warmus et al., 2014; Zhao et al., 2016). These mechanisms include disruption of postsynaptic targeting and anchoring of AMPA and NMDA receptors, mediation of the excitotoxicity induced by Aβ through regulating the postsynaptic targeting of Fyn kinase (Figure 21A) or independently from Aβ (Figure 21B), among others (Hoover et al., 2010; L. M. Ittner et al., 2010; Tracy et al., 2016; Warmus et al., 2014; Zhao et al., 2016).

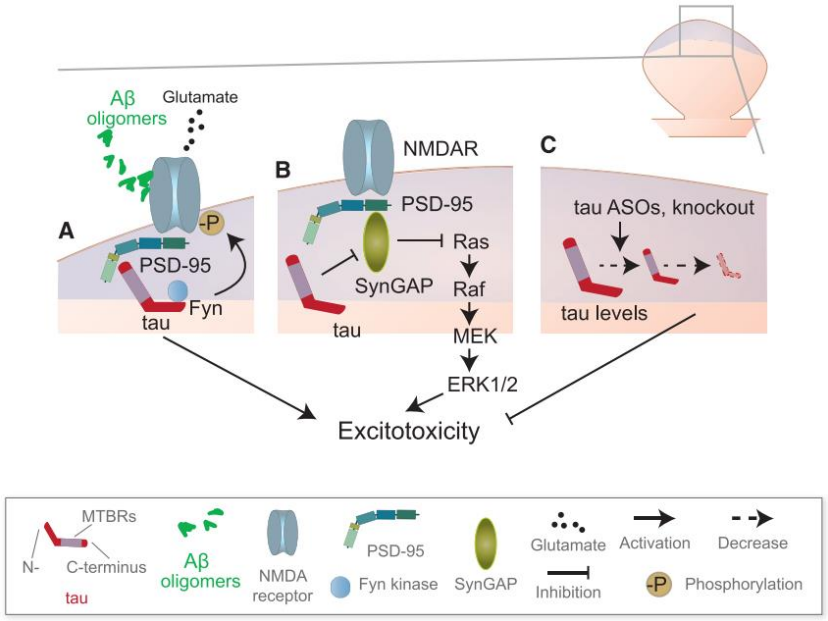

Figure 21. Tau mediates excitotoxicity at the postsynaptic compartment. Tau can exert synaptotoxic effects in several ways, including mediating excitotoxicity induced by Aβ or independently from Aβ through various mechanisms at the postsynaptic compartment. (A) Tau recruits Fyn kinase to PSD-95/NMDA receptor complexes and mediates excitotoxicity induced by Aβ and excess glutamate. Fyn kinase phosphorylates NMDA receptors, enhancing the interaction between them and PSD-95 and, therefore, enabling toxic downstream signaling pathways. (B) Tau limits the binding of SynGAP, a negative regulator of the excitotoxic Ras-Raf-MEK-ERK1/2 pathway, to PSD-95 at PSD-95/NMDA receptor complexes, thus enabling NMDA receptor-mediated activation of this excitotoxic signaling pathway. (C) Reduction of Tau levels by ASOs or knockout prevents excitotoxicity. Abbreviations: ASOs, antisense oligonucleotides; MTBRs, microtubule-binding regions. *Adapted from A. Ittner & Ittner, 2018 (A–C).*

Mitochondria are also pivotal players in the molecular mechanisms leading to synaptic dysfunction and loss, owing to the importance of mitochondria for synaptic physiology. For instance, mitochondrial transport to presynaptic and postsynaptic compartments is impaired due to the pathological changes in Tau that disrupt axonal transport (Eckert et al., 2010). Moreover, mitochondrial dynamics and function are altered by both Aβ and Tau pathologies through several pathways (Eckert et al., 2014; Quintanilla et al., 2012; X. Wang et al., 2009).

In Alzheimer's disease, the initial synaptic dysfunction and subsequent synaptic loss result in impaired synaptic transmission, turning molecular alterations into cellular

abnormalities and, eventually, into neural circuits defects. In this way, it has been proposed that synaptic dysfunction leads to alterations in higher levels of organization by causing the dysfunction of specific circuits and, ultimately, giving rise to general network abnormalities.

In Alzheimer's disease patients and animal models, aberrant network activity has been observed (Kazim et al., 2021; Palop & Mucke, 2016; Vico Varela et al., 2019; Zott et al., 2018). These network abnormalities manifest as anomalous increases in network excitability and, specifically, as hippocampal hyperactivity (Bookheimer et al., 2000; Busche et al., 2012; Palop et al., 2007; Quiroz et al., 2010; Reiman et al., 2012; Zott et al., 2019). Moreover, alterations in oscillatory activity significantly contribute to these network abnormalities (Ahnaou et al., 2017; Goutagny et al., 2013; Hollnagel et al., 2019; Stoiljkovic et al., 2019; Verret et al., 2012). Furthermore, epileptiform activity is frequent in Alzheimer's disease patients and animal models (Amatniek et al., 2006; Horváth et al., 2016; Imfeld et al., 2013; Mendez et al., 1994; Palop et al., 2007; Verret et al., 2012; Vossel et al., 2013, 2016). Aberrant network activity in Alzheimer's disease could stem from an imbalance between excitation and inhibition and, eventually, lead to cognitive impairment (Kazim et al., 2021; Lauterborn et al., 2021; Palop & Mucke, 2016; Vico Varela et al., 2019; Zott et al., 2018). Hence, it has been proposed that the imbalance between excitation and inhibition and subsequent aberrant network activity and cognitive deficits are associated with dysfunction of the GABAergic system (Ambrad Giovannetti & Fuhrmann, 2019; Hollnagel et al., 2019; Mably & Colgin, 2018; Palop & Mucke, 2016; Petrache et al., 2019; Shimojo et al., 2020; Verret et al., 2012; Villette & Dutar, 2016).

It is worth mentioning that the majority of data on synaptic dysfunction and loss in Alzheimer's disease refers to glutamatergic synapses. In contrast, data on GABAergic synapses are limited, especially in human studies. Given the prominent role proposed for the GABAergic system in network abnormalities and cognitive deficits in Alzheimer's disease, further research on the state of GABAergic synapses is needed.

2.1.2.2 Cholinergic deficits

The basal forebrain cholinergic projection system comprises cholinergic neurons located in the MSDB complex and the nucleus basalis of Meynert—in humans—or the nucleus basalis magnocellularis—in rodents (Ashwell & Mai, 2012). Cholinergic neurons of the basal forebrain project to cortical regions, such as the neocortex and hippocampus, and limbic regions, like the amygdala (Zaborszky et al., 2012). These projections exert a neuromodulatory effect by tonically increasing the excitability of their target regions (Gulyás et al., 1990; Krnjević & Ropert, 1982).

As previously mentioned for cholinergic neurons of the septohippocampal pathway, cholinergic neurons are studied through detection of the enzymes acetylcholinesterase and choline acetyltransferase. These are used as markers to determine the state of the cholinergic system in various contexts, including Alzheimer's disease (Zaborszky et al., 2012).

One of the earliest features of Alzheimer's disease described, aside from senile plaques and neurofibrillary tangles, is the disruption of the cholinergic system. Cholinergic dysfunction is supported by the reduction of presynaptic markers of cholinergic neurons like choline acetyltransferase, the degeneration of cholinergic neurons of the basal forebrain, and the demonstration that cholinergic antagonists impair learning and memory whereas agonists yield the inverse effect (Davies & Maloney, 1976; Drachman, 1974; Whitehouse et al., 1982). Altogether, these findings led to the proposal of the cholinergic hypothesis of Alzheimer's disease-related memory dysfunction (Bartus, 2000; Bartus et al., 1982). This hypothesis posits that the cholinergic deficit resulting from the loss of cholinergic neurons of the basal forebrain and their axons that project to cortical and limbic regions, namely the cholinergic denervation of these regions, has a causal role in the pathogenesis of Alzheimer's disease.

Of the four currently approved pharmacological approaches specific to Alzheimer's disease, three are cholinesterase inhibitors—donepezil, rivastigmine, and galantamine—and the remaining one is an NMDA receptor antagonist—memantine (Knopman et al., 2021). Cholinesterase inhibitors aim to prevent acetylcholine degradation to sustain its activity at cholinergic synapses (Hampel et al., 2018). However, these treatments only provide modest benefits, delaying symptom progression by 6 months, and are palliative, lacking disease-modifying effects (Fink et al., 2018; Marucci et al., 2021).

The current perspective on the role of the cholinergic system in Alzheimer's disease is that dysfunction and loss of cholinergic transmission and neurons occurs but does not entirely explain the pathogenesis and clinical symptoms of Alzheimer's disease (Davies, 1999; Mesulam, 2004). Therefore, exceptional efforts are needed to ascertain the pathological mechanisms underlying Alzheimer's disease and to, at last, develop effective disease-modifying treatments.

2.1.2.3 The septohippocampal pathway in Alzheimer's disease

The basal forebrain is severely compromised in Alzheimer's disease. In particular, cholinergic neurons of the basal forebrain are selectively vulnerable (Davies & Maloney, 1976; Whitehouse et al., 1982). As mentioned above, early studies led to the proposal of the cholinergic hypothesis of Alzheimer's disease-related memory dysfunction (Bartus, 2000; Bartus et al., 1982). Dysfunction and loss of cholinergic neurons of the basal forebrain lead

to progressive denervation of cortical and limbic regions, which has been corroborated in rodent models of Alzheimer's disease (Aucoin et al., 2005; Colom et al., 2010).

Numerous studies have examined the cholinergic component of the basal forebrain, whereas its GABAergic counterpart has been disregarded, especially in human studies. This other component, namely the GABAergic septohippocampal pathway, has been described in rhesus monkeys and, analogous to rodents, originates in the MSDB complex and terminates specifically on GABAergic hippocampal interneurons (Gulyás et al., 1991). In humans, the MSDB complex is functionally connected with the hippocampus (Yuan et al., 2019). However, the state of the GABAergic septohippocampal pathway has not been expressly studied in humans.

The GABAergic system has been considered relatively spared in Alzheimer's disease (Canas et al., 2014; Mitew et al., 2013; Reinikainen et al., 1988; Rissman et al., 2007). However, Alzheimer's disease patients and animal models present aberrant network activity, particularly in the form of altered oscillatory activity, hyperexcitability, and epileptiform activity (Horváth et al., 2016; Palop et al., 2007; Quiroz et al., 2010; Stoiljkovic et al., 2019; Verret et al., 2012). Evidence support that these network abnormalities arise from an imbalance between excitation and inhibition and that this imbalance could underlie cognitive deficits (Kazim et al., 2021; Lauterborn et al., 2021; Palop & Mucke, 2016; Vico Varela et al., 2019; Zott et al., 2018). Dysfunction of the GABAergic system has been proposed to play a key role in the imbalance between excitation and inhibition and, thus, in aberrant network activity and cognitive impairment (Ambrad Giovannetti & Fuhrmann, 2019; Mably & Colgin, 2018; Palop & Mucke, 2016; Petrache et al., 2019; Shimojo et al., 2020; Verret et al., 2012; Villette & Dutar, 2016).

The particular cell types and circuits involved in aberrant network activity in Alzheimer's disease remain unclear. Nevertheless, the GABAergic septohippocampal pathway may be a promising candidate. GABAergic septohippocampal neurons specifically innervate GABAergic hippocampal interneurons, which in turn contact hippocampal principal neurons (Freund & Antal, 1988; Freund & Buzsáki, 1996; Gulyás et al., 1990) (see section 1.1.1.2.1). Hence, the rhythmic activity of GABAergic septohippocampal neurons suppresses feedforward inhibition and synchronizes principal neuron activity in the hippocampus, giving rise to oscillatory activity (Gangadharan et al., 2016; Hangya et al., 2009) (see section 1.3).

In animal models of aging, electrophysiological and behavioral data indicate age-related functional alterations in the septohippocampal pathway (Apartis et al., 2000; Flood & E. Morley, 1997). Moreover, results from studies in animal models of Alzheimer's disease point to the specific involvement of the GABAergic septohippocampal pathway and its

hippocampal targets in the disease process. For instance, Aβ injection in the hippocampus leads to reductions in the rhythmic firing of GABAergic septohippocampal neurons, altered hippocampal theta oscillations, and impaired memory processes without loss of GABAergic septohippocampal neurons (Villette et al., 2010). In this animal model of amyloidosis, hippocamposeptal neurons are also affected, compromising the communication and coordination between the hippocampus and the MSDB complex and, thus, the correct functioning of the septo-hippocampo-septal loop (Villette et al., 2012).

Our group has examined the state of the GABAergic septohippocampal pathway in Alzheimer's disease transgenic mouse models through tract-tracing studies (Rubio et al., 2012; Soler et al., 2017). In a first study in J20 mice, which accumulate Aβ in the neocortex and hippocampus, data reveal a premature impairment of the GABAergic septohippocampal pathway at 8 months. This impairment is not caused by the loss of GABAergic septohippocampal neurons or by changes in the number of GABAergic hippocampal interneurons, as both neuron populations are spared, but by a decrease in the number and complexity of GABAergic septohippocampal synaptic contacts on GABAergic hippocampal interneurons (Figure 22A and B). Indeed, the percentage of contacted GABAergic hippocampal interneurons, as well as the number of synaptic boutons constituting the basket-shaped contacts established by GABAergic septohippocampal neurons, are severely reduced in J20 animals (Figure 22A and B). The loss of GABAergic septohippocampal innervation affects the total interneuron population and, more dramatically, the PV-positive interneuron subpopulation. Remarkably, J20 mice exhibit altered hippocampal theta and gamma oscillations, manifested as a reduced spectral power of the theta and gamma frequency bands. These data suggest that the aberrant network activity and cognitive impairment observed in Alzheimer's disease patients may be caused by the loss of GABAergic septohippocampal innervation, owing to its modulation of hippocampal network activity (Rubio et al., 2012; Vega-Flores et al., 2014).

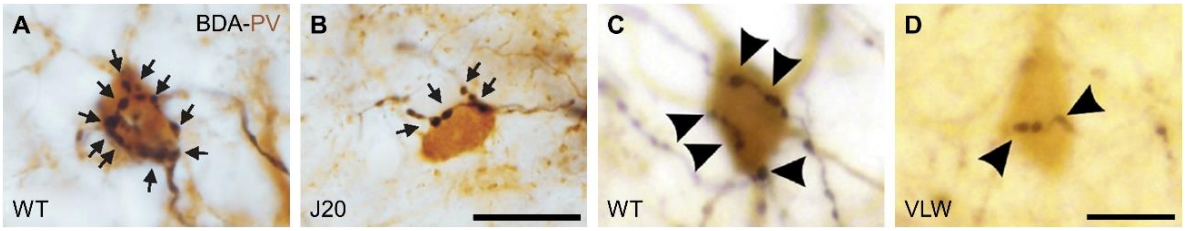

Figure 22. GABAergic septohippocampal innervation in J20 and VLW mice.
GABAergic septohippocampal neurons establish numerous synaptic boutons, labelled through tract-tracing methods (BDA, black), arranged in basket-shaped structures around the soma and proximal dendrites of GABAergic hippocampal interneurons, in this case PV-positive interneurons (brown). (A and B) GABAergic septohippocampal innervation is prematurely altered in J20 mice at 8 months, compared to age-matched WT animals. (C and D) GABAergic septohippocampal innervation is impaired in VLW mice at 8 months, compared to age-matched WT animals. Abbreviations: BDA, biotinylated dextran-amine; WT, wild-type. Scale bar: 20 μm (B, applies to A and B), 15 μm (D, applies to C and D). *Adapted from Vega-Flores et al., 2014 (A and B) and Soler et al., 2017 (C and D).*

In a subsequent study in VLW mice, which accumulate P-Tau in the neocortex and hippocampus, results demonstrate a dramatic impairment of the GABAergic septohippocampal pathway. Analogous to J20 animals, this impairment implies no loss of GABAergic septohippocampal neurons or alterations in the number of GABAergic hippocampal interneurons but is due to a decrease in the number and complexity of GABAergic septohippocampal synaptic contacts on GABAergic hippocampal interneurons (Figure 22C and D). Precisely, the percentage of contacted GABAergic hippocampal interneurons and the number of synaptic boutons composing the basket-shaped contacts established by GABAergic septohippocampal neurons are drastically reduced in VLW mice (Figure 22C and D). The loss of GABAergic septohippocampal innervation affects PV-positive interneurons already at 2 months, and at 8 months it is more severe and mainly affects PV-positive interneurons accumulating P-Tau. These findings suggest that alterations in GABAergic septohippocampal innervation and dysfunction of PV-positive interneurons accumulating P-Tau may contribute to network abnormalities and cognitive deficits in tauopathies, including Alzheimer's disease (Soler et al., 2017).

2.2 Animal models

Animal models are essential tools for Alzheimer's disease research that allow investigating underlying causes, dissecting involved pathophysiological mechanisms, and validating therapeutic approaches. Numerous animal models have been generated in an attempt to reproduce the neuropathological, pathophysiological, and clinical features of Alzheimer's disease (Götz et al., 2018; Vignon et al., 2021).

The most common methods in Alzheimer's disease modeling are genetic strategies, mainly transgenic mouse lines bearing mutations in genes related to familial Alzheimer's disease, including the *APP*, *PSEN1*, and *PSEN2* genes, which encode proteins with a role in Aβ production. There are non-genetic strategies at use as well, such as biochemical, pharmacological, or lesion models (Götz et al., 2018; Mckean et al., 2021).

Rodent models constitute the preferred animal models as they have a short lifespan and rapid reproduction, they are easy and relatively inexpensive to maintain and breed in comparison with other species, there are well-established genetic modification methods to generate rodent models that recapitulate disease features, and there are numerous data, tools, and standardized behavioral tests to evaluate rodent phenotypes (Ferrari & Sorbi, 2021; Mckean et al., 2021).

Currently, 210 rodent models of Alzheimer's disease exist, of which 194 are mouse models and 16 are rat models (*Research Models: Alzheimer's Disease*, n.d.). Although animal models provide insight into mechanistic aspects of Alzheimer's disease, none of the current models reproduce Alzheimer's disease pathology entirely. Notably, the efficacy of pharmacological treatments from rodent data fails to translate to human studies, calling for further efforts in the development of animal models of Alzheimer's disease (Götz et al., 2018; Mckean et al., 2021; Vignon et al., 2021).

2.2.1 J20 transgenic mouse line

The first transgenic mouse model of Alzheimer's disease overexpressed the human *APP* gene carrying the familial Alzheimer's disease Indiana mutation (V717F) and presented Aβ pathology reminiscent of Alzheimer's disease (Games et al., 1995). In subsequent models, multiple mutations were combined on the *APP* gene to achieve earlier and more severe Aβ pathology, neurodegeneration, and cognitive deficits (Sturchler-Pierrat et al., 1997).

The J20 transgenic mouse line was generated by the team of Dr. Mucke at the University of California, San Francisco (United States of America). J20 mice overexpress human APP carrying the Swedish (K670N/M671L) and Indiana (V717F) mutations

(hAPP^{Sw,Ind}) associated with familial Alzheimer's disease (Figure 23A), under the control of the human platelet-derived growth factor subunit β promoter (Mucke et al., 2000). In order to achieve the goals of this thesis, the J20 transgenic mouse model has been used.

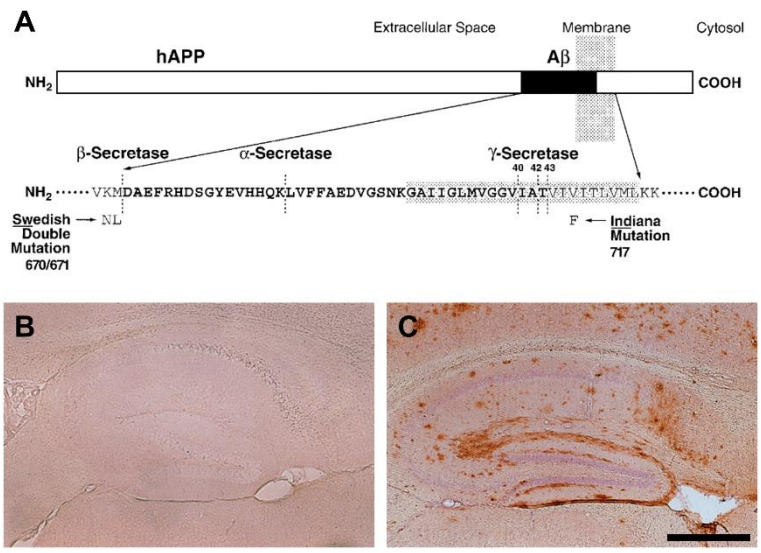

Figure 23. APP mutations and Aβ pathology in J20 mice.
(A) Diagram of hAPP indicating the familial Alzheimer's disease mutations carried by J20 mice: K670N/M671L (Swedish double mutation) and V717F (Indiana mutation). The sequence of Aβ is indicated in bold in single-letter amino acid code. (B and C) Immunodetection of Aβ plaques with 3D6 antibody against amino acids 1–5 of Aβ reveals numerous senile plaques in the hippocampus of J20 mice at 10 months (C) compared to control animals at 15 months (B). Scale bar: 500 μm. *Adapted from Mucke et al., 2000 (A–C).*

In J20 mice, APP is expressed in neurons widespread across different brain regions, reaching maximal levels in the neocortex and hippocampus. These animals present high levels of Aβ and develop plaques at 5–7 months, which at first are diffuse but progressively convert into neuritic plaques and increase in number with age (Figure 23C) (Mucke et al., 2000). The hippocampus of J20 mice presents a reduction in presynaptic and postsynaptic markers and aberrant axonal sprouting (Chin et al., 2004; S. Hong et al., 2016; Mucke et al., 2000; Palop et al., 2007). Moreover, these animals demonstrate functional alterations in hippocampal synapses and circuits, such as deficits in synaptic plasticity and synaptic transmission and electrophysiological alterations in the form of spontaneous epileptiform activity, hyperexcitability, and altered oscillatory activity (Palop et al., 2007; Palop & Mucke, 2016; Rubio et al., 2012; Saganich et al., 2006; Vega-Flores et al., 2014). Furthermore, J20 mice exhibit age-dependent cognitive impairment from 4 months onward, including learning and memory deficits (Cheng et al., 2007; Cissé et al., 2011; J. A. Harris et al., 2010; Palop et al., 2003; Sanchez-Mejia et al., 2008; Wright et al., 2013).

2.2.2 VLW transgenic mouse line

Mutations in the *MAPT* gene have not been linked to Alzheimer's disease. However, transgenic mouse models expressing the mutant *APP* do not develop Tau pathology. Hence, the study of Tau pathology has required the generation of transgenic mouse lines overexpressing the *MAPT* gene carrying familial tauopathy mutations (Götz et al., 2018; Mckean et al., 2021; Vignon et al., 2021).

The first transgenic mouse model of this kind overexpressed the human *MAPT* gene carrying the familial tauopathy P301L mutation and developed Tau pathology mimicking human tauopathies (J. Lewis et al., 2000).

The VLW transgenic mouse line was generated by the team of Dr. Ávila at the *Centro de Biología Molecular Severo Ochoa* (Spain). VLW mice overexpress human Tau protein isoform 2N4R (with two amino-terminal inserts and four tubulin-binding repeats) carrying three mutations (G272V, P301L, and R406W; $hTau^{VLW}$) associated with the familial tauopathy FTDP-17 (Figure 24A), under the control of the murine thymocyte differentiation antigen 1 (Thy1) promoter (Lim et al., 2001). In the development of the present thesis, the VLW transgenic mouse model has been employed.

In VLW mice, pyramidal neurons of the neocortex and hippocampal CA1 and CA3 regions express high levels of mutant human Tau (Lim et al., 2001). High amounts of soluble P-Tau, including both transgenic and endogenous Tau, are present in the same neurons and regions where the transgene is expressed (Figure 24C). This P-Tau is in the form of pre-tangles, namely non-fibrillar structures comprising oligomers of different orders, only assembles paired helical filaments from 18 months and onward, and does not give rise to neurofibrillary tangles (Guerrero et al., 2009; Lim et al., 2001). In addition to pyramidal neurons of the hippocampal CA1 and CA3 regions, mossy cells of the dentate gyrus and PV-positive interneurons of the hippocampus also display soluble P-Tau in VLW animals (Soler et al., 2017). Moreover, VLW mice exhibit electrophysiological alterations, such as spontaneous epileptiform activity and hyperexcitability, and cognitive impairment affecting learning and memory from 6 months onward (García-Cabrero et al., 2013; Navarro et al., 2008; Rossi et al., 2020).

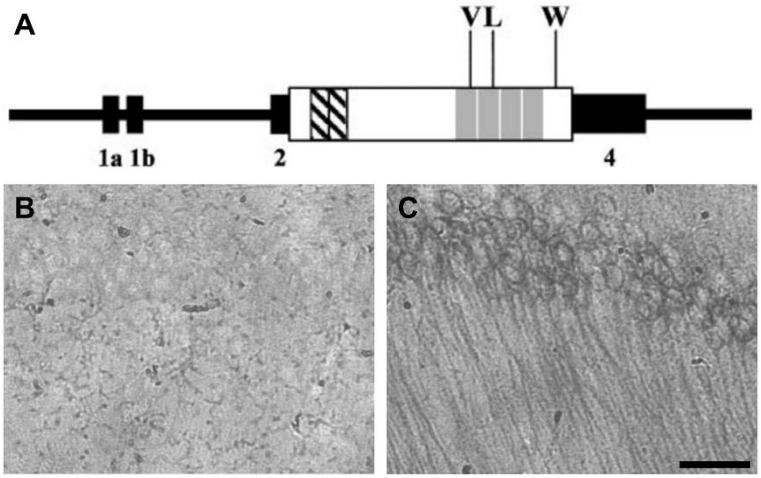

Figure 24. Tau mutations and Tau pathology in VLW mice.
(A) Diagram of the human *MAPT* gene indicating the familial tauopathy FTDP-17 mutations carried by VLW mice: G272V (V), P301L (L), and R406W (W). The sequence of the 2N4R Tau isoform is depicted (open box), containing two amino-terminal inserts (striped boxes) and four microtubule-binding repeats (gray boxes), and is inserted into a murine Thy1 expression cassette between exons 2 and 4 (black boxes). (B and C) Immunodetection of P-Tau with AT-180 antibody against Tau phosphorylated at Thr231 shows strong somatodendritic staining in pyramidal neurons of the hippocampal CA1 region of VLW mice at 10 months (C) compared to aged-matched control animals (B). Scale bar: 100 μm. *Adapted from Lim et al., 2001 (A–C).*

2.2.3 J20/VLW transgenic mouse line

In an attempt to recapitulate the complete spectrum of Alzheimer's disease pathology, mouse lines carrying mutations in more than one gene simultaneously have been bred over the years. Frequently, these double transgenic mouse models bear mutant *APP* and *PSEN1* or *PSEN2* genes. Nevertheless, due to the lack of Tau pathology in animals expressing mutant *APP* and *PSEN1* or *PSEN2* genes, several double transgenic mouse models bearing mutant *APP* and *MAPT* genes have been generated as well (Götz et al., 2018; Mckean et al., 2021; Vignon et al., 2021).

Considering that Aβ and Tau pathologies are central in Alzheimer's disease, it is reasonable to conceive that simultaneously reproducing both hallmarks will result in a more accurate depiction of the disease's neuropathology, pathophysiology, and clinical phenotype and, therefore, in a better chance to explore the disease etiology, associated pathogenic mechanisms, and therapeutic strategies.

In the present thesis, a new double transgenic mouse model was generated by crossing J20 and VLW animals, yielding the J20/VLW mouse line. These animals bear the transgenes of both parent mouse lines and, therefore, express $hAPP^{Sw,Ind}$ and $hTau^{VLW}$. By recapitulating the two central pathologies of Alzheimer's disease, J20/VLW mice could illustrate the complete spectrum of Alzheimer's disease pathology and shed light on potential synergies among its main exponents.

2.3 Cognitive resilience to Alzheimer's disease

Since the description of Alzheimer's disease at the beginning of the twentieth century, the neuropathological hallmarks that define it as a distinct neurodegenerative disease have been senile plaques and neurofibrillary tangles (Alzheimer, 1907; Khachaturian, 1985; McKhann et al., 1984). Although the diagnostic guidelines have been updated over the years, owing to scientific and methodological advances, diagnosis of Alzheimer's disease requires the presence of said neuropathological hallmarks or biomarker measures of the proteins they involve (Hyman et al., 2012; Jack et al., 2018; Montine et al., 2012). Nevertheless, multiple studies suggest that the relationship between senile plaques, neurofibrillary tangles, and cognitive function does not always predict the clinical manifestations at the individual level, implying that these neuropathological changes are not straightforwardly related to dementia (Boyle et al., 2019; Brookmeyer et al., 2016; Jack et al., 2019; Matthews et al., 2009; Sonnen et al., 2007).

The current perspective considers Alzheimer's disease as a continuum, which includes the preclinical stage of normal cognition and the clinical stages of mild cognitive impairment and dementia, with the goal of understanding the progression through the stages over the course of the disease and of developing treatments that stop this progression in preclinical stages, when normal cognitive function is maintained (Dubois et al., 2016; Hyman et al., 2012; Jack et al., 2018; Montine et al., 2012). However, this continuum assumes an invariable linear progression between stages that does not always occur (Kawas & Corrada, 2020).

The neuropathological hallmarks of Alzheimer's disease are frequently found in individuals with a normal cognitive function that never develop dementia (Gomez-Isla, 2021; Jack et al., 2018; Kawas et al., 2021). These individuals are believed to present resilience: they display a level of cognitive functioning higher than predicted based on the existing neuropathological alterations (Montine et al., 2019). Hence, they are referred to as cognitive resilience to Alzheimer's disease (CRAD) or non-demented with Alzheimer's disease neuropathology (NDAN) subjects. Within the current diagnostic guidelines, these subjects devoid of clinical symptoms could be diagnosed with Alzheimer's disease based on postmortem neuropathological changes or *in vivo* biomarker measures (Jack et al., 2018). Their subsequent inclusion in studies as Alzheimer's disease patients would confound research outcomes and hinder the discovery of the substrates of cognitive resilience (Gomez-Isla, 2021). Understanding which factors and mechanisms underlie cognitive resilience is a matter of great importance, as it could have a profound impact on the therapeutic approaches for the prevention and treatment of Alzheimer's disease. Thus, CRAD must be considered a distinct clinical entity.

2.3.1 Neuropathological features

The presence of senile plaques, neurofibrillary tangles, and other manifestations of Aβ and Tau pathologies biologically defines Alzheimer's disease and is necessary for its diagnosis (Hyman et al., 2012; Jack et al., 2018; Montine et al., 2012). However, these features have been found repeatedly in postmortem studies or *in vivo* biomarker analyses in individuals with normal cognition.

Approximately 20% of subjects with normal cognition aged 50 years and older present Aβ and Tau pathologies, and 10% exhibit sufficient amounts to meet neuropathological criteria for Alzheimer's disease (Aizenstein et al., 2008; D. A. Bennett et al., 2006; Jack et al., 2014, 2017; W. J. Jansen et al., 2015; Knopman et al., 2003; Mintun et al., 2006; Price et al., 1991; Riley et al., 2005; Rowe et al., 2010, 2007; J. A. Schneider et al., 2009). This proportion rises with increasing age, surpassing 40% in individuals aged 90 years and older (Beker et al., 2021; Jack et al., 2017; W. J. Jansen et al., 2015; J. L. Robinson et al., 2018; Snitz et al., 2020; Tanprasertsuk et al., 2019).

2.3.2 Functional protection against dementia

The main risk factor for both Alzheimer's disease and dementia is age (Knopman et al., 2021). However, certain subjects maintain proper cognitive function throughout aging, even while bearing the classical Alzheimer's disease pathology (Gomez-Isla, 2021; Jack et al., 2018). These individuals, hereafter referred to as CRAD subjects, are characterized by presenting the neuropathological hallmarks of Alzheimer's disease but not its clinical symptoms. It is believed that CRAD subjects possess intrinsic mechanisms that confer neuroprotection against the cognitive impairment associated with Alzheimer's disease. These mechanisms will be discussed in the following sections.

2.3.2.1 Synaptic preservation

It is widely accepted that Alzheimer's disease can be regarded as a synaptic dysfunction disorder (Knopman et al., 2021; Palop & Mucke, 2016; Spires-Jones & Hyman, 2014). Hence, synaptic preservation is a suitable substrate potentially underlying cognitive resilience.

On the one hand, Aβ and Tau oligomers are absent from synapses in CRAD subjects, contrary to Alzheimer's disease patients, and their absence is associated with maintenance of synaptic integrity and cognitive resilience (Bjorklund et al., 2012; Perez-Nievas et al., 2013; Singh et al., 2020).

CRAD subjects and Alzheimer's disease patients present comparable amounts of Aβ plaques and Aβ monomers, dimers, and oligomers (Figure 25A) (Arnold et al., 2013;

Bjorklund et al., 2012; Perez-Nievas et al., 2013). However, soluble Aβ oligomers, the most synaptotoxic Aβ species, are abundant in the postsynaptic compartment of Alzheimer's disease patients yet absent in the case of CRAD subjects. Moreover, the expression of phosphorylated CREB, a transcription factor essential for synaptic plasticity and memory, is decreased in Alzheimer's disease patients but normal in CRAD subjects (Bjorklund et al., 2012; A. J. Silva et al., 1998).

The levels of neurofibrillary tangles, total Tau, P-Tau, and Tau monomers and oligomers are similar or slightly lower in CRAD subjects compared to Alzheimer's disease patients yet increased in comparison with control individuals (Figure 25B) (Arnold et al., 2013; Perez-Nievas et al., 2013; Singh et al., 2020). Nevertheless, the association of soluble Tau oligomers—typically comprised of P-Tau—with synapses is reduced in CRAD subjects, contrary to Alzheimer's disease patients, and correlates with cognitive status (Perez-Nievas et al., 2013; Singh et al., 2020). Furthermore, synaptic plasticity in the form of LTP is decreased in Alzheimer's disease patients but normal in CRAD subjects (Singh et al., 2020).

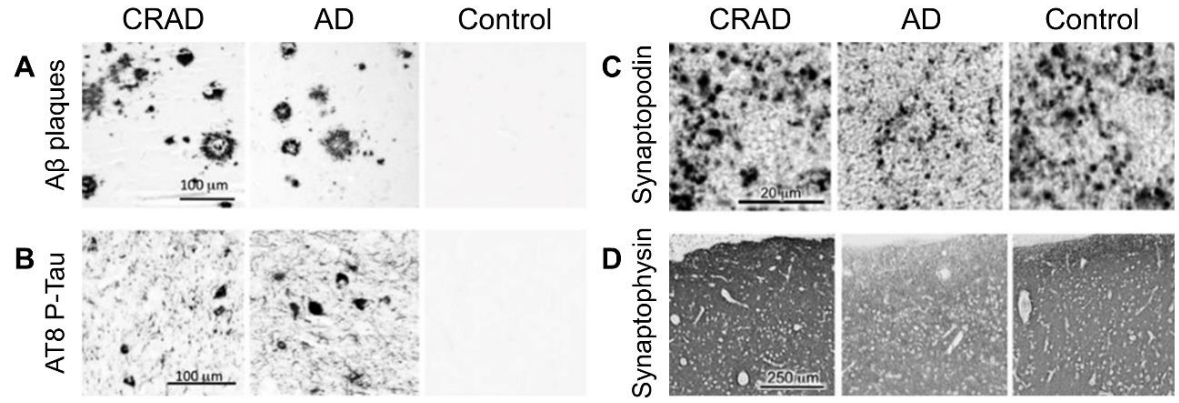

Figure 25. Neuropathology and synaptic markers in CRAD, Alzheimer's disease, and control subjects.
Immunodetection of Aβ plaques (A) and P-Tau (B) reveals similar densities of neuropathology in CRAD subjects and Alzheimer's disease patients, and significantly lower densities in control subjects. In contrast, immunodetection of presynaptic marker synaptophysin (C) and postsynaptic marker synaptopodin (D) shows comparable levels of both synaptic markers in CRAD and control subjects, whereas Alzheimer's disease patients present significantly lower levels. CRAD, Alzheimer's disease, and control subjects were age-matched, and only Alzheimer's disease patients presented cognitive impairment. (A) Immunodetection of Aβ plaques with M0872 antibody against the amino-terminal end of Aβ. (B) Immunodetection of P-Tau with AT8 antibody against Tau phosphorylated at Ser202 and Thr205. (C) Immunodetection of synaptic vesicle protein synaptophysin with M0776 antibody. (D) Immunodetection of postsynaptic density and dendritic spine protein synaptopodin with Q44590M antibody. Panels A–C depict layer III and panel D shows layers I–IV from the middle frontal gyrus (mainly Brodmann area 46, corresponding to the dorsolateral prefrontal cortex). Scale bar: 100 μm (A and B), 20 μm (C), 250 μm (D). Abbreviations: AD, Alzheimer's disease. *Adapted from Arnold et al., 2013 (A–D).*

On the other hand, the essential components of the synaptic machinery that are dysfunctional in Alzheimer's disease are preserved in CRAD subjects, which is considered critical for the maintenance of synaptic and cognitive function (Arnold et al., 2013; Perez-Nievas et al., 2013; Walker et al., 2022; Zolochevska et al., 2018). CRAD subjects present normal expression levels of presynaptic proteins and normal densities of postsynaptic

dendritic spines, whereas both presynaptic and postsynaptic markers of synaptic integrity are reduced in Alzheimer's disease patients (Figure 25C and D) (Arnold et al., 2013; Perez-Nievas et al., 2013; Walker et al., 2022). Additionally, CRAD subjects display a differential gene expression pattern at the postsynaptic compartment of glutamatergic synapses that differs from that of Alzheimer's disease and control subjects (Liang et al., 2010; Walker et al., 2022; Zolochevska et al., 2018). In relation to this, a specific set of miRNAs that regulates genes involved in synaptic function is also differentially expressed in CRAD subjects and could have a role in conferring the distinctive gene expression pattern that possibly provides synaptic protection against Alzheimer's disease pathology (Zolochevska & Taglialatela, 2020).

Collectively, these factors may contribute to the mechanisms that confer synaptic protection and are responsible for preserved synaptic integrity, which is believed to be one of the main exponents underlying cognitive resilience in CRAD.

2.3.2.2 Other factors

Multiple factors have been linked to resilience against the cognitive impairment associated with Alzheimer's disease. To begin with, reduced neuroinflammation and oxidative stress could play a role in cognitive resilience. In contrast to Alzheimer's disease patients, CRAD subjects present a decreased number of activated microglia and reactive astrocytes, a distinctive cytokine expression profile, and a decreased expression of chemokines involved in microglial recruitment (Barroeta-Espar et al., 2019; Perez-Nievas et al., 2013). Moreover, CRAD subjects exhibit reduced levels of oxidative damage markers and functional antioxidant response pathways compared to Alzheimer's disease patients (Fracassi et al., 2021).

Another mechanism potentially underlying cognitive resilience is adult hippocampal neurogenesis. Its impairment has been associated with cognitive deficits. Notably, adult hippocampal neurogenesis is preserved in CRAD subjects (Briley et al., 2016; Moreno-Jiménez et al., 2019). Additionally, CRAD subjects present increased expression of neurotrophic factors compared to Alzheimer's disease patients (Barroeta-Espar et al., 2019).

Further to this, larger cognitive reserve, which is associated with higher educational or occupational attainment, and larger brain reserve, meaning greater hippocampal and brain volumes, are other factors proposed to be involved in cognitive resilience in CRAD subjects (Chételat et al., 2010; Erten-Lyons et al., 2009; Katzman et al., 1988; Stern, 2012; Valenzuela & Sachdev, 2006).

Altogether, reduced neuroinflammation, functional antioxidant mechanisms, preserved adult hippocampal neurogenesis, and larger cognitive and brain reserve together

with additional physiological or molecular mechanisms may contribute to cognitive resilience in CRAD.

The identification of CRAD subjects, presenting Aβ and Tau pathologies but lacking clinical symptoms of dementia, opens a window on the existence of factors and mechanisms able to protect against cognitive impairment and points to the need to identify the processes driving cognitive resilience.

OBJECTIVES

The hallmarks of Alzheimer's disease, senile plaques and neurofibrillary tangles, have long been placed at the center of the disease etiology and pathogenesis. The toxic effects of Aβ and Tau can be either independent or synergistic. Certainly, Tau has a pivotal role in these pathological effects, as it mediates the toxicity induced by Aβ, neurodegeneration, and cognitive decline. In addition, the identification of diverse physiological functions of Tau, together with the importance of phosphorylation for the regulation of its activity, has opened up new avenues for physiological roles of Tau phosphorylation distinct from its pathological effects in Alzheimer's disease.

By depicting the two central pathologies of Alzheimer's disease, the J20/VLW mouse line could recapitulate the complete spectrum of Alzheimer's disease pathology and facilitate the elucidation of potential synergies among Aβ and Tau. Moreover, dysfunction of the GABAergic system is involved in the imbalance between excitation and inhibition that leads to aberrant network activity and cognitive impairment in Alzheimer's disease. Specifically, the GABAergic septohippocampal pathway and GABAergic hippocampal interneurons are relevant for cognitive processes that are altered in this disease. Altogether, the available data evidence the need to explore these subjects in an attempt to fill a knowledge gap.

In the context of this rationale, the following objectives have been set for this thesis.

1. **Characterization of the Tau phosphorylation pattern in hippocampal interneurons in physiological and pathological conditions.**

   1.1 To analyze the Tau phosphorylation pattern in mice and human subjects in physiological conditions.

   1.2 To determine the Tau phosphorylation pattern in Alzheimer's disease mouse models and human patients.

   1.3 To examine the induction of murine Tau phosphorylation in interneurons by mutant human Tau present in pyramidal neurons in the hippocampus.

2. **Histological and functional characterization of a new animal model of Alzheimer's disease: J20/VLW mice.**

   2.1 To analyze Aβ levels and plaque burden in the hippocampus in J20/VLW animals.

2.2 To examine the accumulation of phosphorylated Tau in the hippocampus in J20/VLW mice.

2.3 To study the GABAergic septohippocampal pathway in J20/VLW mice.

2.4 To evaluate hippocampal electrophysiology in J20/VLW animals.

2.5 To analyze the cognitive function of J20/VLW mice.

REPORT ON THE PUBLICATIONS DERIVED FROM THIS THESIS

UNIVERSITAT DE BARCELONA

Departament de Biologia Cel·lular, Fisiologia i Immunologia
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La Dra. Marta Pascual Sánchez, Professora Titular del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Facultat de Biologia de la Universitat de Barcelona, i el Dr. Eduardo Soriano García, Catedràtic del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Facultat de Biologia de la Universitat de Barcelona, com a directors de la Tesi Doctoral de l'Eva Dávila Bouziguet.

Informen

que, fruit de la realització del seu projecte de Tesi Doctoral "*Characterization of a new animal model of Alzheimer's disease: relevance of Tau phosphorylation in hippocampal interneurons*", la candidata a Doctora Eva Dávila Bouziguet ha participat activament en l'elaboració de dos articles publicats en revistes internacionals.

1. **Differential accumulation of Tau phosphorylated at residues Thr231, Ser262 and Thr205 in hippocampal interneurons and its modulation by Tau mutations (VLW) and amyloid-β peptide.**
   Eva Dávila-Bouziguet*, Georgina Targa-Fabra*, Jesús Ávila, Eduardo Soriano, Marta Pascual.
   Neurobiology of Disease (2019); 125: 232–244. doi: 10.1016/j.nbd.2018.12.006.
   *Aquests autors han contribuït per igual.
   Factor d'impacte: 5.332.
   Aquest article constitueix el primer capítol de resultats d'aquesta Tesi Doctoral.

2. **Functional protection in J20/VLW mice: a model of non-demented with Alzheimer's disease neuropathology.**
   Eva Dávila-Bouziguet, Arnau Casòliba-Melich, Georgina Targa-Fabra, Lorena Galera-López, Andrés Ozaita, Rafael Maldonado, Jesús Ávila, José M. Delgado-García, Agnès Gruart, Eduardo Soriano, Marta Pascual.
   Brain (2022); 145 (2): 729–743. doi: 10.1093/brain/awab319.
   Factor d'impacte: 13.501.
   Aquest article constitueix el segon capítol de resultats d'aquesta Tesi Doctoral.

Aquests estudis aporten informació rellevant sobre la fosforilació fisiològica de la proteïna Tau en interneurones GABAèrgiques de l'hipocamp i sobre el seu paper protector de la innervació GABAèrgica septohipocàmpica, l'activitat oscil·latòria de l'hipocamp i la funció cognitiva en la malaltia d'Alzheimer. El nostre article en la revista *Brain* mostra la caracterització d'un nou model animal de la malaltia d'Alzheimer i el presenta com una eina molt valuosa per a futures investigacions sobre els factors responsables de la resiliència cognitiva a la demència associada a la malaltia d'Alzheimer.

L'Eva ha participat activament en l'elaboració de totes les publicacions. En el primer article ha contribuït en la planificació i realització dels experiments, les quantificacions, l'obtenció d'imatges, el muntatge de figures, l'escriptura i la revisió de la publicació. En el segon article ha participat en la planificació i realització dels experiments, les quantificacions, l'obtenció d'imatges, el muntatge de figures, l'escriptura i la revisió de la publicació. Tot això justifica la seva posició com a primera coautora en el primer article i com a primera autora en el segon article.

I, perquè així consti per a la presentació de la Tesi Doctoral de l'Eva Dávila Bouziguet, signen el present informe.

Atentament,

Dra. Marta Pascual Sánchez

Dr. Eduardo Soriano García

RESULTS

Differential accumulation of Tau phosphorylated at residues Thr231, Ser262 and Thr205 in hippocampal interneurons and its modulation by Tau mutations (VLW) and amyloid-β peptide

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*Aquests autors han contribuït per igual.

RESUM

La malaltia d'Alzheimer es caracteritza per l'acumulació de pèptid β-amiloide (Aβ) i proteïna Tau hiperfosforilada (P-Tau). Les nostres dades recents mostren una acumulació diferencial de proteïna Tau fosforilada al residu Thr231 (pThr231 Tau) en diferents neurones de l'hipocamp en els ratolins VLW, un model que sobreexpressa Tau humana mutant. Aquí demostrem que, en els ratolins VLW, l'acumulació de P-Tau humana a les cèl·lules piramidals indueix la fosforilació de la Tau murina al residu Thr231 a les interneurones de l'hipocamp. A més, mostrem que la Tau fosforilada al residu Ser262 (pSer262 Tau) i la Tau fosforilada al residu Thr205 (pThr205 Tau) estan presents específicament al soma d'algunes interneurones de l'hipocamp en els ratolins control. Els animals VLW mostren una densitat

d'interneurones de l'hipocamp que acumulen pThr205 Tau menor que els ratolins control. En canvi, la densitat d'interneurones que acumulen pThr205 Tau en els ratolins J20, un model que acumula Aβ, està incrementada en comparació amb els animals control, i coincideix amb les regions de l'hipocamp amb major acumulació de plaques d'Aβ, suggerint que la pThr205 Tau és induïda per l'Aβ. No hem trobat diferències significatives en la densitat d'interneurones de l'hipocamp positives per la pSer262 Tau en els ratolins VLW o J20 en comparació amb els animals control. També mostrem que la pSer262 Tau i la pThr205 Tau estan presents al soma d'algunes interneurones de l'hipocamp que contenen parvalbúmina, calbindina o calretinina en els ratolins control, VLW i J20. Addicionalment, els nostres resultats indiquen que algunes interneurones de l'hipocamp de pacients de la malaltia d'Alzheimer i d'individus control acumulen pSer262 Tau i pThr205 Tau. En conjunt, aquestes dades apunten a un paper específic de la pSer262 Tau i la pThr205 Tau en el soma de les interneurones de l'hipocamp en condicions fisiològiques i patològiques.

Aquest article ha estat publicat a la revista *Neurobiology of Disease*:

Dávila-Bouziguet, E., Targa-Fabra, G., Ávila, J., Soriano, E., & Pascual, M. (2019). Differential accumulation of Tau phosphorylated at residues Thr231, Ser262 and Thr205 in hippocampal interneurons and its modulation by Tau mutations (VLW) and amyloid-β peptide. *Neurobiology of Disease*, 125, 232–244. doi: 10.1016/j.nbd.2018.12.006.

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Differential accumulation of Tau phosphorylated at residues Thr231, Ser262 and Thr205 in hippocampal interneurons and its modulation by Tau mutations (VLW) and amyloid-β peptide

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ABSTRACT

Alzheimer's disease (AD) is characterized by the accumulation of amyloid-β peptide (Aβ) and hyperphosphorylated Tau protein (P-Tau). Our recent data showed a differential accumulation of Tau protein phosphorylated at residue Thr231 (pThr231) in distinct hippocampal neurons in VLW mice—a model that overexpresses mutated human Tau. Here we demonstrate that, in VLW mice, the accumulation of human P-Tau in pyramidal cells induces the phosphorylation of murine Tau at residue Thr231 in hippocampal interneurons.

In addition, we show that pSer262 and pThr205 Tau are present specifically in the soma of some hippocampal interneurons in control mice. Analysis of J20 mice—a model that accumulates Aβ—and of VLW animals showed that the density of hippocampal interneurons accumulating pThr205 Tau is lower in VLW mice than in controls. In contrast, the density of interneurons accumulating pThr205 Tau in J20 mice was increased compared to controls in hippocampal regions with a higher Aβ plaque load, thereby suggesting that pThr205 Tau is induced by Aβ. No significant differences were found between the density of hippocampal interneurons positive for pSer262 Tau in VLW or J20 mice compared to control animals.

We also show that pSer262 and pThr205 Tau are present in the soma of some hippocampal interneurons containing Parvalbumin, Calbindin or Calretinin in control, VLW, and J20 mice. Moreover, our results reveal that some interneurons in human hippocampi of cases of AD and control cases accumulate pSer262 and pThr205 Tau. Taken together, these data point to a specific role of pSer262 and pThr205 Tau in the soma of hippocampal interneurons in control and pathological conditions.

1. Introduction

Tau is a microtubule (MT)-associated protein that participates in tubulin cytoskeleton assembly and stabilization (Drubin and Kirschner, 1986; Weingarten et al., 1975), and also in axonal transport regulation and axon development, as well as in other less well-known functions in the synapse (Ittner et al., 2010) and the nucleus (Sultan et al., 2011; Violet et al., 2014). Certain factors can affect the unfolded conformation of Tau and promote its assembly in paired helical filaments (PHFs) (Kidd, 1963), which leads to aggregation in neurofibrillary tangles (NFTs), a feature of several neurodegenerative diseases (Lee et al., 2001). Together with amyloid-β peptide (Aβ) species and plaques, Tau in NFTs is one of the main histopathological hallmarks of Alzheimer's disease (AD), the most common form of dementia (Ballatore et al., 2007; Bloom, 2014; Haass and Selkoe, 2007).

Mutations can stimulate Tau aggregation, as is the case of familial tauopathies like frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Hutton et al., 1998). In addition, a wide range of post-translational modifications of Tau, notably hyperphosphorylation, can facilitate its aggregation (Kopke et al., 1993). Despite these observations, data show that soluble Tau oligomers, not NFTs, are the most toxic structures for neurons and synapses (Flach et al., 2012;

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SantaCruz et al., 2005; Tian et al., 2013; Yoshiyama et al., 2007).

In mature neurons, Tau is found mainly in the axon (Binder et al., 1985) and only small amounts localize to dendrites (Papasozomenos and Binder, 1987). However, the presence of Aβ oligomers, or Tau hyperphosphorylation, mutations, or overexpression can cause the mislocalization of this protein to the somatodendritic compartment and may even drive it to postsynaptic spines under resting conditions, hence producing excitatory synapse dysfunction (Frandemiche et al., 2014; Götz et al., 1995; Hoover et al., 2010; Thies and Mandelkow, 2007). In this regard, Tau appears to be required for Aβ-induced toxicity, as seen both *in vitro* (Frandemiche et al., 2014; Jin et al., 2011; Zempel et al., 2013) and *in vivo* (Ittner et al., 2010; Roberson et al., 2007, 2011). For instance, missorted dendritic Tau mediates the synaptic impairment induced by Aβ (Frandemiche et al., 2014; Ittner et al., 2010; Zempel et al., 2013). In addition, a reduction in endogenous Tau levels in human amyloid precursor protein (APP)-overexpressing mice protects them against synaptic, network, and cognitive deficits (Roberson et al., 2007, 2011).

Tau is a complex protein with more than 85 putative phosphorylation sites (Hanger et al., 2007; Morishima-Kawashima et al., 1995). Certain phosphoepitopes in Tau are recognized by antibodies that are used as diagnostic markers of AD, including pThr205, pThr231 and pSer262 (Goedert et al., 1994; Šimić et al., 2016). Phosphorylation at residue Ser262 in the repeat domain of Tau greatly reduces its affinity for MTs, but also prevents it from assembling into PHFs (Drewes et al., 1995; Schneider et al., 1999; Sengupta et al., 1998). The effect of pThr231 on the affinity of Tau for microtubules is less prominent; primarily, this phosphoepitope in the proline-rich domain of Tau regulates the assembly of tubulin in MTs (Amniai et al., 2009; Cho and Johnson, 2004; Sengupta et al., 1998). In addition, phosphorylation at residues Thr231 and Ser262 may abnormally target Tau to dendritic spines (Xia et al., 2015). Surprisingly, a protective effect against Aβ toxicity has recently been proposed for pThr205 Tau (Ittner et al., 2016). Therefore, the detailed functions of these phosphorylated residues in Tau are still unknown.

Our recent data demonstrate the presence of pThr231 Tau in hippocampal GABAergic interneurons containing Parvalbumin (PV) in VLW animals, a mouse model accumulating P-Tau (Lim et al., 2001; Soler et al., 2017). Our results suggest that a dysfunction in hippocampal interneurons underlies the cognitive deficits associated with tauopathies such as AD (Soler et al., 2017). In this regard, the dysfunction of GABAergic transmission was recently proposed as one of the key factors in the pathogenesis of network dysfunction in AD (Busche et al., 2015; Palop and Mucke, 2010), with PV-positive interneurons possibly playing a prominent role. The function of neural circuits depends on the synchronous activity of their components, and deregulation of the excitatory/inhibitory balance seems to contribute to the cortical network alterations and cognitive dysfunction associated with AD (Palop and Mucke, 2016; Verret et al., 2012; Villette and Dutar, 2016).

Here we compared the distribution of several P-Tau forms, namely pThr231, pSer262 and pThr205 Tau, in the hippocampus in normal and pathological conditions in wild-type (WT), and in VLW and J20 mice, which accumulate P-Tau and Aβ respectively (Lim et al., 2001; Mucke et al., 2000), as well as in human samples. The soma of hippocampal interneurons accumulated pSer262 and pThr205 in normal and pathological conditions, thereby suggesting that Aβ induces Tau phosphorylation. These results point to a new role of P-Tau in hippocampal GABAergic interneurons. This neuronal population thus emerges as a new target for therapeutic approaches in AD.

2. Material and methods

2.1. Animals

To conduct the histological procedures, we used WT adult male mice (C57BL/6J strain; 8-month-old (mo); $n = 4$) and also 8 mo transgenic adult male mice from two different lines presenting AD features and with the same genetic background. In this regard, we used VLW mice, which overexpress human Tau protein (hTau) with 4 tubulin-binding repeats and 3 mutations related to FTDP-17: G272V (V), P301L (L) and R406W (W) under the control of the Thy-1 promoter ($n = 4$). The second line was J20 mice, which overexpress the human APP carrying two mutations, namely Swedish (K670N/M671L) and Indiana (V717F) familial AD mutations ($n = 4$).

All animals were kept on a 12-h light-dark schedule with access to food and water *ad libitum*. All the animal experiments were performed in accordance with the European Community Council Directive and the National Institute of Health guidelines for the care and use of laboratory animals. The local ethical committees also approved these experiments.

2.2. Human samples

Human samples from control subjects ($n = 2$; 61 and 66 years old) and AD patients ($n = 2$; 73 and 91 years old, Braak IV-V) were provided by Dr. Isidre Ferrer, from the Brain Bank of the Institute of Neuropathology at Bellvitge Hospital in Barcelona (for more detailed information see Supplementary Table 1). After the post-mortem interval (PMI) indicated in Supplementary Table 1, the tissue was fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 24 h, and then cryoprotected with phosphate-buffered saline (PBS) with 30% sucrose. It was then frozen by a quick immersion in 2-methylbutane at −50 °C, and 30 µm coronal sections were obtained by cryomicrotomy. Human samples were treated in accordance with the European Community Council Directive and the National Institute of Health guidelines.

2.3. Immunodetection

To obtain brain tissue, animals were deeply anesthetized by administrating a lethal dose (400 µL per 60 g of weight) of a solution 10:1 of Ketolar® (ketamine hydrochloride 50 mg/mL, Parke-Davis)/Rompun® (2% xylazine-thiazine hydrochloride, Bayer). They were then immediately perfused with 4% PFA in 0.1 M PB. Next, the brains were post-fixed for 48 h in 4% PFA and then cryoprotected with PBS with 30% sucrose at 4 °C. They were then frozen by a quick immersion in 2-methylbutane at −50 °C, and 30 µm coronal sections were cut. These were stored in a cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1 M PB) at −20 °C until use.

To detect the accumulation of hTau, pThr231, pSer262 or pThr205 Tau, tissue sections from WT, VLW and J20 mice were blocked for 2 h and incubated overnight with HT7 mouse anti-hTau (ThermoFisher Scientific; 1/500), AT-180 mouse anti-phosphothreonine 231 (Innogenetics; 1/300), S262 rabbit anti-phosphoserine 262 (Invitrogen™; 1/100), or T205 rabbit anti-phosphothreonine 205 antibodies (Invitrogen™; 1/1000) at 4 °C. Primary antibodies were visualized by sequential incubation with biotinylated secondary antibodies and ABC complex (2 h each; Vector Laboratories). The peroxidase reaction was developed with diaminobenzidine (DAB), together with nickel ammonium sulfate, cobalt chloride and H_2O_2 to intensify the final signal. The sections were mounted onto gelatinized slides, dehydrated, and coverslipped with Eukitt® (O.Kindler). The same procedure followed for the S262 and T205 antibodies was performed using human samples from control subjects and AD patients.

Some mouse brain sections were also stained with 3D6 mouse anti-APP antibody (obtained from the supernatant of cultured Murine Hybridoma Cell Line, RB96 3D6.32.2.4 (PTA-5130), American Type Culture Collection; 1/200) in order to detect the presence of senile plaques in J20 mice. The primary antibody was visualized by sequential incubation with DAB to produce a brown end product.

To determine whether hippocampal interneurons accumulated P-Tau, several double fluorescent immunostainings were conducted using

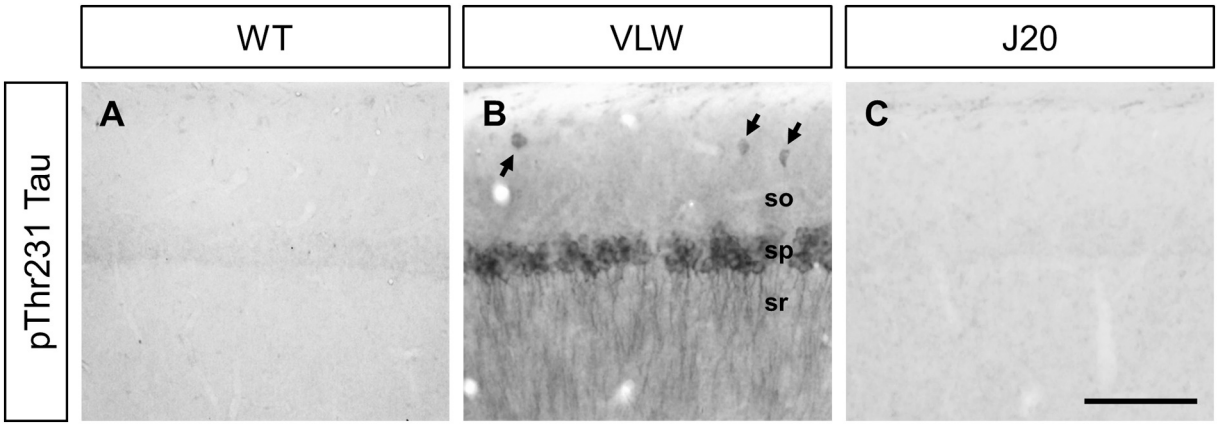

Fig. 1. pThr231 Tau is present in pyramidal neurons and hippocampal interneurons exclusively in VLW mice. Immunodetection of pThr231 Tau-immunopositive cells in hippocampal sections from 8 mo WT, VLW and J20 mice. Tau protein phosphorylated at residue Thr231 detected with AT-180 antibody is not present in WT (A) and J20 (C) hippocampi, whereas VLW mice (B) display pThr231 Tau-positive staining in the sp. and in several hippocampal neurons (arrows). Abbreviations: so, *stratum oriens*; sp., *stratum pyramidale*; sr, *stratum radiatum*. Scale bar: 100 µm.

S262 or T205 and PV, Calbindin (CB) or Calretinin (CR) primary antibodies. First, brain sections were blocked for 2 h and incubated overnight simultaneously with goat anti-PV (1/3000), goat anti-CR (1/3000) or mouse anti-CB (Swant®; 1/3000) antibodies and S262 or T205. They were then incubated with Alexa Fluor 568 donkey anti-goat or anti-mouse IgG against interneuron primary antibodies, whereas P-Tau primary antibodies were targeted with Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen™; 1/1000), for 2 h. Finally, brain sections were mounted onto slides and coverslipped with Mowiol® (Merck).

To assess P-Tau induction, double fluorescent immunostainings using AT-180 or HT7 antibodies combined with anti-PV antibody were conducted.

Some sections were stained with T205 together with AT-180 antibodies to detect the presence of both P-Tau forms in a single cell.

2.4. Image acquisition

Optical microscopy (Nikon E600, Nikon Corporation) observations were focused on the immunohistochemically stained brain sections. Images were acquired through a digital camera (Olympus DP72, Olympus Corporation) coupled to the microscope and were processed by Cell F^ software (Olympus Corporation).

Confocal microscopy (Leica TCS SP5, Leica Microsystems) observations were performed to acquire images from the immunofluorescence-stained samples, using LAS AF software (Leica Microsystems). The images were then processed by Fiji software (Schindelin et al., 2012) to observe possible colocalization between the interneurons and P-Tau markers.

2.5. Analysis of histological sections

To estimate the density of interneurons accumulating pSer262 or pThr205 Tau, the samples stained with S262 or T205 antibodies were scanned with a NanoZoomer 2.0HT whole slide imager (Hamamatsu Photonics) at 20×. The density of pSer262 and pThr205 Tau-immunopositive interneurons was quantified in various regions and layers of the hippocampal area (CA1, *stratum oriens* (so), *stratum radiatum* (sr), *stratum lacunosum moleculare* (slm); CA3, so, sr; and DG, *stratum moleculare* (sm), *stratum granulare* (sg), hilus) of each section (4 animals/genotype, 3–4 sections/animal). The cells and area comprising each hippocampal region were quantified using Fiji software. The density of immunopositive hippocampal cells was defined as the density of cells per square millimeter.

To assess the percentage of Aβ plaque load in each hippocampal region, the samples stained with 3D6 antibody were scanned with a NanoZoomer 2.0HT whole slide imager at 20×. Later, the Trainable Weka Segmentation plugin from Fiji software was applied to the images by using a set of machine learning algorithms with a collection of image features selected by the user to produce pixel-based segmentations. All images were processed using a macro provided by Sebastién Tosi (Institute for Research in Biomedicine, Barcelona) to identify and quantify the amount of plaque present in a given hippocampal section (4 animals, 4 sections/animal).

2.6. Statistical analysis of histological data

Histological data were processed for statistical analysis with GraphPad Prism 6 (GraphPad Software Incorporated). To assess differences between the experimental groups, overall comparisons, namely one-way ANOVA and Kruskal-Wallis tests, were used when the samples fitted a normal distribution or not, respectively. To determine correlation between Aβ plaque load and density of pThr205 Tau-positive cells, the Pearson's *r* test was used. Significance was set at $p < .05$: $^{*}p < .05$, $^{**}p < .01$, $^{****}p < .0001$. Statistical values are presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Tau phosphorylated at residue Thr231 is present in the soma of hippocampal interneurons specifically in VLW mice

To analyze the pattern of Tau phosphorylation in the hippocampus in control and pathological conditions, we first performed immunodetection of pThr231 Tau on hippocampal sections from 8 mo WT mice, VLW mice, which accumulate hyperphosphorylated forms of Tau, and J20 animals, which accumulate Aβ. As previously described (Lim et al., 2001; Soler et al., 2017), VLW mice showed intense immunostaining in the soma and apical dendrites of pyramidal neurons in CA1-3. In addition, as we previously described (Soler et al., 2017), the soma of some hippocampal interneurons located mainly in the so also accumulated pThr231 Tau (Fig. 1B). In contrast, WT and J20 mice did not show pThr231-positive cells in any region of the hippocampus (Fig. 1A, C). These data suggest that the overexpression of mutated hTau in VLW mice has a direct effect on the accumulation of pThr231 Tau in hippocampal interneurons, absent in control mice and in J20 animals.

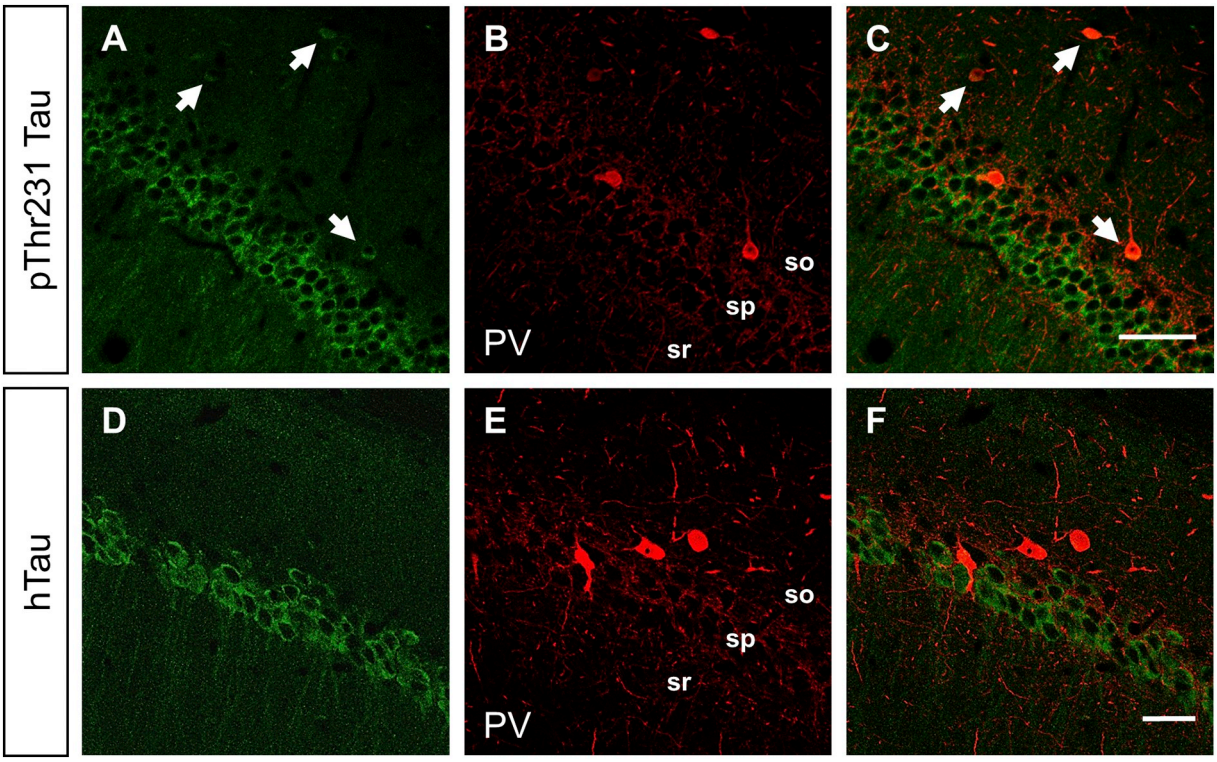

Fig. 2. Human P-Tau accumulated in pyramidal neurons induces murine pThr231 Tau in PV-positive hippocampal interneurons in VLW mice. Double immunofluorescent detection of P-Tau or hTau and interneuron marker PV in the CA1 region in hippocampal sections from 8 mo VLW mice. (A) Accumulation of Tau phosphorylated at residue Thr231 in hippocampal cells, detected with AT-180 antibody (arrows). (B and E) Detection of PV-positive hippocampal interneurons. (C) Colocalization of pThr231 Tau with PV-positive interneurons (arrows). (D) Expression of hTau in hippocampal cells, detected with HT7 antibody. (F) No colocalization of hTau and PV-positive interneurons. Abbreviations: PV, Parvalbumin; so, *stratum oriens*; sp., *stratum pyramidale*; sr, *stratum radiatum*. Scale bars: in C, 50 µm (applies to A-C); in F, 25 µm (applies to D-F).

3.2. Accumulation of pThr231 hTau in pyramidal cells induces pThr231 murine Tau in PV-positive hippocampal interneurons in VLW mice

VLW mice overexpress mutated hTau under the Thy-1 promoter, which leads to the accumulation of P-Tau mainly in pyramidal neurons in the hippocampus (Lim et al., 2001). Moreover, we previously demonstrated an accumulation of pThr231 Tau in PV-positive hippocampal interneurons in VLW mice (Soler et al., 2017).

To determine whether the presence pThr231 Tau in hippocampal interneurons in VLW mice is due to an overexpression of mutated hTau in these cells or to intercellular P-Tau induction, we first detected specifically hTau by the use of HT7 antibody in WT, VLW and J20 hippocampi. Our data confirmed that WT and J20 mice did not accumulate hTau (Supplementary Fig. 1A-B, E-F), and indicated that only pyramidal neurons in CA1–3 expressed hTau in VLW animals (Supplementary Fig. 1C, D). No signal was observed in neurons outside the pyramidal cell layer, indicating that hippocampal interneurons did not express hTau in VLW mice. In order to confirm this observation, we performed double fluorescent immunodetection on hippocampal sections from 8 mo VLW mice. Using an anti-PV antibody to detect hippocampal interneurons combined with anti-pThr231 Tau or with HT7, which specifically detects hTau, we found that PV-positive interneurons accumulated pThr231 Tau but not hTau (Fig. 2A–F). These observations indicate that the accumulation of mutated hTau in pyramidal neurons induces the phosphorylation of endogenous Tau at residue Thr231 in PV-positive interneurons in VLW mice, thereby demonstrating an intercellular spread of P-Tau *in vivo*.

3.3. Hippocampal interneurons accumulate P-Tau at residues Ser262 and Thr205 in their soma in non-pathological conditions

Tau hyperphosphorylation is associated with AD and other neurodegenerative conditions (Iqbal et al., 2016; Khan and Bloom, 2016); however, the physiological functions of Tau are also regulated by phosphorylation. In fact, Tau phosphorylation at different sites could have different outcomes for each Tau function (Avila, 2009; Medina et al., 2016). Some phosphorylated Tau residues are related to neurodegenerative processes in humans, but their importance in non-pathological conditions is not clear. To analyze the phosphorylation of Tau in physiological conditions, we performed immunohistochemistry against pSer262 and pThr205 Tau on hippocampal sections from 8 mo WT mice. Our results revealed that some hippocampal neurons located in the so and sr of CA1-3 (Fig. 3A, B) and also scattered in the sg and in the hilus of the dentate gyrus (DG, Fig. 3C) showed pSer262 Tau-immunopositive staining in their soma. In addition, some pThr205 Tau-positive cells were also present in the so and sr of CA1-3, and dispersed in the sg, sm and in the hilus of the DG (Fig. 3D-F). The distribution and morphology of these cells appeared to correspond to hippocampal interneurons. Using double immunodetection of pSer262 and pThr205 Tau with hippocampal GABAergic neuron markers such as PV, CB and CR, we show that the pSer262 and pThr205 Tau-positive cells correspond to GABAergic hippocampal cells containing PV, CB and CR (Fig. 4A-I). We quantified the presence of P-Tau forms in PV-immunopositive cells, the main population of GABAergic hippocampal cells. In the PV-positive cell population, 62% of the cells accumulated pSer262 Tau and 58% of them presented pThr205 Tau. These data demonstrate that pSer262 and pThr205 Tau accumulate in the soma of hippocampal interneurons in non-pathological conditions.

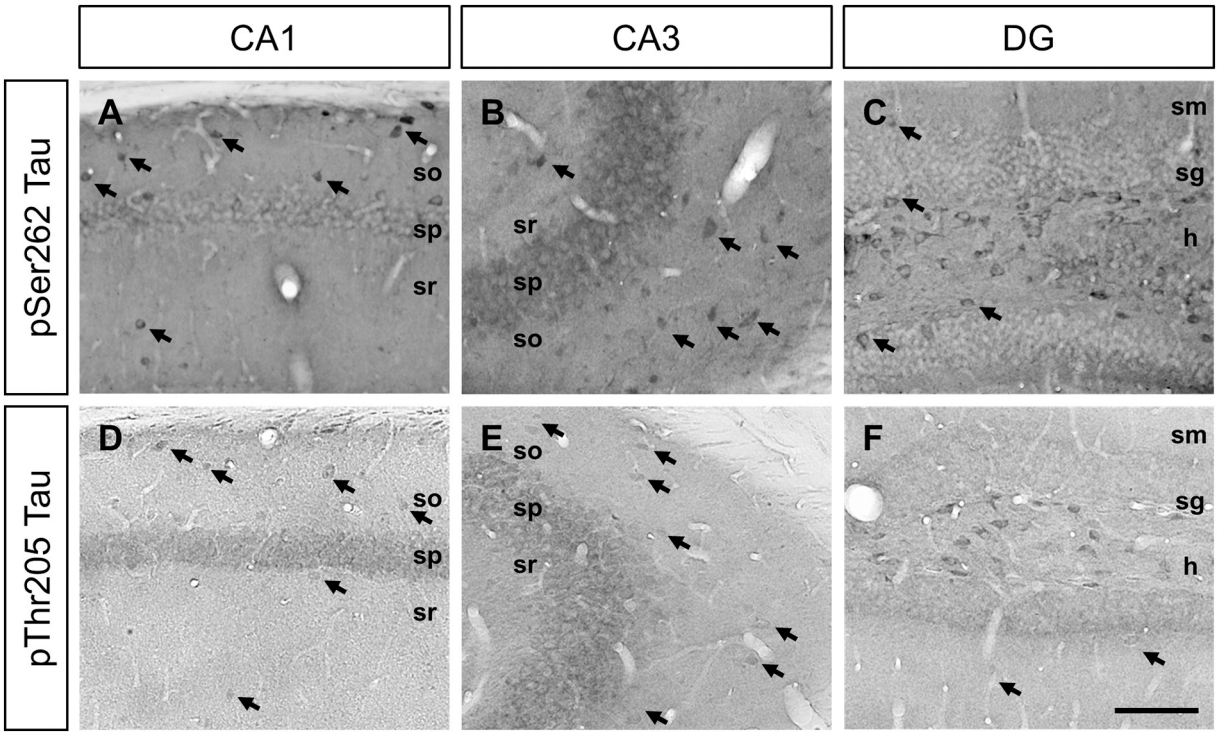

Fig. 3. Distribution pattern of pSer262 and pThr205 Tau-positive cells in WT mice. Immunodetection of pSer262 and pThr205 Tau-immunopositive cells in hippocampal sections from 8 mo WT mice. (A-C) The soma of hippocampal cells in the CA1 (A), CA3 (B) and DG (C) regions accumulate pSer262 Tau. (D-F) The soma of hippocampal cells in the CA1 (D), CA3 (E) and DG (F) regions accumulate pThr205 Tau. Abbreviations: h, hilus; sg, *stratum granulare*; sm, *stratum moleculare*; so, *stratum oriens*; sp., *stratum pyramidale*; sr, *stratum radiatum*. Scale bar: 100 μm.

3.4. The distribution and density of pSer262 Tau-positive hippocampal interneurons are similar in WT, VLW and J20 mice

To study whether pSer262 Tau accumulation in the soma of hippocampal interneurons is also present in pathological conditions, we next compared pSer262 Tau immunodetection on hippocampal sections from WT animals with those of VLW and J20 mice. Like the hippocampi of WT animals, those of VLW and J20 mice showed some scattered neurons in the so and sr of CA1-3 (Fig. 5B-C, F-G), and in the hilus, sg and sm of the DG (Fig. 5J-K). Therefore, we studied the density of pSer262 Tau-positive cells by comparing WT mice with VLW and J20 animals. No differences in the density of interneurons accumulating pSer262 Tau in the hippocampus were detected between the three animal models. Neither were differences observed when CA1, CA3 and the DG were analyzed separately (Fig. 5D, H, L).

Thus, our data show that there are no differences in the distribution pattern of pSer262 Tau or in the density of cells accumulating this P-Tau form in the hippocampus between normal and pathological conditions. These results indicate that pSer262 accumulation in the soma of hippocampal interneurons is independent of the Aβ or P-Tau present in the hippocampus.

3.5. The density of pThr205 Tau-immunopositive hippocampal interneurons decreases in VLW mice

Next, we compared pThr205 Tau accumulation in the hippocampus of VLW and WT mice. In contrast to data on pSer262 Tau, differences were detected in the distribution of pThr205 Tau-positive cells between the normal and the pathological models. In this regard, there was a clear accumulation of pThr205 Tau in VLW pyramidal cells while this was not observed in WT animals (Fig. 6A, B). VLW mice overexpress mutated hTau mostly in these cells, thus accumulating P-Tau in the pyramidal layer (Lim et al., 2001; Soler et al., 2017).

In addition to pyramidal cells, some mossy cells and several interneurons, mainly in the so and sr, accumulated pThr205 Tau in the hippocampus of VLW mice (Fig. 6A-B, E-F, I-J). We then analyzed the number of pThr205 Tau-positive cells in distinct hippocampal regions. The density of pThr205 Tau-positive cells in VLW mice was lower than in age-matched WT mice, mainly in CA1 and CA3 regions (45% and 47% of reduction respectively, Fig. 6D, H, L). Nevertheless, regarding the density of pThr205 Tau-positive cells in the DG, no differences were found between the WT and VLW mice (Fig. 6L; 112.5 ± 12.6 and 87.6 ± 9.4, respectively).

Our previous data (Soler et al., 2017) demonstrated that some PV-positive interneurons, the most abundant subtype of hippocampal interneurons (Freund and Buzsaki, 1996; Matyas et al., 2004), accumulated pThr231 Tau in VLW mice (Soler et al., 2017). It has been described that Tau phosphorylation at specific residues depends on phosphorylation at other Tau epitopes (Ando et al., 2016). We therefore examined whether the decrease in Thr205 Tau phosphorylation in VLW mouse interneurons was due to the presence of pThr231 Tau in PV-positive interneurons. By means of double immunodetection using the AT-180 antibody against pThr231, and the T205 antibody against pThr205 (Fig. 7), we demonstrated colocalization between T205 and AT-180 staining in some hippocampal interneurons located mainly in the so, but also in pyramidal neurons (Fig. 7A-C). Therefore, some neurons in VLW mice accumulated pThr231 and pThr205 Tau simultaneously. This observation thus suggests that pThr231 Tau does not repress the phosphorylation of Tau at residue Thr205.

3.6. The density of pThr205 Tau-immunopositive hippocampal interneurons increases in the presence of Aβ accumulation in J20 animals

Analysis of the hippocampi of J20 mice subsequently demonstrated the presence of intensely immunostained pThr205 Tau-positive cells in the so and sr of the CA1-3 and in the hilus and sm of the DG (Fig. 6C, G, K). Comparison of the number of pThr205 Tau-positive interneurons in J20 and WT animals showed an almost 1.5-fold increase in the density

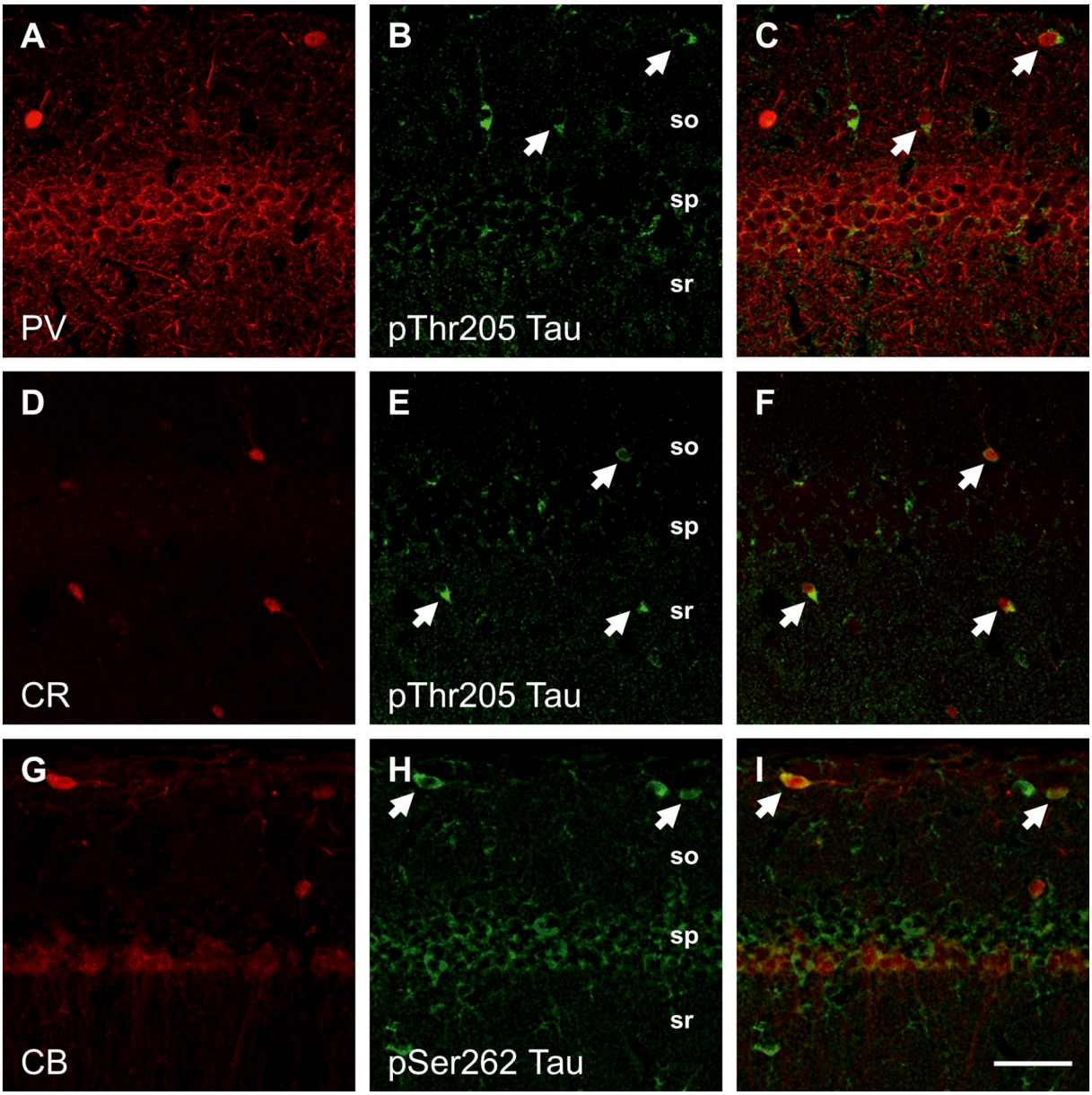

Fig. 4. WT mice accumulate pSer262 and pThr205 Tau in PV-, CR- and CB-positive hippocampal interneurons. Double immunofluorescent detection of pSer262 or pThr205 Tau and interneuron markers PV, CR and CB in hippocampal sections from 8 mo WT mice. (A-F) PV-, CR- and CB-positive hippocampal interneurons (red) accumulate Tau phosphorylated at residue Thr205 (green) in 8 mo WT mice. Colocalization of pThr205 Tau with PV- (arrows in C) and CR-positive (arrows in F) interneurons. (G-I) PV-, CR- and CB-positive hippocampal interneurons (red) accumulate Tau phosphorylated at residue Ser262 (green) in 8 mo WT mice. Colocalization of pSer262 Tau with CB-positive (arrows in I) interneurons. Abbreviations: CB, Calbindin; CR, Calretinin; PV, Parvalbumin; so, *stratum oriens*; sp., *stratum pyramidale*; sr, *stratum radiatum*. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of these cells in the so of the CA1 (Fig. 6D; WT: 263.7 ± 7.6 and J20: 393.7 ± 39.3; $p = .0104$) and a 2-fold increase in the DG (Fig. 6L; WT: 112.5 ± 12.6 and J20: 224.5 ± 35.0; $p = .0203$) of J20 mice. No differences were found in the sr + slm of the CA1 or in the CA3 when compared with WT data (Fig. 6D, H). Furthermore, neither were differences in the density of pThr205 Tau-positive cells detected in the hilus (WT: 606.5 ± 77.1 and J20: 833.8 ± 139.1; $p = .194$). Given that mossy cells are the most abundant population in this region, the differences observed in the DG appear to correspond mainly to an increase in the density of pThr205 Tau-positive interneurons.

A number of studies have demonstrated how Aβ influences Tau hyperphosphorylation (Forner et al., 2017; Götz et al., 2001; Ittner and Götz, 2011). Given that the increase in the density of pThr205 Tau-positive cells in J20 mice with respect to WT animals was not homogeneous in all hippocampal regions (Fig. 6D, L), the percentage of increase was calculated in CA1, CA3 and DG independently. Considering that the so showed statistical differences only in the CA1 (Fig. 6D), the distinct strata of this region were studied separately. Comparison of J20 mice with control animals revealed that the CA1 so (33.0 ± 1.9%) and the DG (45.4 ± 5.6%) displayed the highest percentage of increase in the density of pThr205 Tau-positive cells, while the CA1 sr, together with the CA1 slm (sr + slm) and the CA3, showed only a slight increase (7.1 ± 3.1% and 5.5 ± 3.9%, respectively). Statistical analysis showed a significant increase in the so of the CA1 and in the DG compared with the sr + slm of the CA1 and with the CA3 (Fig. 8A). These data indicate a differential increase in the density of pThr205 Tau-positive cells in distinct hippocampal regions.

To determine whether these specific increases in the DG and so of

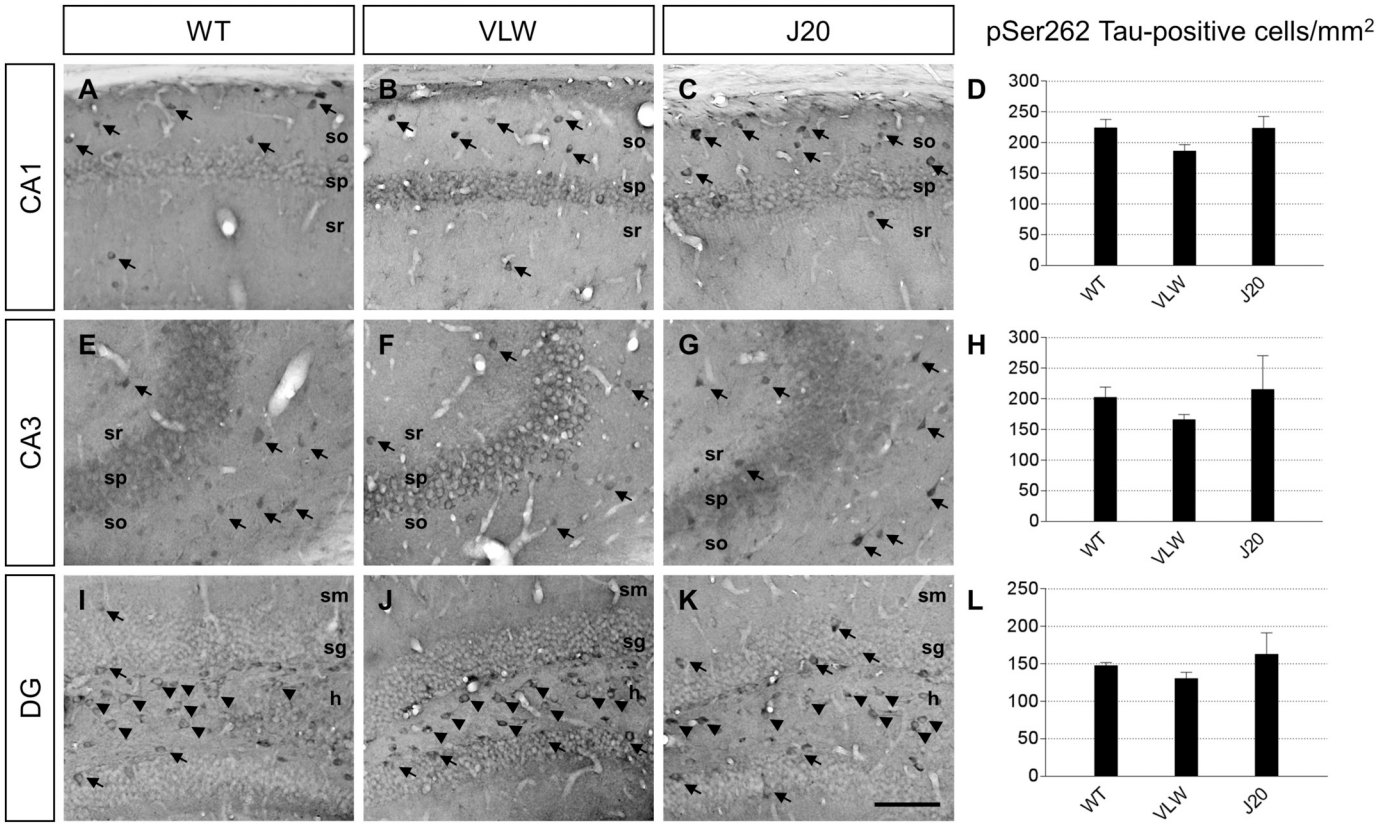

Fig. 5. Distribution pattern and cell density quantification of pSer262 Tau-positive cells in WT, VLW and J20 mice. Immunodetection and cell density quantification of pSer262 Tau-immunopositive cells in hippocampal sections from 8 mo WT, VLW and J20 mice. (A-C, E-G and I-K) pSer262 Tau accumulation in hippocampal interneurons (arrows) located in different areas of the CA1 (A-C), CA3 (*E*-G) and DG (I-K) regions, and in mossy cells (arrowheads) of the DG (I-K), noting a stronger immunostaining in the cells located in the so of J20 mice (C) compared to WT mice (A). (D, H and L) Density quantification of immunopositive interneurons in each region, cell densities being generally higher in WT and J20 mice compared to VLW mice. For (D), (H) and (L): one-way ANOVA. $n = 4$ animals/genotype, 3–4 sections/animal. Error bars represent SEM. Abbreviations: h, hilus; sg, *stratum granulare*; sm, *stratum moleculare*; so, *stratum oriens*; sp., *stratum pyramidale*; sr, *stratum radiatum*. Scale bar: 100 µm.

the CA1 region correlate with the presence of Aβ plaques, we performed 3D6 immunostaining. As previously described (Furcila et al., 2018), Aβ plaques accumulated in the CA1 and DG regions (Fig. 8C), accounting for 35% and 47% of the Aβ in the hippocampus (Fig. 8B). Our results showed a high degree of correlation between the increase in pThr205 Tau-positive cells and the presence of Aβ plaques (Fig. 8D).

Taken together, these results demonstrate that the regions that showed an increase in cells accumulating pThr205 Tau correlated with those with a higher Aβ plaque load, thereby pointing to an inductive effect of Aβ in the phosphorylation of Tau at residue Thr205.

3.7. The soma of PV-, CR- and CB-positive hippocampal interneurons accumulate pSer262 and pThr205 Tau in VLW and J20 mice

Our data established that pSer262 and pThr205 Tau were present in the soma of some neurons scattered throughout the hippocampus in normal and pathological conditions. To identify the hippocampal interneuron populations that accumulated both P-Tau forms, we performed a double immunodetection using PV, CB or CR interneuron markers and S262 or T205 antibodies in brain sections from VLW and J20 mice. Our results revealed colocalization between the hippocampal interneuron markers PV, CR and CB and the S262 (Fig. 9D-F, G-I) or T205 (Fig. 9A-C, J-L) antibody signal.

These findings therefore indicate that both pSer262 and pThr205 Tau accumulate in hippocampal PV-, CR- and CB-positive interneurons in VLW and J20 mice, as previously observed in WT animals.

3.8. pSer262 and pThr205 Tau accumulate in human hippocampal interneurons in normal and pathological conditions

Our data demonstrated the presence of pSer262 and pThr205 Tau in the soma of hippocampal interneurons in both WT animals and pathological mouse models of AD. We therefore addressed whether human hippocampal interneurons also accumulate pSer262 Tau and pThr205 Tau. To this end, we analyzed human hippocampal sections from two control subjects and from two AD patients (Braak IV-V). Using immunohistochemistry against pSer262 and pThr205 Tau, we demonstrated that several human hippocampal neurons showed an immunopositive signal for pSer262 (Fig. 10A, B) or pThr205 Tau (Fig. 10C, D) in normal and pathological conditions. The pSer262 and pThr205 Tau-positive cells were large multipolar neurons located outside the pyramidal and granular cell layers, mainly in the sr and so. The location and morphological characteristics of cells accumulating pSer262 or pThr205 Tau seemed to correspond to interneurons. Although the staining in hippocampal interneurons differed from the NFTs observed in pyramidal neurons in AD samples, these P-Tau forms had different cellular distributions. The pSer262 Tau staining was spread evenly within the entire neuron and was higher in AD samples. In contrast, the pThr205 Tau staining was homogeneously distributed, but it also formed somatic clusters both in control and AD samples.

On the basis of our findings, we conclude that pSer262 and pThr205 Tau accumulate in hippocampal interneurons in mice and human subjects in normal and pathological conditions.

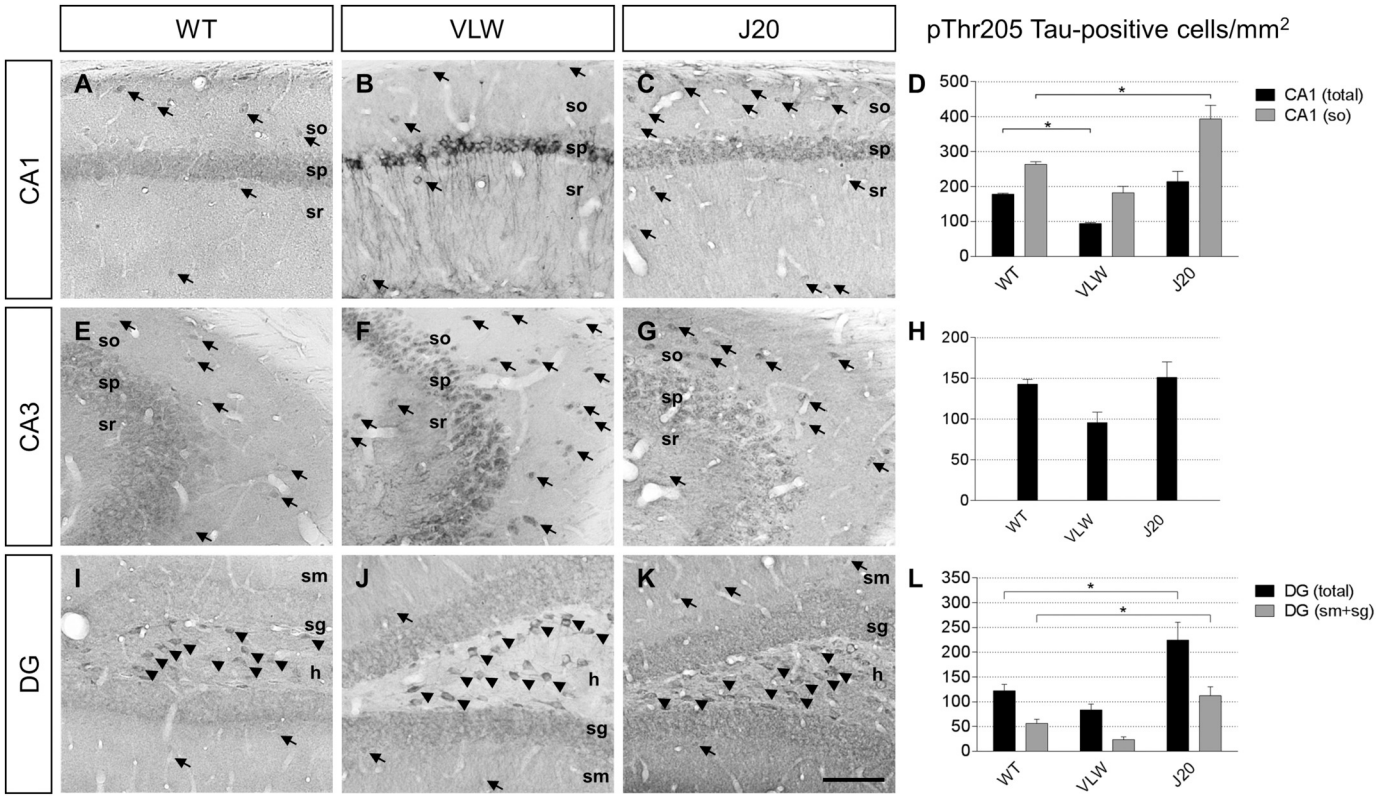

Fig. 6. Distribution pattern and cell density quantification of pThr205 Tau-positive cells in WT, VLW and J20 mice. Immunodetection and cell density quantification of pThr205 Tau-immunopositive cells in hippocampal sections from 8 mo WT, VLW and J20 mice. (A-C, E-G and I-K) pThr205 Tau accumulation in hippocampal interneurons (arrows) located in different areas of the CA1 (A-C), CA3 (E-G) and DG (I-K) regions, and in mossy cells (arrowheads) of the DG (I-K), noting a stronger immunostaining in the sp of VLW mice (B) compared to WT mice (A). (D, H and L) Density quantification of immunopositive interneurons in each region, cell densities being generally higher in WT and J20 mice compared to VLW mice. For (D), (H) and (L): one-way ANOVA, $*p < .05$. $n = 4$ animals/genotype, 3–4 sections/animal. Error bars represent SEM. Abbreviations: h, hilus; sg, *stratum granulare*; sm, *stratum moleculare*; so, *stratum oriens*; sp., *stratum pyramidale*; sr, *stratum radiatum*. Scale bar: 100 µm.

4. Discussion

4.1. pThr231 hTau in pyramidal cells induces pThr231 murine Tau in PV-positive hippocampal interneurons specifically in VLW mice

Our previous studies (Soler et al., 2017) demonstrated that hippocampal neuron populations accumulate distinct forms of P-Tau in VLW mice, an animal model that overexpresses mutated hTau. This model shows an accumulation of pThr231 Tau in pyramidal cells, mossy cells and PV-positive interneurons. Our present data indicate that only pyramidal neurons in VLW mice express hTau; in addition, we show that the pThr231 Tau-positive signal is absent in WT hippocampi, thereby suggesting a specific effect of P-Tau accumulation on the phosphorylation of Tau in hippocampal interneurons.

P-Tau aggregates spread and replicate in a prion-like manner. It has been shown, mainly in cell culture experiments, that the uptake of pathological Tau seeds causes hyperphosphorylation, misfolding and aggregation of monomeric Tau in recipient cells (Clavaguera et al.,

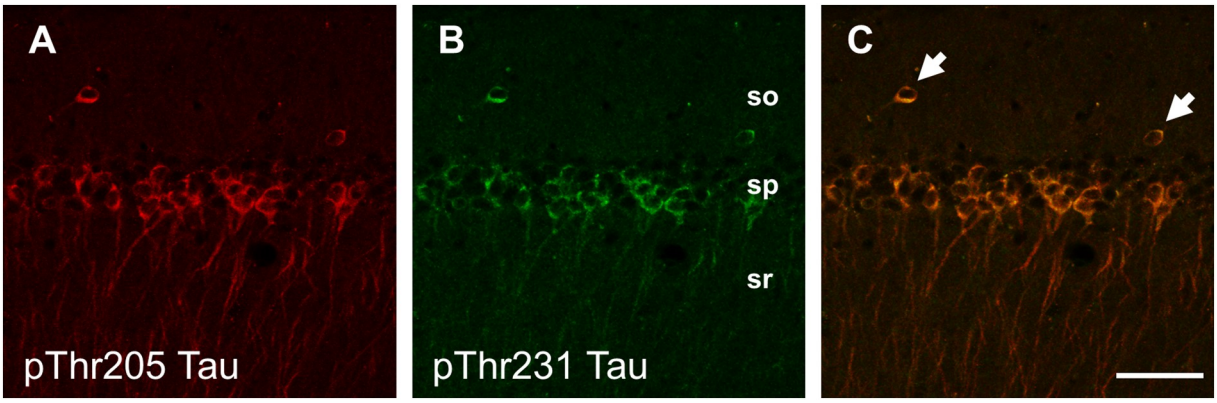

Fig. 7. Hippocampal interneurons and pyramidal cells accumulate both pThr231 and pThr205 Tau in 8 mo VLW mice. Double immunofluorescent detection of pThr231 and pThr205 Tau in 8 mo VLW mice showed hippocampal interneurons located in the *stratum oriens* (so) and pyramidal neurons accumulating Tau phosphorylated at residue Thr205 (red, A) and pThr231 (green, B). Colocalization of pThr205 with pThr231 Tau (arrows in C) in hippocampal interneurons. Scale bar: 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

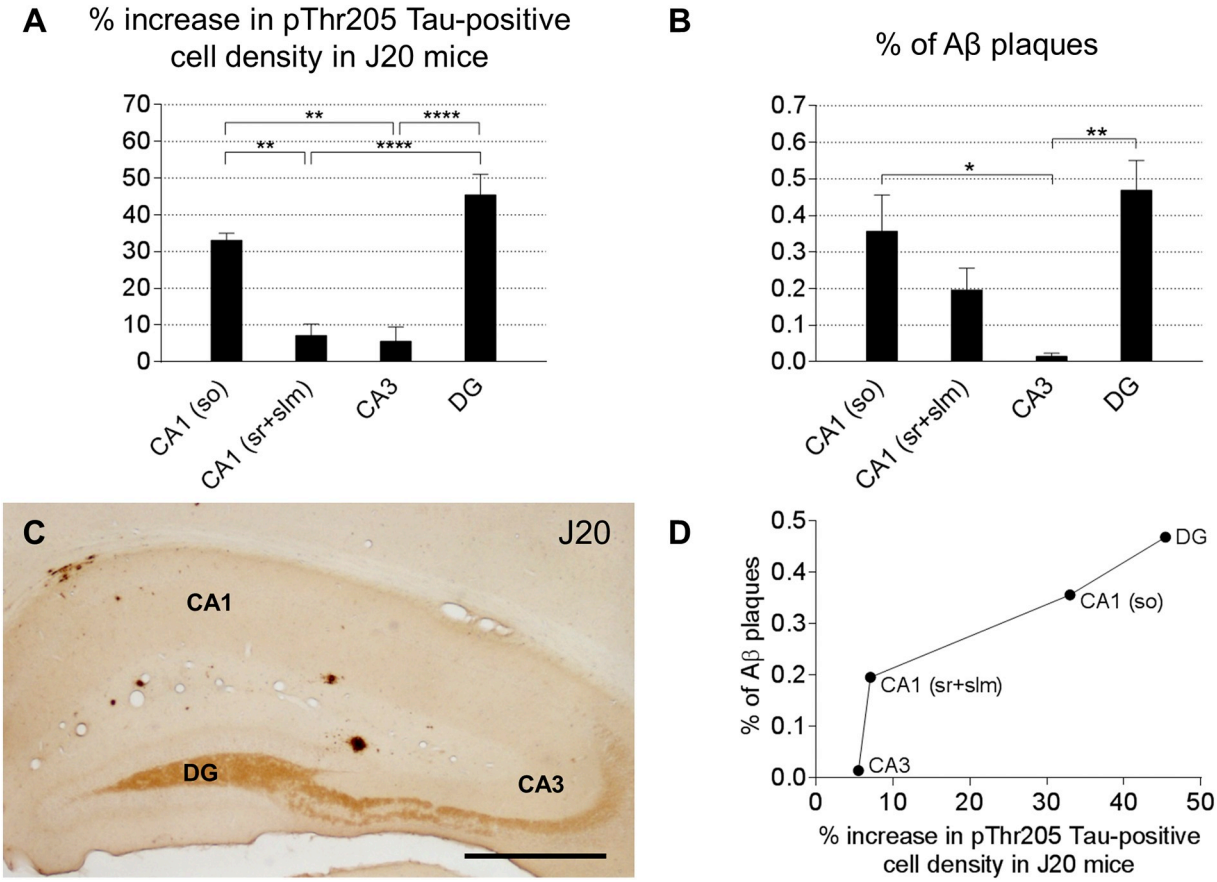

Fig. 8. Increases in the density of pThr205 Tau-positive cells and correlation to the distribution pattern of Aβ plaques in J20 mice. Subfield-specific increases in density of pThr205 Tau-positive cells and Aβ plaque load in hippocampal sections from 8 mo J20 mice. (A) Quantification of the percentage increase in the density of pThr205 Tau-positive cells, compared to WT mice, in distinct hippocampal subfields. (B) Quantification of the percentage of Aβ plaques in distinct hippocampal subfields. (C) Aβ accumulation in different areas of the hippocampus, detected with the 3D6 antibody, displaying the presence of higher plaque loads in the so of CA1 and in the DG. (D) Correlation analysis between percentages of Aβ plaques and increases in the density of pThr205 Tau-positive cells in different areas of the hippocampus. For (A) and (B): one-way ANOVA, $*p < .05$, $**p < .01$, $****p < .0001$. For (D): Pearson's $r = 0{,}989$. $n = 4$ animals/genotype, 4 sections/animal. Error bars represent SEM. Abbreviations: slm, *stratum lacunosum moleculare*; so, *stratum oriens*; sr, *stratum radiatum*. Scale bar: 500 μm.

2009; Polanco et al., 2016; Wang et al., 2017). Here we describe *in vivo* that overexpression of mutated hTau in pyramidal neurons induces the phosphorylation of murine Tau at residue Thr231 in PV-positive hippocampal interneurons.

4.2. Hippocampal interneurons and mossy cells accumulate pSer262 Tau in control and pathological conditions

Our results show that pSer262 Tau accumulates in the soma of PV-, CR- and CB-positive interneurons and mossy cells in WT, VLW and J20 mice. Nevertheless, Tau is normally located in the axon, although lower levels are also present in the somatodendritic compartment and nucleus (Avila et al., 2004; Hernández and Avila, 2007). Many studies have reported the development of non-typical functions of Tau protein alongside the dendritic spines or even in the nucleus. These functions include maintenance of the integrity of genomic DNA, and cytoplasmic and nuclear RNA, as well as the regulation of NMDA receptors and synaptic plasticity (Guo et al., 2017; Sotiropoulos et al., 2017).

In addition, pSer262 Tau has been described as pathological and used as a diagnostic marker for AD (Braak et al., 2011; Šimić et al., 2016; Wang and Mandelkow, 2016). Several studies have reported the capacity of Aβ to induce Tau hyperphosphorylation (Bolmont et al., 2007; Götz et al., 2001; Stancu et al., 2014), as well as the relevance of the same P-Tau forms in promoting the same effect on other Tau molecules, hence increasing the P-Tau burden (Šimić et al., 2016). Given that our data suggest that the phosphorylation of residue Ser262 is not influenced by the presence of hyperphosphorylated forms of Tau in VLW animals or by the accumulation of Aβ in J20 mice, it would appear that pSer262 Tau in the soma of hippocampal interneurons and mossy cells has a specific function that is independent of AD pathology.

On the basis of our results, we propose that Tau shows distinct patterns of phosphorylation in hippocampal interneurons and mossy cells, possibly in order to exert functions other than those performed exclusively in the axon. Our data reinforce the notion that, despite being considered pathological (Guo et al., 2017), pSer262 Tau is not induced by the accumulation of P-Tau or Aβ.

4.3. The presence of P-Tau and Aβ influences the accumulation of pThr205 Tau in hippocampal interneurons

Our results show that the soma of PV-, CR- and CB-positive interneurons and mossy cells accumulate pThr205 Tau in WT, VLW and J20 mice. These data point to a physiological role of pThr205 Tau in the somatic region of hippocampal interneurons and mossy cells. In addition, our findings reveal a significant decrease in the density of pThr205 Tau-positive cells in the CA1 and CA3 regions of VLW mice. As previously described, pThr231 Tau accumulates only in PV-positive cells in the hippocampal interneuron population (Soler et al., 2017). Likewise, other studies have stated that the phosphorylation of specific residues may influence—by inducing or repressing—phosphorylation in other regions or domains of Tau (Ando et al., 2016). Our results reveal that pThr231 and pThr205 Tau coexist in a single cell, thereby suggesting

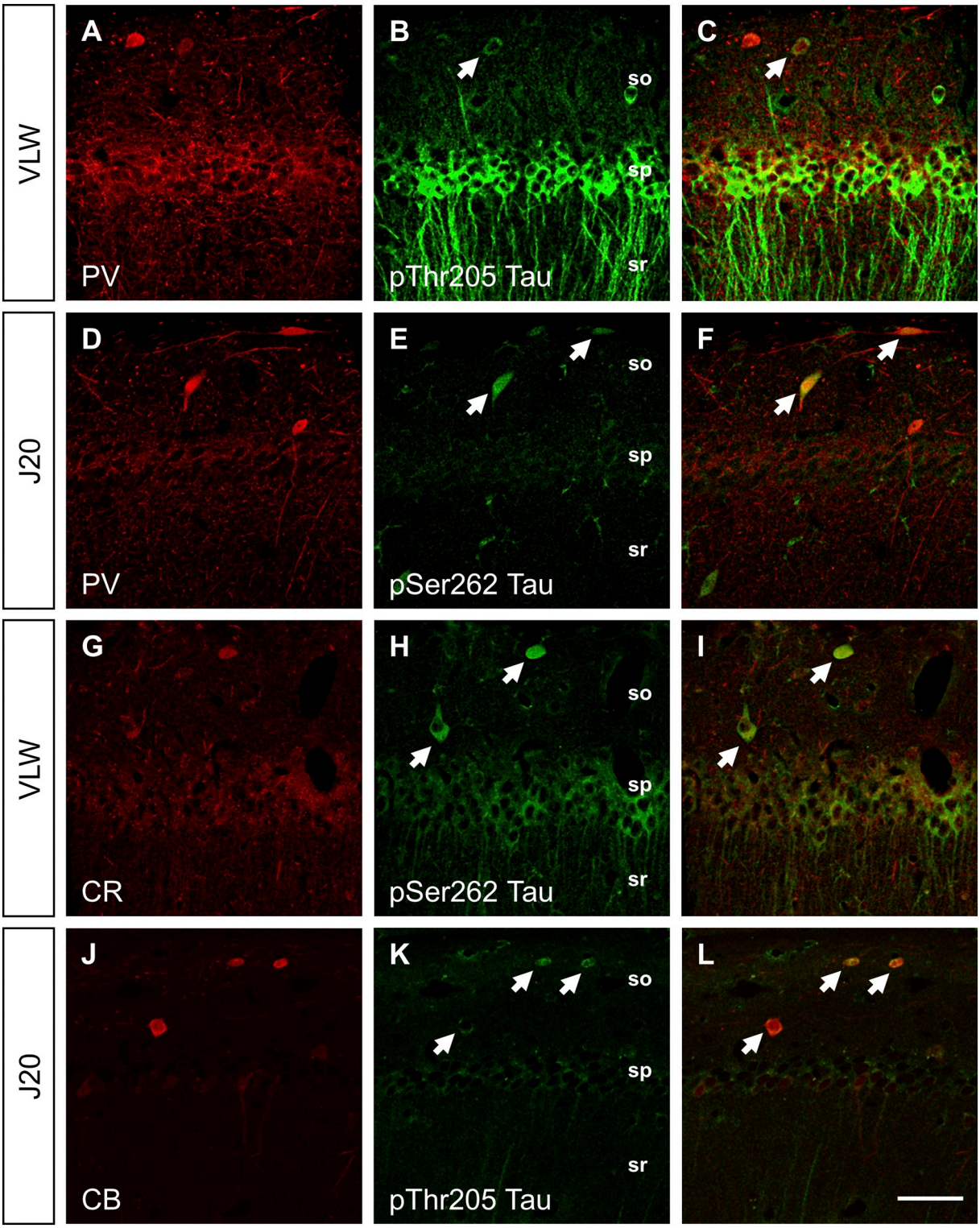

Fig. 9. VLW and J20 mice accumulate pSer262 and pThr205 Tau in PV-, CR- and CB-positive hippocampal interneurons. Double immunofluorescent detection of pSer262 or pThr205 Tau and interneuron markers PV, CR and CB in hippocampal sections from 8 mo VLW and J20 mice. (A-C and J-L) PV-, CR- and CB-positive hippocampal interneurons (red) accumulate Tau phosphorylated at residue Thr205 (green). Colocalization of pThr205 Tau with PV- (arrows in C) and CB-positive (arrows in L) interneurons. (D-I) PV-, CR- and CB-positive hippocampal interneurons (red) accumulate Tau phosphorylated at residue Ser262 (green). Colocalization of pSer262 Tau with PV- (arrows in F) and CR-positive (arrows in I) interneurons. Abbreviations: CB, Calbindin; CR, Calretinin; PV, Parvalbumin; so, *stratum oriens*; sp., *stratum pyramidale*; sr, *stratum radiatum*. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

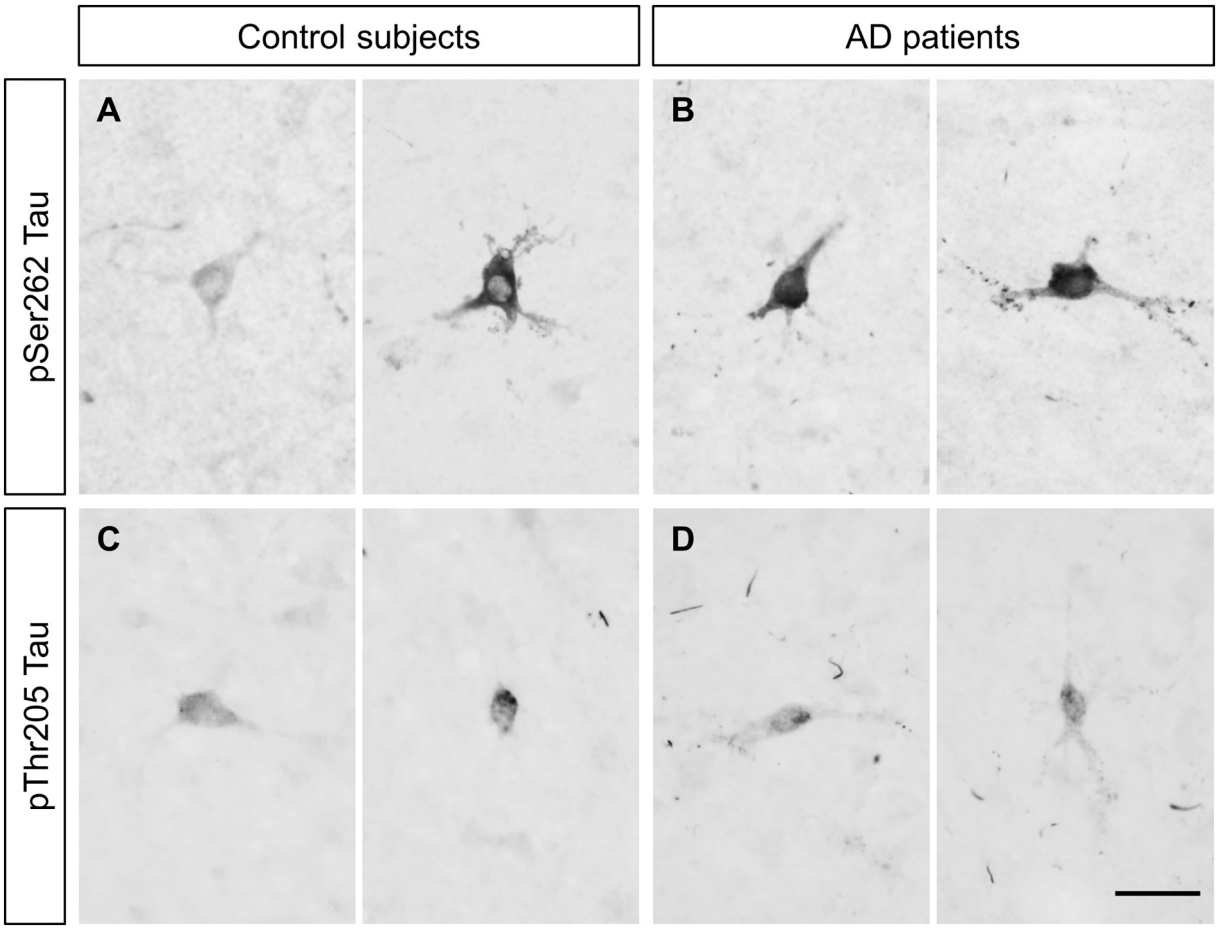

Fig. 10. pSer262 and pThr205 Tau accumulate in the soma of hippocampal interneurons in non-pathological and AD human subjects. Immunodetection of pSer262 or pThr205 Tau in the sr and slm in hippocampal sections from non-pathological and AD human subjects. (A and C) The soma of interneurons in non-pathological hippocampi accumulate pSer262 (A) and pThr205 (C) Tau. (B and D) The soma of hippocampal interneurons of AD patients accumulate pSer262 (B) and pThr205 (D) Tau. Scale bar: 50 μm.

that the former does not suppress the latter.

Our present data demonstrate that hTau expressed in pyramidal cells in VLW mice induces the phosphorylation of murine Tau at residue Thr231 in PV-positive interneurons. Therefore, we cannot discard the possibility that hyperphosphorylated hTau accumulated in the pyramidal cells of VLW mice represses the phosphorylation of murine Tau at residue Thr205 in hippocampal interneurons, thereby reducing the density of pThr205 Tau-positive interneurons.

In addition, many Tau residues, such as Thr205, are dependent on Glycogen Synthase Kinase 3 (GSK3) (Guo et al., 2017), a kinase that requires the previous (primed) phosphorylation of other Ser/Thr sites on its substrates, following a certain pattern, to exert its function (Medina et al., 2011; Sutherland, 2011). Hence, it cannot be discarded that VLW mice, a model of Tau hyperphosphorylation, show altered priming patterns of Tau phosphorylation, thus impeding GSK3 from correctly phosphorylating Tau at residue Thr205.

In contrast to the results obtained in VLW mice, J20 animals showed a significant increase in the density of cells accumulating pThr205 Tau in the CA1 so and DG with respect to WT mice.

Aβ induces Tau hyperphosphorylation (Bolmont et al., 2007; Götz et al., 2001; Stancu et al., 2014). Our data indicate that the regions that show an increase in the number of cells accumulating pThr205 Tau correlate with those that display a higher Aβ plaque load, thereby suggesting an inductive effect of Aβ in the phosphorylation of Tau at residue Thr205. Thus, these data imply that the increase in the density of hippocampal interneurons accumulating pThr205 Tau in J20 mice could be a consequence of an inductive effect of Aβ on Tau phosphorylation. It has recently been described that the specific phosphorylation of Tau at residue Thr205 inhibits Aβ neurotoxicity (Ittner et al., 2016). This observation contrasts with the current view that Tau phosphorylation downstream of Aβ toxicity is a pathological response. Thus, given that GABAergic hippocampal neurons appear to be resistant to neurotoxicity in AD (Canas et al., 2014; Mitew et al., 2013; Reinikainen et al., 1988; Rissman et al., 2007), our data showing an inductive effect of Aβ on Tau phosphorylation at residue Thr205 in hippocampal interneurons could reflect a reaction in hippocampal interneurons to protect themselves from neurotoxicity.

4.4. The soma of some human hippocampal interneurons accumulate pSer262 and pThr205 Tau in control subjects and AD patients

After observing that pSer262 and pThr205 Tau are present in hippocampal interneurons in physiological conditions, and that the number of cells accumulating pThr205 Tau increases in the presence of Aβ, we analyzed human hippocampal interneurons.

Our data show that human control and AD samples show few neurons accumulating pSer262 and pThr205 Tau in their soma in the sr and so. Despite the limited number of human samples analyzed, the location and morphology of pSer262 and pThr205 Tau-positive human neurons seem to correspond to hippocampal interneurons. Therefore, these results suggest that human hippocampal interneurons accumulate pSer262 and pThr205 Tau in control and pathological conditions. Thus, the presence of pSer262 and pThr205 Tau in the soma of interneurons in murine hippocampi is also detected in control and pathological

human samples.

Our results reveal a differential pattern of phosphorylation and distribution of P-Tau in hippocampal interneurons and mossy cells. The data suggest that Tau protein acts differently in these neuronal populations, developing yet unknown additional functions in the soma. In addition, the presence of P-Tau and Aβ influences the accumulation of pThr205 Tau in hippocampal interneurons, thereby supporting the notion that pThr205 Tau probably protects against Aβ in AD.

Recently, various therapeutic approaches to AD have attempted to decrease the P-Tau as a strategy to prevent Aβ toxicity (Medina, 2018; Pedersen and Sigurdsson, 2015). Our present data point to a physiological role of pSer262 and pThr205 Tau in the soma of hippocampal interneurons and that these phosphorylations are modulated by the presence of Aβ. Thus, our results evidence that caution must be taken when pharmacological modulation of Tau phosphorylation is proposed as a treatment for AD. A deeper understanding of the dynamics of Tau phosphorylation on the distinct hippocampal neuronal types may contribute to the design of new therapies for AD and other tauopathies. In this context, research into the behavior of Tau in hippocampal interneurons may broaden current knowledge of Tau function and impairment, and may also help to determine the potential of interneurons as a therapeutic target to halt the cognitive impairment associated with AD.

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Disclosure statement

The authors have no actual or potential conflicts of interest to disclose.

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Supplementary Material

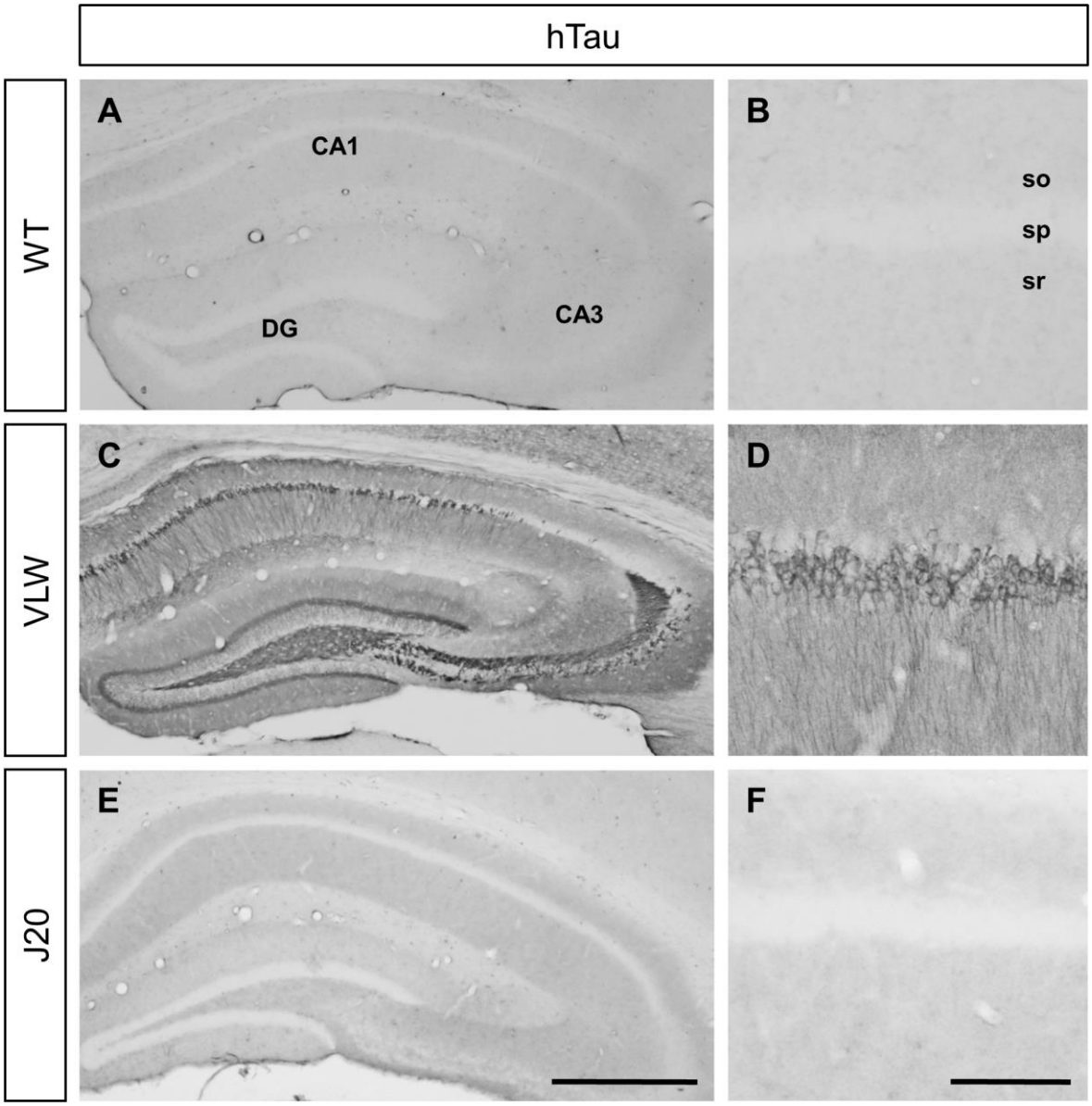
for

Differential accumulation of Tau phosphorylated at residues Thr231, Ser262 and Thr205 in hippocampal interneurons and its modulation by Tau mutations (VLW) and amyloid-β peptide

Eva Dávila-Bouziguet et al.

Supplementary Figure 1

Supplementary Table 1



Supplementary Fig. 1. hTau is present in pyramidal neurons exclusively in VLW mice. Immunodetection of hTau-positive cells in hippocampal sections from 8 mo WT, VLW and J20 mice. hTau protein detected with HT7 antibody is not present in WT (A, B) and J20 (E, F) hippocampi, whereas the hippocampi of VLW mice (C, D) display hTau-positive staining in the sp. Abbreviations: so, *stratum oriens*; sp., *stratum pyramidale*; sr, *stratum radiatum*. Scale bar: in E, 500 μm (applies to A, C, E); in F, 100 μm (applies to B, D, F).

Supplementary Table 1. Detailed information about the human samples used in this study.

	Age	Gender	Diagnosis		PMI
			Neurofibrillary pathology	Amyloid-β deposition	
AD-1	73	Male	AD: Braak Stage V	Stage C phase 4	4:30
AD-2	91	Female	AD: Braak Stage IV	Stage C phase 2	9:00
C-1	61	Male	Without lesions	-	2:45
C-2	66	Male	Without lesions	-	5:00

AD, Alzheimer's disease; C, control; PMI, post-mortem interval. PMI is indicated in hours:minutes.

Functional protection in J20/VLW mice: a model of non-demented with Alzheimer's disease neuropathology

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RESUM

En la malaltia d'Alzheimer coincideixen l'acumulació de β-amiloide i Tau hiperfosforilada amb una activitat neuronal alterada, ritmes oscil·latoris aberrants i dèficits cognitius. La malaltia d'Alzheimer no associada a demència defineix una nova entitat clínica amb la presència de les patologies de β-amiloide i Tau però amb la cognició preservada. Els mecanismes subjacents a aquesta neuroprotecció continuen indeterminats i actualment no hi ha models animals de la malaltia d'Alzheimer no associada a demència. En aquest treball, demostrem que els ratolins J20/VLW (que acumulen β-amiloide i Tau hiperfosforilada) presenten una activitat rítmica de l'hipocamp i una cognició conservades, a diferència dels animals J20 i VLW, que mostren alteracions significatives en aquests paràmetres. A més, mostrem que la sobreexpressió de Tau humana mutant junt amb l'acumulació de β-amiloide en els ratolins J20/VLW generen un patró de fosforilació de Tau diferencial a les interneurones de l'hipocamp. La connexió GABAèrgica septohipocàmpica, responsable de

l'activitat rítmica de l'hipocamp, es conserva en els ratolins J20/VLW, en contrast amb els mutants senzills. Les nostres dades assenyalen els ratolins J20/VLW com un nou model animal adequat per explorar els mecanismes que afavoreixen la preservació cognitiva en la malaltia d'Alzheimer no associada a demència. Addicionalment, els nostres resultats suggereixen que un patró de fosforilació de Tau diferencial a les interneurones de l'hipocamp prevé la pèrdua de la innervació GABAèrgica septohipocàmpica i les alteracions en l'electrofisiologia de l'hipocamp, evitant així dèficits cognitius.

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BRAIN
ORIGINAL ARTICLE

Functional protection in J20/VLW mice: a model of non-demented with Alzheimer's disease neuropathology

Eva Dávila-Bouziguet,[1,2] Arnau Casòliba-Melich,[1,2] Georgina Targa-Fabra,[1,2] Lorena Galera-López,[3] Andrés Ozaita,[3] Rafael Maldonado,[3] Jesús Ávila,[2,4] José M. Delgado-García,[5] Agnès Gruart,[5] Eduardo Soriano[1,2] and Marta Pascual[1,2]

Alzheimer's disease comprises amyloid-β and hyperphosphorylated Tau accumulation, imbalanced neuronal activity, aberrant oscillatory rhythms and cognitive deficits. Non-demented with Alzheimer's disease neuropathology defines a novel clinical entity with amyloid-β and Tau pathologies but preserved cognition. The mechanisms underlying such neuroprotection remain undetermined and animal models of non-demented with Alzheimer's disease neuropathology are currently unavailable.

We demonstrate that J20/VLW mice (accumulating amyloid-β and hyperphosphorylated Tau) exhibit preserved hippocampal rhythmic activity and cognition, as opposed to J20 and VLW animals, which show significant alterations. Furthermore, we show that the overexpression of mutant human Tau in coexistence with amyloid-β accumulation renders a particular hyperphosphorylated Tau signature in hippocampal interneurons. The GABAergic septohippocampal pathway, responsible for hippocampal rhythmic activity, is preserved in J20/VLW mice, in contrast to single mutants.

Our data highlight J20/VLW mice as a suitable animal model in which to explore the mechanisms driving cognitive preservation in non-demented with Alzheimer's disease neuropathology. Moreover, they suggest that a differential Tau phosphorylation pattern in hippocampal interneurons prevents the loss of GABAergic septohippocampal innervation and alterations in local field potentials, thereby avoiding cognitive deficits.

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Abbreviations: CA = cornu ammonis; GAD = glutamic acid decarboxylase; hTau = human Tau protein; LFP = local field potential; MSDB = medial septum and diagonal band of Broca; NDAN = non-demented with Alzheimer's disease neuropathology; P-Tau = hyperphosphorylated Tau protein; PV = parvalbumin; SH = septohippocampal

Introduction

The histopathological hallmarks of Alzheimer's disease include neurodegeneration, extracellular deposits of amyloid-β peptide (i.e. senile plaques) and intracellular neurofibrillary tangles of hyperphosphorylated Tau protein (P-Tau).[1] Moreover, altered neural activity, such as synaptically driven hyperactivity, occurs in mouse models of Alzheimer's disease,[2,3] and functional MRI studies have also revealed hippocampal hyperactivity in asymptomatic individuals at genetic risk of Alzheimer's disease.[4,5] Furthermore, epileptiform activity is more common in individuals with this disease than in controls.[6] Theta and gamma oscillations of local field potentials (LFPs) are reduced in Alzheimer's disease patients and animal models, thereby further indicating an imbalance between excitatory and inhibitory circuits.[7–10] Theta and gamma oscillations are regulated by fast-spiking, parvalbumin (PV)-positive hippocampal interneurons.[11,12] At the circuit level, these cells provide perisomatic inhibition onto glutamatergic pyramidal and granular neurons and regulate spike timing.[13] Consistent with a decrease in PV output in Alzheimer's disease, pyramidal neurons show reduced spontaneous GABAergic currents, and optogenetic stimulation of PV-positive cells improves gamma oscillations and reduces amyloid-β load and P-Tau levels in mouse models of Alzheimer's disease.[14,15] Additional work suggests that loss of GABAergic tone partly underlies network dysfunction in Alzheimer's disease and tauopathies.[16–18]

The medial septum and diagonal band of Broca (MSDB) complex and the nucleus basalis of Meynert innervate the cerebral cortex and the hippocampus. Along with cholinergic fibres, the septohippocampal (SH) pathway consists of another key element, namely the GABAergic SH pathway. This component involves inhibitory long-range projection neurons that terminate specifically on GABAergic hippocampal interneurons,[19,20] which in turn govern the activity of pyramidal neurons. The activation of GABAergic SH neurons results in the selective inhibition of inhibitory interneurons, hence enabling the synchronous activation of a large number of pyramidal neurons.[21,22] Therefore, the GABAergic SH pathway has been proposed to be responsible for producing the correct levels of excitation, as well as regulating synchronous neuronal activity, including theta and gamma oscillations, which are crucial for memory and cognition.[23–26]

Alterations in the GABAergic SH pathway have been associated with Alzheimer's disease. For instance, J20 mice expressing human amyloid precursor protein (hAPP) with Swedish and Indiana mutations of familial Alzheimer's disease[27] present a marked deterioration, manifested as a reduced number and complexity of GABAergic SH axon terminals. This decline also correlates with electrophysiological changes within the hippocampal network.[9,28] Similarly, VLW mice expressing human Tau protein (hTau) with three mutations related to frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17)[29] show a decrease in GABAergic SH innervation.[30] Moreover, VLW mice present epilepsy and $GABA_A$ receptor-mediated hyperexcitability.[31]

Interestingly, individuals presenting the histopathological hallmarks of Alzheimer's disease (namely amyloid-β plaques and P-Tau tangles) without cognitive impairment have been reported.[32–37] This clinical entity, called non-demented with Alzheimer's disease neuropathology (NDAN), is thought to have intrinsic mechanisms conferring neuroprotection against the classical degeneration of Alzheimer's disease. Several factors have been proposed as responsible for this protection, such as increased neurogenesis,[33] increased hippocampal and total brain volume (which could indicate a larger cognitive reserve),[34] neuronal and nuclear hypertrophy,[35] and preservation of the essential elements of the synaptic machinery that are dysfunctional in Alzheimer's disease.[32,36] A differential pattern of cytokine expression, linked to reduced glial activation in human resilience to Alzheimer's disease, together with the absence of soluble P-Tau accumulation in synapses, might contribute to the prevention of neuronal dysfunction in NDAN patients.[38,39] These individuals also have a differential gene expression pattern at the postsynaptic density that differs from that of Alzheimer's disease and control participants and may contribute to the mechanisms that confer synapse protection against Alzheimer's disease.[37] The lack of animal models mimicking NDAN hinders its characterization and identification of the mechanisms involved in preventing cognitive decline.

To assess the synergic or opposing effects of amyloid-β and P-Tau on hippocampal neuron physiology, hippocampal activity rhythms and cognition, we crossed J20 ($hAPP^{Sw,Ind}$) and VLW ($hTau^{VLW}$) mice to generate a double transgenic mouse model with both amyloid-β and Tau pathologies, which are characteristic of Alzheimer's disease. The amyloid-β plaque load in the resulting J20/VLW mice did not differ from that in single transgenic J20 animals. Analysis of Tau phosphoepitopes revealed high levels of Tau phosphorylated at Thr231 (pThr231) and Thr205 (pThr205) in cornu ammonis (CA) 1 pyramidal neurons in J20/VLW mice, similar to VLW animals. In contrast, J20/VLW mice presented higher densities of hippocampal interneurons accumulating pThr205 and pSer262 Tau than VLW animals, thereby indicating an interneuron-specific modulation of Tau phosphorylation by amyloid-β. Surprisingly, GABAergic SH innervation on hippocampal interneurons in J20/VLW mice did not differ from that in control animals. Examination of hippocampal electrophysiology revealed partial recovery of hippocampal theta oscillations in J20/VLW animals, while these oscillations were greatly impaired in J20 and VLW mice. Furthermore, recognition memory deficits associated with amyloid-β accumulation were prevented in the double transgenic mouse model. These data render the J20/VLW mouse line a suitable animal model in which to further study the cognitive preservation in human NDAN participants, and they support the maintenance of the GABAergic system as a mechanism underlying functional hippocampal electrophysiological activity and cognitive neuroprotection in NDAN.

Materials and methods

Animals

A double transgenic mouse line was generated by crossing J20 and VLW lines in a C57BL/6J genetic background. The offspring included double mutant J20/VLW, hemizygous J20, heterozygous VLW and non-transgenic wild-type mice that were used as controls. J20 animals overexpress $hAPP^{Sw,Ind}$, hAPP carrying two familial Alzheimer's disease mutations, namely Swedish (K670N/M671L) and Indiana (V717F), under the control of the platelet-derived

growth factor subunit β promoter.[27] VLW mice overexpress hTau^{VLW}, hTau with four tubulin-binding repeats and three mutations related to FTDP-17 (G272V, P301L and R406W) under the control of the Thy-1 promoter.[29]

For all experiments, *n* denotes the number of animals. Animals were mainly littermates and all of them were derived from the same colony. For the histological procedures, wild-type (8 and 12 months old; $n = 4$–5 per group], J20 (8 and 12 months old; $n = 4$ per group), VLW (8 months old; $n = 3$–4 per group) and J20/VLW (8 and 12 months old; $n = 4$–5 per group) mice were used. For the biochemical analyses, 6–8-month-old wild-type ($n = 6$), J20 ($n = 2$–6), VLW ($n = 6$) and J20/VLW ($n = 2$) mice were used. For the electrophysiological study, 8-month-old wild-type, VLW and J20/VLW mice were used ($n = 4$ per group). For the behavioural tests, 8-month-old wild-type ($n = 9$), J20 ($n = 6$), VLW ($n = 7$) and J20/VLW ($n = 5$) mice were used.

Animals were kept on a 12-h light/dark schedule with access to food and water *ad libitum*. Experimenters were blinded to the genotype of mice until data acquisition was completed. Experiments were performed in accordance with the European Community Council Directive and the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the local ethical committees.

Detection of septohippocampal fibres

Animals were anaesthetized with a 10/1 mixture of Ketolar® (50 mg/ml ketamine chlorhydrate, Parke-Davis)/Rompun® (2% xylidine-thiazine chlorhydrate, Bayer) and stereotaxically injected with an anterograde tracer, 10% biotinylated dextran-amine (BDA; 10 000 MW, Molecular Probes), in the MSDB complex. Each animal received midline injections of the tracer into the MSDB complex at one anteroposterior (AP) level and two dorsoventral (DV) injection sites by iontophoresis. Stereotaxic coordinates in millimetres were (from Bregma): AP 0.7 and DV –3.0 and –3.7.[40] This protocol results in intense BDA labelling in the MSDB complex, which contains the highest proportion of GABAergic SH neurons.[41] After 5–6 days, animals were anaesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were post-fixed for 48 h in 4% paraformaldehyde, cryoprotected in PBS with 30% sucrose, frozen and 30-μm coronal sections were cut and stored in a cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1 M phosphate buffer) at –20°C until use.

Immunodetection

Sections from iontophoretically injected animals were processed for double immunodetection of BDA and interneuron or P-Tau markers.[41] Sections were incubated simultaneously with the ABC complex (Vector Laboratories; 1/100) to visualize BDA, and rabbit polyclonal antibodies against PV (Swant®; 1/3000) or glutamic acid decarboxylase isoforms 65 and 67 (GAD, Chemicon International; 1/2000) for interneuron markers, or AT-180 mouse anti-phosphothreonine 231 (Innogenetics; 1/300), T205 rabbit anti-phosphothreonine 205 (Invitrogen™; 1/1000) or S262 rabbit anti-phosphoserine 262 (Invitrogen™; 1/100) antibodies for P-Tau residues. BDA was developed with hydrogen peroxide and diaminobenzidine, nickel ammonium sulphate and cobalt chloride, yielding a black end product in SH fibres. Primary antibodies were visualized by sequential incubation with biotinylated secondary antibodies and the ABC complex (Vector Laboratories). Peroxidase activity was developed with hydrogen peroxide and diaminobenzidine to produce a brown end product. Sections were mounted onto gelatinized slides, dehydrated and coverslipped with Eukitt® (O. Kindler).

To detect pThr231, pThr205, pSer262 Tau or hTau, sections were incubated with AT-180, T205, S262 or HT7 mouse anti-hTau (Invitrogen™; 1/500) antibodies. Subsequent steps were performed as described.

To detect amyloid-β plaques or hAPP, sections were incubated with 3D6 mouse anti-amyloid-β (amino acids 1–5) [obtained from the supernatant of cultured Murine Hybridoma Cell Line, RB96 3D6.32.2.4 (PTA-5130)], American Type Culture Collection; 1/200) or 6E10 mouse anti-amyloid-β and hAPP (amino acids 1–16) (Covance; 1/500) antibodies. Subsequent steps were performed as described.

Analysis of histological sections

Microscopic observations focused on sections corresponding to the medial septum and to dorsal (sections between AP –1.6 and –2.3 mm from Bregma) and ventral (sections between AP –2.9 and –3.4 mm from Bregma) hippocampal levels, following the atlas reported by Paxinos and Franklin.[40]

To estimate the density of hippocampal interneurons and the percentage of these cells contacted by GABAergic SH fibres, the density of interneurons containing GAD, PV, pThr231, pThr205 or pSer262 Tau and the percentage of these receiving BDA-positive pericellular baskets was calculated in distinct regions of the hippocampal area (CA1, CA3 and dentate gyrus) of each section (8-month-old wild-type, J20, VLW and J20/VLW mice and 12-month-old wild-type and J20/VLW mice; $n = 4$–5 animals per group, three sections per animal). The area comprising the hippocampal region of each section was quantified using Fiji software.[42] Because of the large number of GAD-immunopositive cells, several sample areas were selected for each section (8-month-old wild-type, J20, VLW and J20/VLW mice and 12-month-old wild-type and J20/VLW mice; $n = 4$ animals per group, three sections per animal). The selected samples (125-μm wide stripes) contained all hippocampal layers (perpendicularly from the ventricle to the pial surface), and each section included the CA1, the CA3 and the dentate gyrus. Data were represented as density of interneurons per square millimetre and percentage of GAD-, PV-, pThr231, pThr205 or pSer262 Tau-positive cells contacted by GABAergic SH fibres. To assess the complexity of GABAergic SH contacts, synaptic boutons around the soma of GAD-, PV-, pThr231, pThr205 or pSer262 Tau-positive cells were counted in the same sample areas under an optical microscope (Nikon E600, Nikon Corporation), and data were expressed as number of boutons per basket.

To estimate the density of interneurons accumulating pThr231, pThr205 or pSer262 Tau, and to compute the mean grey value of the pThr231 and pThr205 signals in pyramidal neurons, samples immunodetected with AT-180, T205 or S262 antibodies were scanned with a NanoZoomer 2.0HT whole slide imager (Hamamatsu Photonics) at ×20. The density of pThr231, pThr205 and pSer262 Tau-immunopositive interneurons was quantified in distinct regions of the hippocampal area (CA1, CA3 and dentate gyrus) of each section (8-month-old wild-type, J20, VLW and J20/VLW mice; $n = 4$ animals per group, three sections per animal) and data were expressed as density of cells per square millimetre. The cells and area comprising each hippocampal region were quantified using Fiji software.[42] The mean grey value of the pThr231 and pThr205 Tau signals was calculated in 10-mm^2 stripes in matching regions of the pyramidal layer of the CA1 after thresholding the images to exclude background signal (8-month-old VLW and J20/VLW mice; $n = 3$–4 animals per group, three sections per animal) using Fiji software.[42]

To assess the percentage of amyloid-β plaques in the hippocampus, samples immunodetected with 3D6 antibody were scanned with a NanoZoomer 2.0HT whole slide imager (Hamamatsu Photonics) at ×20. The Trainable Weka

Segmentation plugin[43] from Fiji software[42] was applied to the images by using a set of machine-learning algorithms with a collection of image features selected by the user to produce pixel-based segmentations. Images were processed using a macro provided by Sebastién Tosi (Institute for Research in Biomedicine, Barcelona) to identify and quantify amyloid-β plaques (8- and 12-month-old J20 and J20/VLW mice; $n = 4$ animals per group, three sections per animal).

Biochemical extraction

To obtain protein extracts from brain tissue, animals were sacrificed by cervical dislocation and brains were removed. Hippocampal and other cortical regions were dissected and frozen in liquid nitrogen. Frozen tissue was homogenized in lysis buffer (50 mM HEPES, pH 7.5, 150 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% glycerol and 1% Triton™ X-100) containing cOmplete™ Mini Protease Inhibitor Cocktail (Roche) and phosphatase inhibitors (10 mM tetrasodium pyrophosphate, 200 μM sodium orthovanadate and 10 mM sodium fluoride). Homogenates were centrifuged at 16 000*g* for 20 min at 4°C, and supernatants were stored at –80°C until use.

Western blot

To remove endogenous immunoglobulins, samples were incubated with 20 μl of protein G beads (GE Healthcare) for 2 h at 4°C, and cleaned supernatants were recovered by centrifugation. Samples were diluted 1:3 with 3× loading buffer (150 mM Tris-HCl, pH 6.8, 300 mM glycerol, 10% β-mercaptoethanol, 3% SDS, 0.075% bromophenol blue) and boiled for 10 min at 95°C. They were then resolved by 10% SDS–PAGE gels and electrotransferred to 0.45-μm nitrocellulose membranes (GE Healthcare). Membranes were incubated with AT-180 (Innogenetics; 1/1000), T205 (Invitrogen™; 1/1000), K9JA rabbit anti-total Tau (DAKO; 1/20 000), and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Invitrogen™; 1/10 000) primary antibodies. After sequential incubation with HRP-labelled secondary antibodies (DAKO), membranes were developed with ECL™ Western Blotting Detection Reagents (GE Healthcare). For quantification of western blot, a densitometric analysis of protein bands of interest was performed using Gel-Pro Analyzer v.3.1 software (Media Cybernetic) and was normalized for total Tau (K9JA) and the loading control (GAPDH).

Surgery for the chronic recording of hippocampal local field potentials

Mice were anaesthetized with 0.8–3% halothane delivered from a calibrated Fluotec 5 vaporizer (Datex-Ohmeda) at a flow rate of 1–2 l/min oxygen. Animals were implanted with a recording electrode aimed at the ipsilateral stratum radiatum underneath the CA1 area. Stereotaxic coordinates in millimetres were (from Bregma): AP –2.2, lateromedial (LM) 1.2, and DV –1.0 to –1.5.[40] These electrodes were made of 50-μm Teflon-coated tungsten wire (Advent Research Materials Ltd). A bare silver wire (0.1 mm) was affixed to the skull as a ground. Wires were soldered to a 6-pin socket, which was fixed to the skull with the help of two small screws and dental cement.[44]

Recording procedures

The electroencephalographic field activity of the CA1 area was recorded with the help of Grass P511 differential amplifiers. LFP recordings were carried out with the awake animal placed in either a small box (5 × 5 × 5 cm) to prevent walking movements or a large box (20 × 15 × 15 cm) in which the animal could move freely. Recordings were carried out for 20 min, of which up to 5 min of recording free of unwanted artefacts were selected for spectral analysis.

Data analysis of electrophysiological studies

Hippocampal activity was stored digitally on a computer through an analogue/digital converter (CED 1401 Plus, CED) at a sampling frequency of 11–22 kHz and an amplitude resolution of 12 bits. Commercial computer programs (Spike 2 and SIGAVG, CED) were modified to represent recorded LFPs.

The power spectrum of hippocampal LFPs collected was computed with the help of MATLAB v.7.4.0 software (MathWorks), using the fast Fourier transform with a Hanning window, expressed as relative power and averaged across each session. This average was analysed and compared using the wide-band model, considering the following bands: delta (<4 Hz), theta (4.1–8 Hz), alpha (8.1–12 Hz), beta (12.1–26 Hz) and gamma (26.1–100 Hz).[45,46]

Novel object-recognition test

Object-recognition memory was assessed following a protocol previously described.[47] On Day 1, mice were habituated to a V-shaped maze (each corridor measuring 30 cm long × 4.5 cm wide × 15 cm high) for 9 min. On Day 2, two identical objects (familiar objects) were placed at the end of each corridor for 9 min, and the time that the mice spent exploring each object was measured. Twenty-four hours later, one of the familiar objects was replaced by a new object (novel object) (Fig. 1A). The time spent exploring each of the objects was computed to establish a discrimination index, which was calculated as the difference between the time spent exploring the novel object minus the time exploring the familiar object divided by the total exploration time (addition of the time exploring both objects). Object exploration was defined as the orientation of the nose towards the object at a distance of <2 cm. Mice that explored both objects for <10 s or one object for <3 s were excluded from the analysis. A higher discrimination index is considered to reflect greater memory retention for the familiar object. Total exploration time was considered a measure of general activity during the test.

Statistical analysis

Data were processed for statistical analysis with GraphPad Prism 8 (GraphPad Software Incorporated). Data were tested for normal distribution. To examine differences between two experimental groups, an unpaired two-tailed Student's *t*-test or Welch's *t*-test was used when the samples had equal variances or not, respectively. To assess differences between >2 experimental groups, one-way ANOVA was used. *Post hoc* comparisons were performed by Tukey's test or Fisher's LSD test when a significant main effect of one-way ANOVA was revealed. Significance levels were set at $P < 0.05$: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistical values of continuously distributed data are presented as mean ± standard error of the mean (SEM) along with all data-points.

Data availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

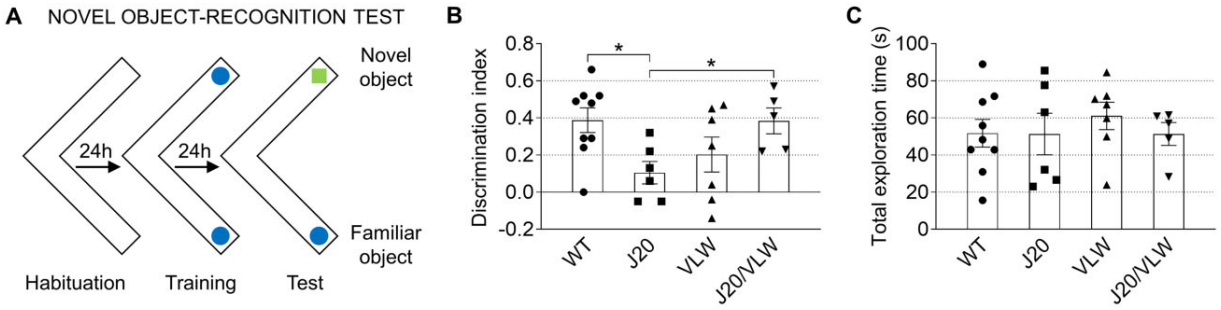

Figure 1 No recognition memory deficits are present in J20/VLW mice. (**A**) Protocol for the novel object recognition test. The time spent exploring each of the objects during the test phase was computed to calculate a discrimination index. (**B**) Discrimination index in the novel object recognition test. A higher discrimination index is considered to reflect greater memory retention for the familiar object. (**C**) Total exploration time in the novel object recognition test was considered a measure of general activity. (**B** and **C**) One-way ANOVA and Fisher's LSD *post hoc*, $^{*}P < 0.05$. 8-month-old wild-type ($n = 9$), J20 ($n = 6$), VLW ($n = 7$) and J20/VLW ($n = 5$) mice. Each dot represents the mean value per animal. Error bars represent SEM.

Results

Cognitive recovery of J20/VLW mice in contrast to J20 animals

J20 and VLW mice show considerable cognitive deficits, mainly in spatial memory.[48–50] To characterize double transgenic J20/VLW mice, which accumulate both amyloid-β and P-Tau, we performed various tests to analyse long-term memory, comparing 8-month-old wild-type, J20, VLW and J20/VLW animals. First, we determined that there were no major differences in either locomotion or anxiety between the four experimental groups (Supplementary Fig. 1). Next, we performed the Y-maze test to study spatial working memory. Our data show that J20/VLW mice presented a higher percentage of correct alternations than J20 and VLW mice (Supplementary Fig. 2). Unexpectedly, alterations in the performance of J20 animals in the novel object recognition test (Fig. 1A), which have been previously described,[48,49] were not present in J20/VLW mice, which showed a behaviour equivalent to that of wild-type mice (Fig. 1B). Surprisingly, these data suggest that the overexpression of mutant hTau, in coexistence with amyloid-β accumulation, protects against the recognition memory deficits associated with amyloid-β. Such specific differences were not related to exploratory behaviour or motility, since total exploration times in the novel object recognition test were similar in the four experimental groups (Fig. 1C).

Hippocampal rhythmic activity preservation in J20/VLW mice

We next analysed the oscillatory activity of hippocampal circuits in 8-month-old behaving wild-type, J20, VLW and J20/VLW mice. Chronically implanted electrodes recorded hippocampal field activity in behaving animals placed in either small or large boxes, to determine the contribution of overt motor activity to the power spectra of the theta and gamma bands (Fig. 2A and B). As previously described,[9] the spectral analysis of LFP recordings showed a clear decrease in the spectral power of the theta band in J20 mice placed in small and large boxes (43% and 38%, respectively) compared to age-matched wild-type animals (Fig. 2C). In addition, a considerable reduction in theta spectral power was present in VLW animals placed in small and large boxes (33% and 42%, respectively). In contrast, J20/VLW mice showed only a slight decrease in theta spectral power when located in small and large boxes (8% and 22%, respectively). We also examined the spectral power of the gamma band (Fig. 2D). In a previous study,[9] we found a 50% reduction in gamma spectral power in J20 animals placed in large and small boxes compared to wild-type mice. The results presented herein demonstrate that the gamma band in VLW animals was equivalent to that of wild-type mice. Interestingly, our data indicate a minor reduction (33%) in this band in J20/VLW mice compared to age-matched wild-type animals. We conclude that both theta and gamma oscillations are rescued in J20/VLW mice. Thus, the coexistence of amyloid-β and P-Tau correlates with preserved hippocampal rhythmic activity.

No changes in amyloid-β accumulation in J20/VLW animals compared to J20 mice

To study changes in amyloid-β accumulation in J20/VLW mice compared to single transgenic J20 animals, we first performed immunodetection with 6E10 antibody (reactive to amino acids 1–16 of amyloid-β and hAPP). No differences were observed in the distribution of 6E10-positive cells or degree of immunoreactivity when comparing the J20 and J20/VLW hippocampus (Supplementary Fig. 3), thus indicating the absence of changes in the level of hAPP expression in double transgenic mice. Subsequently, we measured the concentration of soluble forms of amyloid-β (amino acids 1–42) by ELISA. No differences were observed between 6-month-old J20 and J20/VLW mice (Supplementary Fig. 3). Finally, by immunodetection with 3D6 antibody (which specifically detects amino acids 1–5 of amyloid-β), we analysed the presence of amyloid-β plaques. Our data confirm no changes in the accumulation of amyloid-β plaques in the hippocampus of J20/VLW mice compared to J20 animals at 8 month of age (Fig. 3A–C). Given that there are only a small number of plaques at this maturation stage, we then analysed 12-month-old animals. The percentage of amyloid-β plaques in the hippocampus of 12-month-old J20 and J20/VLW littermates was similar (Fig. 3D–F), suggesting that the cognitive and physiological improvements observed in J20/VLW animals are not a consequence of a reduction in soluble amyloid-β levels or a diminution in amyloid-β plaque load.

Differential Tau phosphorylation signature in hippocampal GABAergic interneurons of J20/VLW mice

Amyloid-β induces Tau phosphorylation both *in vivo* and *in vitro*.[51,52] Therefore, we analysed soluble Tau phosphorylated at Thr231 and Thr205 by western blot of hippocampal and cortical protein extracts from 6–8-month-old wild-type, J20, VLW and J20/VLW mice. Our data show no major changes in the levels of pThr231 Tau between the four experimental groups (Fig. 4A). In contrast, the levels of pThr205 Tau showed a clear

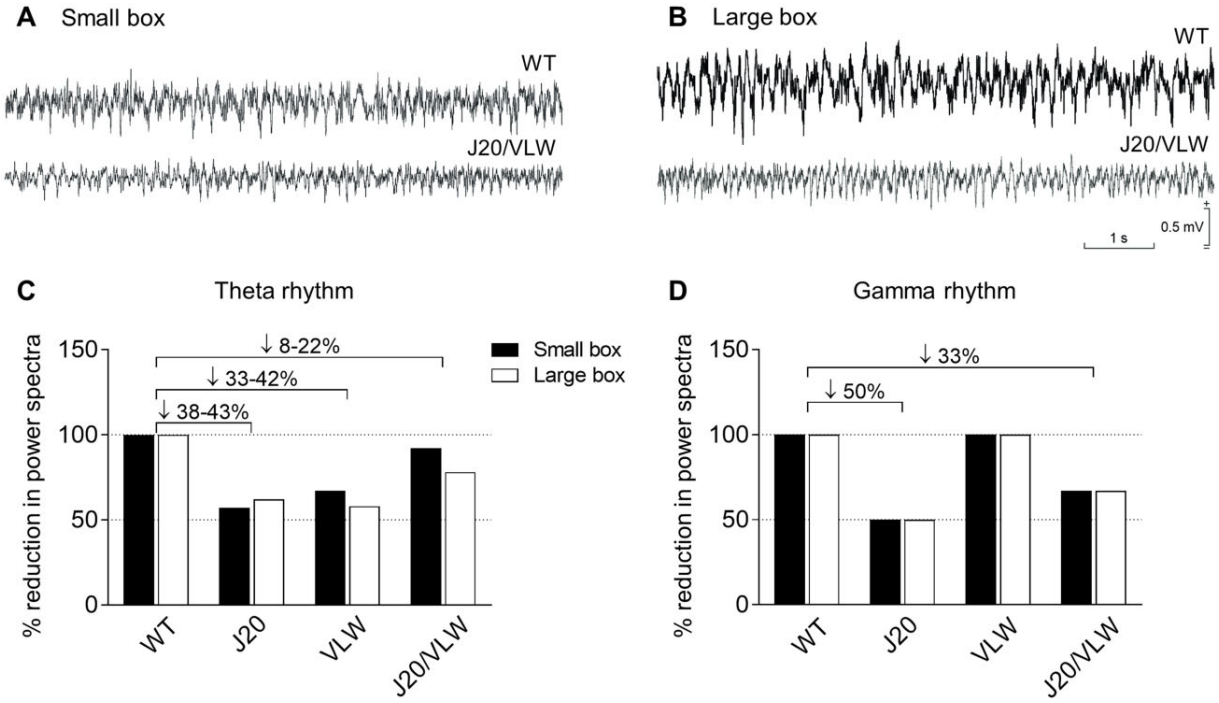

Figure 2 Hippocampal oscillations, which are markedly impaired in J20 and VLW animals, are partially rescued in J20/VLW mice. (**A** and **B**) Representative examples of LFP activity recorded in the CA1 region of the hippocampus of 8-month-old wild-type (WT) and J20/VLW mice placed in either a small (**A**) or a large (**B**) box. (**C** and **D**) Spectral powers were computed from similar records collected from 8-month-old wild-type, J20, VLW and J20/VLW mice. Histograms representing the percentage decrease in spectral power for theta (**C**) and gamma (**D**) bands, comparing J20, VLW and J20/VLW mice to wild-type animals. $n = 4$ animals per group.

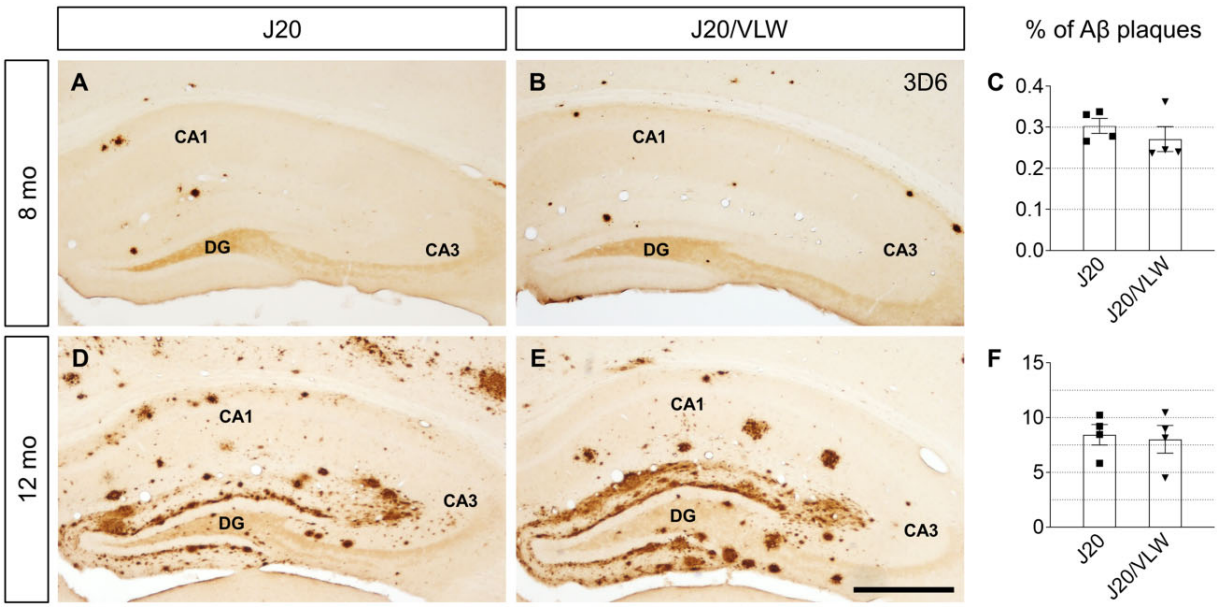

Figure 3 Amyloid-β plaque load of J20/VLW mice is similar to that of J20 animals. Immunodetection of amyloid-β (Aβ) plaques with 3D6 antibody in hippocampal sections from 8- and 12-month-old J20 and J20/VLW mice. (**A**, **B**, **D** and **E**) Accumulation of amyloid-β plaques in the hippocampus of 8- (**A** and **B**) and 12- (**D** and **E**) month-old J20 and J20/VLW mice. (**C** and **F**) Quantification of the percentage of amyloid-β plaques in the hippocampus of 8- (**C**) and 12- (**F**) month-old J20 and J20/VLW animals. (**C** and **F**) Student's *t*-test. $n = 4$ animals per group, three sections per animal. Each dot represents the mean value per animal. Error bars represent SEM. Scale bar = 500 μm.

upwards trend in J20 and J20/VLW mice when compared to wild-type and VLW animals (Fig. 4B), suggesting an effect of amyloid-β on the phosphorylation of Tau at Thr205, as previously described.[53,54]

Next, we examined the pattern of Tau phosphorylation in 8-month-old mice by immunodetection. Our previous data determined that VLW mice overexpress mutant hTau specifically in pyramidal neurons and that phosphorylated hTau in these

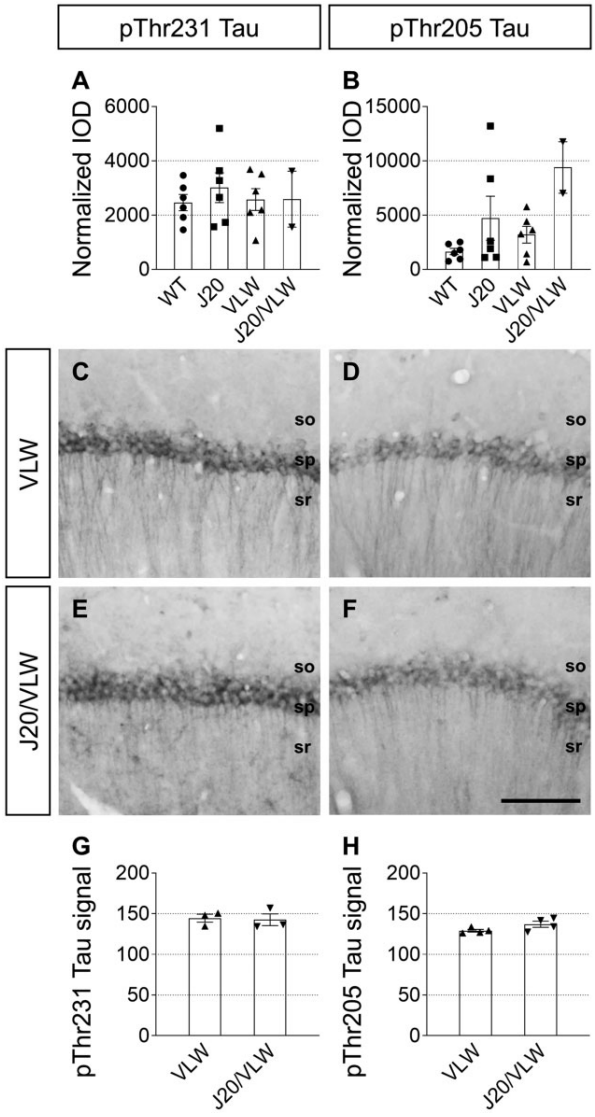

Figure 4 Global levels of pThr231 and pThr205 Tau are not changed in J20/VLW mice. Western blot, immunodetection and quantification of pThr231 and pThr205 Tau. (**A** and **B**) Western blot against pThr231 (**A**) and pThr205 Tau (**B**) in hippocampal and cortical protein extracts from 6–8-month-old wild-type (WT), J20, VLW and J20/VLW mice. (**C**–**F**) pThr231 (**C** and **E**) and pThr205 (**D** and **F**) Tau accumulation in pyramidal neurons in the CA1 region of the hippocampus of 8-month-old VLW and J20/VLW mice. (**G** and **H**) Quantification of the mean grey value of pThr231 (**G**) and pThr205 (**H**) Tau signal in the pyramidal layer of VLW and J20/VLW animals. (**A** and **B**) One-way ANOVA and Tukey's *post hoc*. $n = 2$–6 animals per group. (**G** and **H**) Student's *t*-test. $n = 3$–4 animals per group, three sections per animal. Each dot represents the mean value per animal. Error bars represent SEM. so = stratum oriens; sp = stratum pyramidale; sr = stratum radiatum. Scale bar = 100 μm. IOD = integrated optical density.

cells induces the phosphorylation of murine Tau in hippocampal interneurons.[54] To analyse the distribution of hTau in J20/VLW hippocampal neurons, we performed immunodetection with HT7 antibody. As in VLW animals, hTau expression in J20/VLW mice was restricted to pyramidal neurons (Supplementary Fig. 4).

Subsequently, we show that there are no changes in the accumulation of Tau phosphorylated at Thr231 or Thr205 in the somatodendritic compartment of pyramidal neurons when comparing VLW and J20/VLW mice (Fig. 4C–F). No differences in the pThr231 and pThr205 Tau signals in the CA1 pyramidal layer were detected between VLW and J20/VLW animals (Fig. 4G and H). No pSer262 Tau was detected in the pyramidal layer of either VLW or J20/VLW mice.

We next compared the distribution and density of interneurons accumulating P-Tau in VLW and J20/VLW mice, as recent data reports the presence of murine pThr231, pThr205 and pSer262 Tau in the somas of hippocampal interneurons in VLW mice.[54] Some hippocampal interneurons located mainly in the stratum oriens of the CA1 accumulated pThr231 Tau both in VLW and J20/VLW mice (Fig. 5A and D). Although no statistical differences in the density of pThr231 Tau-positive interneurons were found between the VLW and J20/VLW hippocampus, a clear upwards trend was observed in the double transgenic mice (Fig. 5G). Subsequently, we examined the density and distribution of pThr205 Tau-positive cells. In agreement with our previous studies,[54] amyloid-β in J20 mice induced an increase in the density of pThr205 Tau-positive hippocampal interneurons in comparison with wild-type mice, and P-Tau accumulation in VLW mice induced a reduction in the density of these interneurons (Fig. 5B and H). The density of pThr205 Tau-positive GABAergic interneurons in J20/VLW mice was higher than in VLW animals, lower than in J20 mice and similar to that of wild-type animals (Fig. 5E and H). Thus, our data indicate that the increase in the density of pThr205 Tau-positive interneurons induced by amyloid-β and the decrease observed in the VLW hippocampus are abolished by the simultaneous presence of amyloid-β and P-Tau in J20/VLW animals (Fig. 5B, E and H). Finally, we show a considerable increase in the density of interneurons accumulating pSer262 Tau in J20/VLW mice compared to VLW animals (Fig. 5C, F and I). This observation suggests a synergic effect of P-Tau and amyloid-β in the induction of Tau phosphorylation at Ser262 in hippocampal interneurons. We thus conclude that the coexistence of amyloid-β and P-Tau in J20/VLW mice confers a particular Tau phosphorylation signature in GABAergic hippocampal interneurons but not in hippocampal pyramidal neurons.

To characterize the interneuron subtypes accumulating P-Tau, we performed double fluorescent immunodetection of pThr231, pThr205 and pSer262 Tau, combined with detection of the interneuron markers PV, calretinin and calbindin, on J20/VLW hippocampal slices. As described in VLW animals, pThr231 Tau accumulated specifically in some PV-positive interneurons throughout the hippocampus (Supplementary Fig. 5A–C), while some PV-, calretinin- and calbindin-positive interneurons scattered in different hippocampal layers and regions accumulated pThr205 and pSer262 Tau (Supplementary Fig. 5D–L). In agreement with previous data in human Alzheimer's disease,[55] only a few PV-positive cells with pThr205 Tau in their soma were present in the J20/VLW hippocampus.

GABAergic septohippocampal pathway preservation in J20/VLW mice

The GABAergic SH pathway is crucial for the activity of hippocampal interneurons and hippocampal physiology, rhythmic activity and cognition.[14,25] Given that the previous data demonstrate the preservation of cognition and hippocampal rhythmic activity in J20/VLW mice and an alteration of the pattern of Tau phosphorylation specifically in interneurons, we next analysed GABAergic SH innervation on interneurons accumulating P-Tau. To map the GABAergic SH pathway, we performed stereotaxic injections of an anterograde tracer (BDA) in the MSDB complex.[9,30,41] By double immunodetection of the tracer and pThr231, pThr205 and pSer262 Tau, we determined the percentage of cells accumulating P-Tau and that are innervated by septal GABAergic fibres, and the

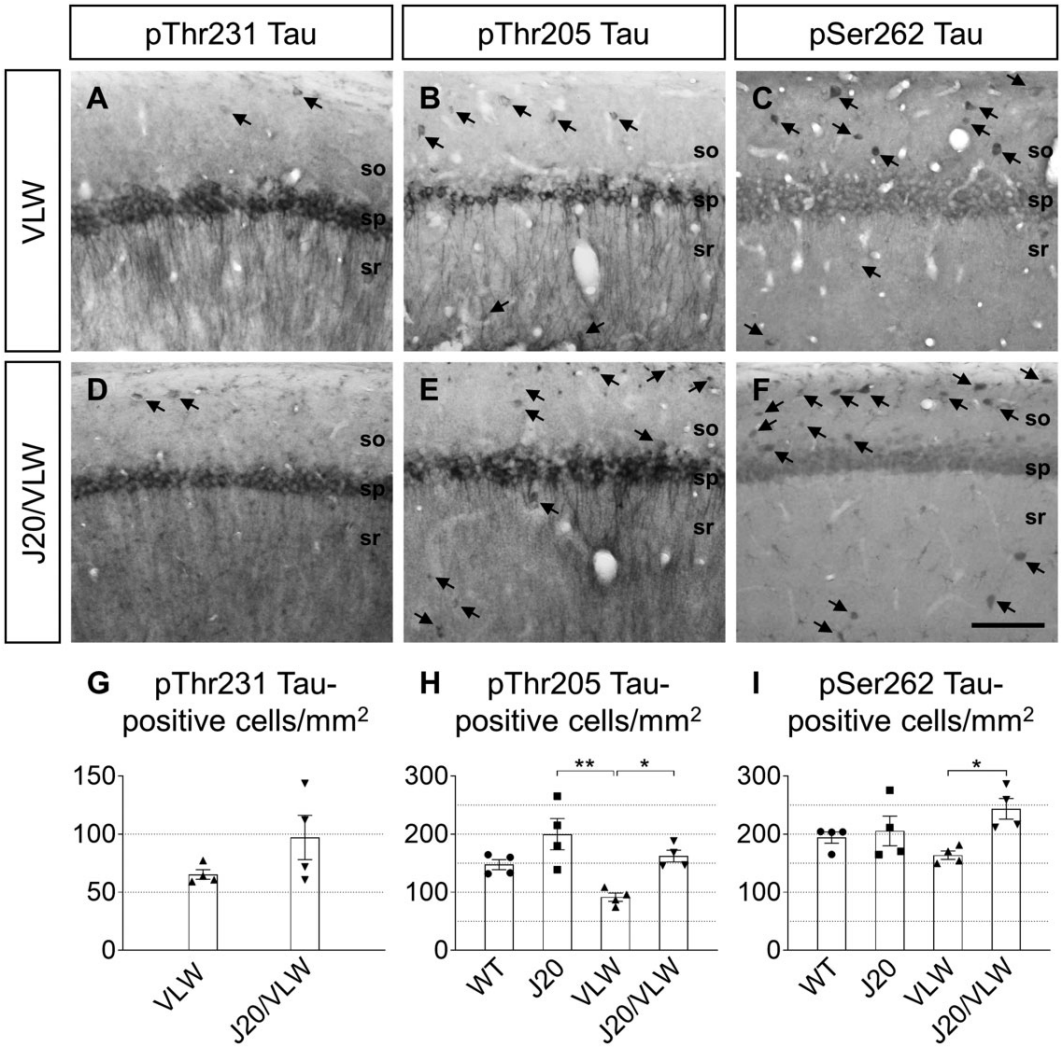

Figure 5 Increased density of hippocampal interneurons accumulating P-Tau in J20/VLW mice, which accumulate amyloid-β peptide, compared to VLW animals. Immunodetection and cell density quantification of pThr231, pThr205 and pSer262 Tau-positive cells in hippocampal sections from 8-month-old wild-type (WT), J20, VLW and J20/VLW mice. (**A**–**F**) Accumulation of pThr231 (**A** and **D**), pThr205 (**B** and **E**) and pSer262 (**C** and **F**) Tau in hippocampal interneurons (arrows) in the CA1 region of the hippocampus of VLW and J20/VLW mice. (**G**) Density quantification of pThr231 Tau-positive interneurons in the hippocampus of VLW and J20/VLW mice. (**H** and **I**) Density quantification of pThr205 (**H**) and pSer262 (**I**) Tau-positive interneurons in the hippocampus of wild-type, J20, VLW and J20/VLW mice. For (**G**): Welch's *t*-test. (**H** and **I**) One-way ANOVA and Tukey's *post hoc*, $^{*}P < 0.05$, $^{**}P < 0.01$. $n = 4$ animals per group, three sections per animal. Each dot represents the mean value per animal. Error bars represent SEM. so = stratum oriens; sp = stratum pyramidale; sr = stratum radiatum. Scale bar = 100 μm.

complexity of GABAergic SH contacts (number of boutons per cell). Our data indicate that the percentage of pThr205 Tau-positive interneurons contacted by GABAergic SH fibres was significantly decreased in J20 and VLW animals compared to wild-type and J20/VLW mice. Moreover, the complexity of the contacts was also reduced in J20 and VLW mice (Supplementary Fig. 6A–F). In contrast, we found that the percentage of pSer262 Tau-positive interneurons contacted by GABAergic SH fibres and the complexity of the contacts were similar in wild-type, J20, VLW and J20/VLW animals (Supplementary Fig. 6G–L). We then studied GABAergic SH innervation on pThr231 Tau-positive interneurons present in VLW and J20/VLW mice. No differences in either the percentage of contacted pThr231 Tau-positive interneurons or the complexity of the contacts were detected between VLW and J20/VLW animals (Supplementary Fig. 6M–P).

Thereafter, we studied whether the GABAergic SH network is preserved in J20/VLW mice by double immunodetection of the tracer BDA and interneuron markers. In agreement with our previous studies,[9,30] single transgenic J20 and VLW mice showed a significant reduction in the percentage of GABAergic hippocampal interneurons (GAD-immunopositive) innervated by GABAergic SH fibres, respectively, compared to wild-type animals (Fig. 6G). Also, both groups showed a decrease in the complexity of GABAergic SH synaptic contacts on GAD-positive hippocampal cells (Fig. 6H).[9] These findings suggest that the impairment of GABAergic SH innervation, which modulates hippocampal network activity, may be linked to the cognitive deficits present in J20 and VLW mice.[9,30] We subsequently characterized the GABAergic SH pathway in J20/VLW animals. Neither the distribution nor percentage of GAD-positive neurons contacted by septal GABAergic fibres, nor the complexity of the GABAergic SH contacts were altered in 8-month-old J20/VLW mice compared to wild-type animals (Fig. 6). We next studied GABAergic SH innervation on PV-positive hippocampal interneurons in J20/VLW animals, the hippocampal interneuron

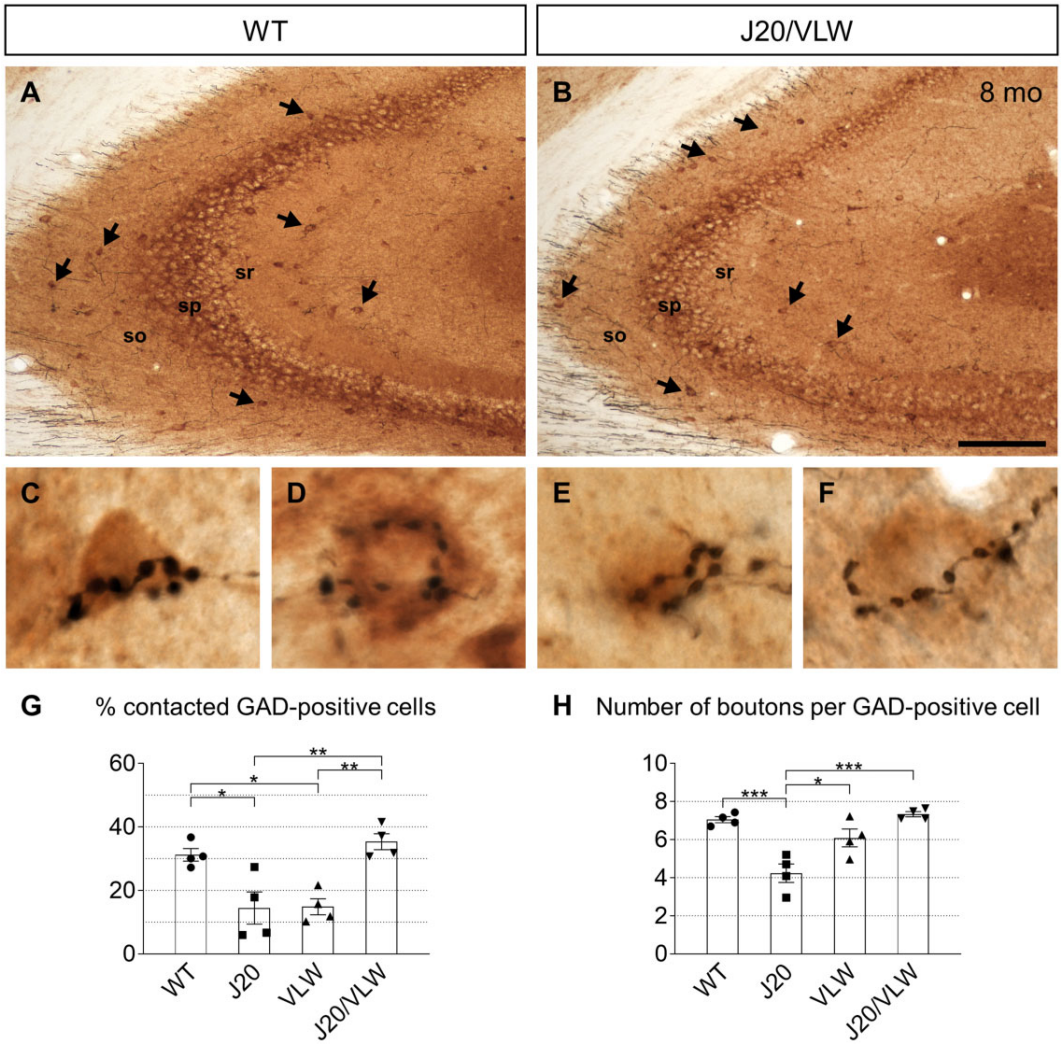

Figure 6 GABAergic SH innervation on GAD-positive cells is preserved in 8-month-old J20/VLW mice. Double immunodetection of GABAergic SH fibres and GAD-positive cells in hippocampal sections from 8-month-old wild-type (WT) and J20/VLW mice. (**A** and **B**) GABAergic SH fibres contacting GAD-positive cells (arrows) in the CA3 region of wild-type (**A**) and J20/VLW (**B**) mice. (**C**–**F**) GABAergic SH baskets forming synaptic boutons (black) on the soma of GAD-positive cells (brown) in wild-type (**C** and **D**) and J20/VLW (**E** and **F**) mice. (**G** and **H**) Quantification of the percentage of GAD-positive cells contacted by GABAergic SH fibres (**G**) and the complexity of GABAergic SH contacts (**H**) in wild-type, J20, VLW and J20/VLW mice. (**G** and **H**) One-way ANOVA and Tukey's *post hoc*, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. $n = 4$ animals per group, three sections per animal. Each dot represents the mean value per animal. Error bars represent SEM. so = stratum oriens; sp = stratum pyramidale; sr = stratum radiatum. Scale bars = 150 μm (**A** and **B**), 10 μm (**C**–**F**).

population most affected by the loss of GABAergic SH innervation in J20 and VLW mice.[9,30] These two groups exhibited a dramatic reduction in the percentage of PV-positive cells contacted by GABAergic SH fibres. Moreover, the complexity of the GABAergic SH synaptic contacts on these cells was diminished in both J20 and VLW mice (Fig. 7G and H).[9,30] In contrast, the distribution and the percentage of PV-containing interneurons contacted by GABAergic SH fibres were spared in J20/VLW mice, and only a slight decrease in the complexity of the GABAergic SH contacts on PV-positive cells occurred (Fig. 7).

To determine whether the improvement in the GABAergic SH innervation in J20/VLW animals is due to a permanent recovery or a delay in the impairment of the GABAergic SH pathway, we analysed 12-month-old mice. Neither a reduction in the percentage of GAD- and PV-positive neurons contacted by GABAergic SH fibres nor in the number of synaptic boutons per GAD- and PV-positive cell was observed in 12-month-old J20/VLW mice, compared to age-matched wild-type animals (Fig. 8).

These data indicate that the maintenance of correct GABAergic SH innervation in J20/VLW mice may contribute to the preservation of cognitive and physiological functions in this double transgenic mouse model. Our findings also suggest that the presence of Tau with a specific phosphorylation pattern, together with amyloid-β accumulation, in J20/VLW mice confers neuroprotection against the GABAergic SH denervation associated with individual amyloid-β and P-Tau pathologies.

To assess the GABAergic cell population in J20/VLW animals, we analysed the density and distribution of hippocampal and septal GABAergic neurons in 8- and 12-month-old mice by immunodetection. The distribution and density of hippocampal GABAergic neurons in J20/VLW mice were similar to those of wild-type counterparts. GAD-positive cells were located throughout distinct

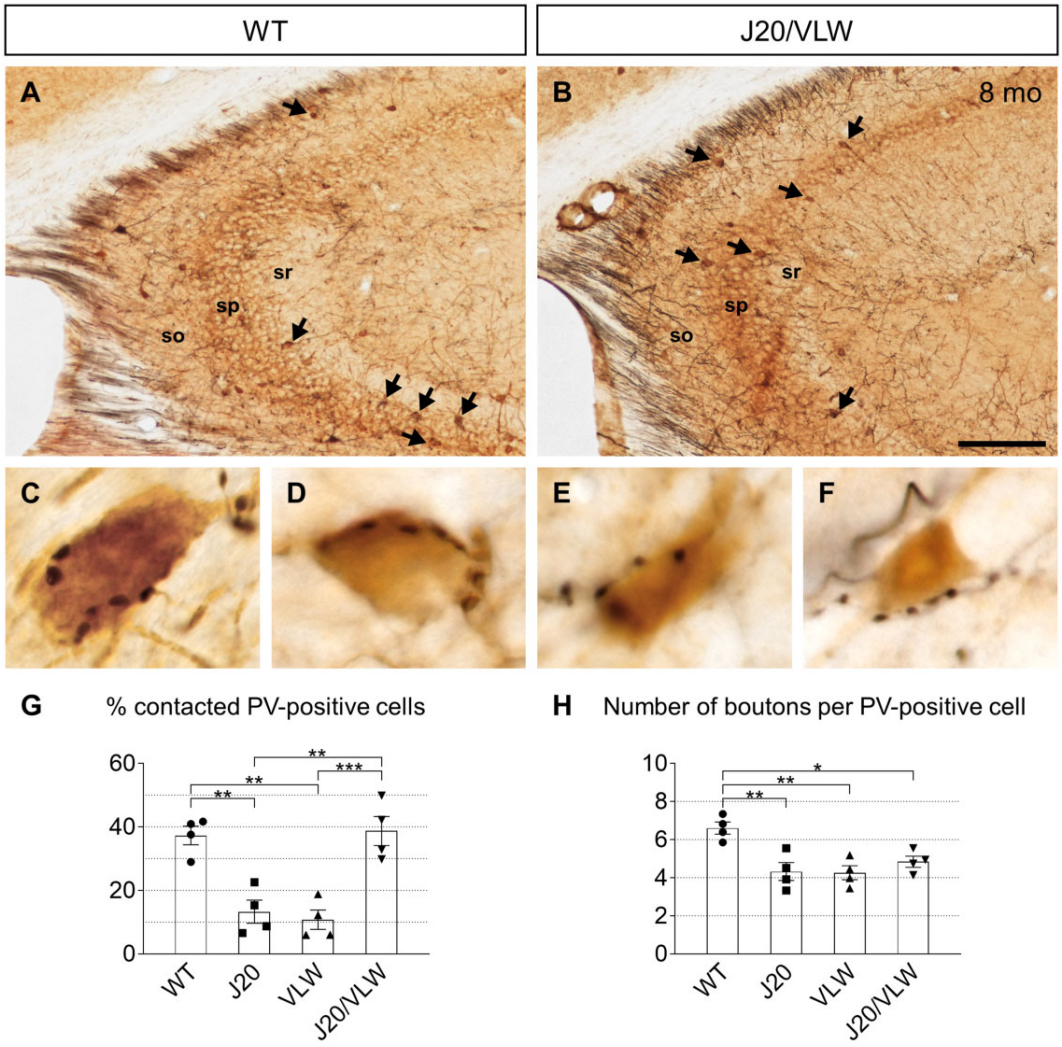

Figure 7 GABAergic SH innervation on PV-positive cells is preserved in 8-month-old J20/VLW mice. Double immunodetection of GABAergic SH fibres and PV-positive cells in hippocampal sections from 8-month-old wild-type (WT) and J20/VLW mice. (**A** and **B**) GABAergic SH fibres contacting PV-positive cells (arrows) in the CA3 region of wild-type (**A**) and J20/VLW (**B**) mice. (**C**–**F**) GABAergic SH baskets forming synaptic boutons (black) on the soma of PV-positive cells (brown) in wild-type (**C** and **D**) and J20/VLW (**E** and **F**) mice. (**G** and **H**) Quantification of the percentage of PV-positive cells contacted by GABAergic SH fibres (**G**) and the complexity of GABAergic SH contacts (**H**) in wild-type, J20, VLW and J20/VLW mice. (**G** and **H**) One-way ANOVA and Tukey's *post hoc*, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. $n = 4$ animals per group, three sections per animal. Each dot represents the mean value per animal. Error bars represent SEM. so = stratum oriens; sp = stratum pyramidale; sr = stratum radiatum. Scale bars = 150 μm (**A** and **B**), 10 μm (**C**–**F**).

layers and areas of the hippocampus (Supplementary Fig. 7A–D). Examination of the PV-positive subtype of interneurons revealed no alterations in either their distribution or density in the J20/VLW hippocampus (Supplementary Fig. 7E–H).

Finally, we studied the GABAergic SH neurons in the septal region by PV immunodetection. The distribution and density of PV-positive cells were preserved in the J20/VLW MSDB complex, compared to age-matched wild-type animals (Supplementary Fig. 8). We conclude that J20/VLW mice do not show an altered distribution or loss of septal and hippocampal GABAergic neurons.

Discussion

Growing evidence indicates that Tau and amyloid-β have opposing effects on neuronal excitability and circuit activity.[56,57] However, the coexistence of Tau- and amyloid-related pathologies has been proposed to act synergistically to impair neural circuit function, and recent studies suggest that Tau has a dominating effect over amyloid-β.[56,57] Moreover, Tau appears to be required for amyloid-β-induced toxicity and endogenous Tau reduction has been shown to improve cognitive deficiencies in J20 mice.[58,59] Conversely, the phosphorylation of Tau at Thr205 inhibits neurotoxicity.[53] A major finding of the present study is that the alterations in theta and gamma rhythms and cognitive deficits observed in single transgenic J20 and VLW animals are not present in J20/VLW mice. Thus, besides displaying amyloid-β and P-Tau pathologies, J20/VLW mice are protected against cognitive and electrophysiological impairments. This finding points J20/VLW mice as a model of NDAN, in which to investigate the fine mechanisms that mediate this relevant clinical entity. Our subsequent analyses suggest that the simultaneous presence of Tau phosphorylated at specific residues in hippocampal interneurons and of amyloid-β accumulation preserves hippocampal function in the double transgenic mouse model by maintaining a functional GABAergic SH pathway.

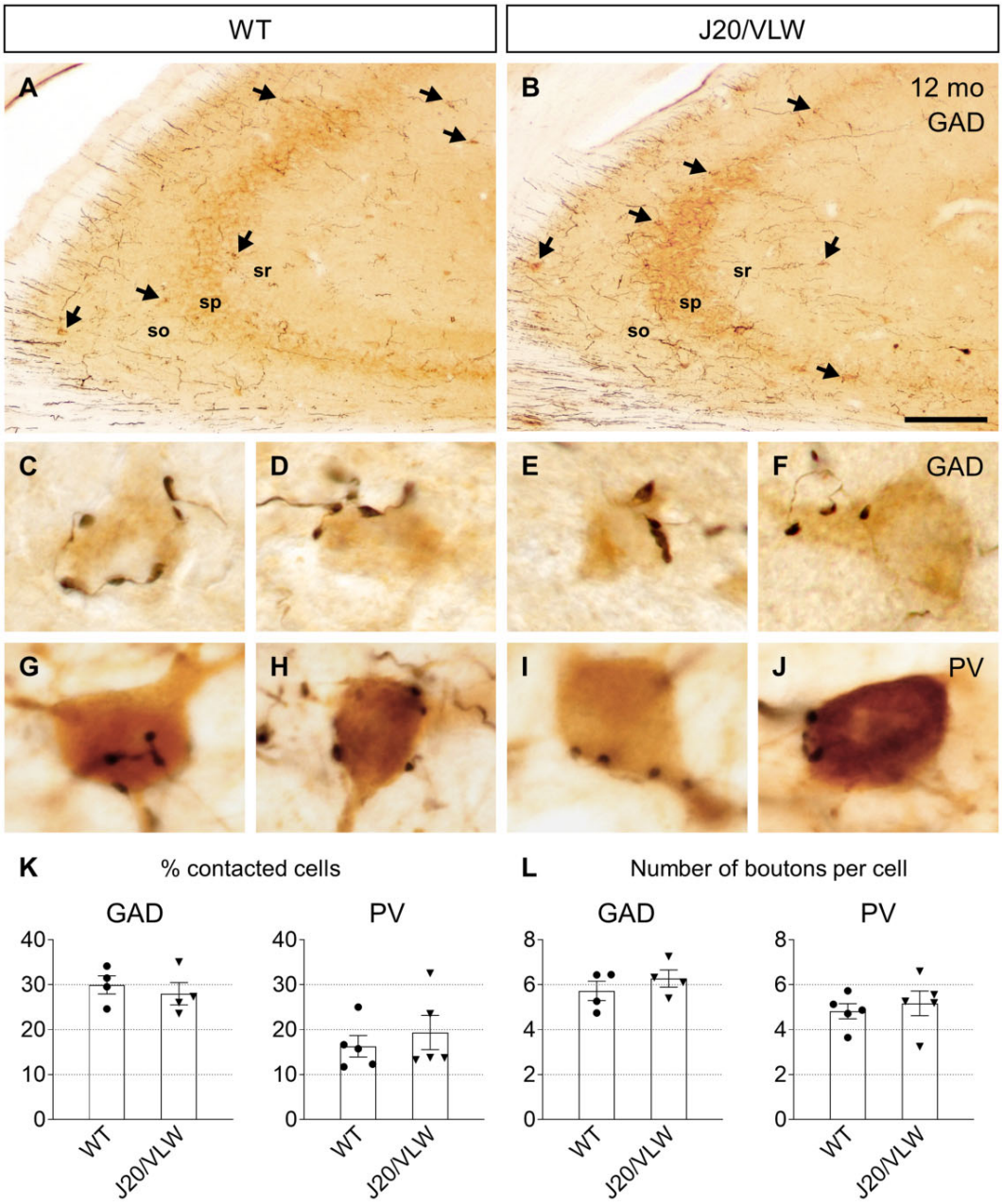

Figure 8 No alterations in GABAergic SH innervation are observed in 12-month-old J20/VLW mice. Double immunodetection of GABAergic SH fibres and GAD- or PV-positive cells in hippocampal sections from 12-month-old wild-type (WT) and J20/VLW mice. (**A** and **B**) GABAergic SH fibres contacting GAD-positive cells (arrows) in the CA3 region of wild-type (**A**) and J20/VLW (**B**) mice. (**C–F**) GABAergic SH baskets forming synaptic boutons (black) on the soma of GAD-positive cells (brown) in wild-type (**C** and **D**) and J20/VLW (**E** and **F**) mice. (**G–J**) GABAergic SH baskets forming synaptic boutons (black) on the soma of PV-positive cells (brown) in wild-type (**G** and **H**) and J20/VLW (**I** and **J**) mice. (**K** and **L**) Quantification of the percentage of GAD- and PV-positive cells contacted by GABAergic SH fibres (**K**) and the complexity of GABAergic SH contacts (**L**) in wild-type and J20/VLW mice. (**K** and **L**) Student's *t*-test. $n = 4–5$ animals per group, three sections per animal. Each dot represents the mean value per animal. Error bars represent SEM. so = stratum oriens; sp = stratum pyramidale; sr = stratum radiatum. Scale bars = 150 μm (**A** and **B**), 10 μm (**C–J**).

While amyloid-β may cause Tau phosphorylation and Tau may increase amyloid-β toxicity, there are conflicting lines of evidence as to whether Tau leads to an increase in amyloid deposition. Certain APP/Tau mice overexpressing hAPP^{Sw} together with hTau P301L (JNPL3/Tg2576 and APP23/B6P301L) show no differences in amyloid-β plaque load compared to single transgenic hAPP^{Sw} mice.[60,61] However, 16-month-old Tg2576/VLW mice displayed enhanced amyloid deposition.[62] Our data reveal no changes in soluble amyloid-β or amyloid-β deposition in J20/VLW mice compared to J20 animals.

Previous studies demonstrated that amyloid-β increases Tau phosphorylation.[63–65] No major changes in the levels of soluble pThr231 Tau were detected in the four experimental groups analysed. In contrast, the levels of pThr205 Tau showed a clear upwards trend in J20 and J20/VLW mice when compared to wild-type and VLW animals. This observation suggests an effect of

amyloid-β on Tau phosphorylation at Thr205, as previously described.[53,54] Also, we did not detect changes in the levels of Tau phosphorylation at either Thr231 or Thr205 in hippocampal pyramidal neurons when comparing VLW and J20/VLW mice, or at pSer262 Tau, which is absent in pyramidal neurons in both animal models. In addition, P-Tau mislocalization to the somatodendritic compartment of pyramidal neurons described previously in VLW mice[29,30,54] occurred in the J20/VLW hippocampus.

Thus, we conclude that the cognitive preservation observed in J20/VLW animals is not due to a reduction in amyloid-β load or to changes in the levels of P-Tau in pyramidal neurons.

In addition to the presence of P-Tau in pyramidal neurons,[66,67] the somas of hippocampal interneurons accumulate P-Tau in control and pathological conditions.[54] As in hAPP^{Sw}/VLW mice,[65] our data indicate that the presence of amyloid-β enhanced Tau microtubule-binding domain phosphorylation at non-proline directed phosphorylation sites such as Ser262 in J20/VLW interneurons in comparison to VLW mice. These findings reveal that amyloid-β facilitates Tau phosphorylation at these sites specifically in GABAergic neurons. The phosphorylation of Tau at Ser262 induces its detachment from microtubules.[68] Moreover, it has been reported that amyloid-β induction of pThr231 Tau is dependent on pSer262.[68] Thus, the clear upwards trend in the density of pThr231 Tau-positive interneurons in J20/VLW mice may result from the increased number of pSer262 Tau-positive interneurons in these animals. Taken together, these data suggest that, in the J20/VLW hippocampus, the increase in Tau phosphorylated at both Ser262 and Thr231 in GABAergic neurons facilitates the somatic localization of Tau, thereby favouring a novel function of this protein in the soma of hippocampal interneurons in this mouse model.

Amyloid-β species cause synaptic loss and dysfunction in both glutamatergic and GABAergic synapses.[9,69] We previously described that amyloid-β accumulation in J20 mice and presence of P-Tau in VLW animals induce abnormal GABAergic SH innervation in these two animal models.[9,30] Our results indicate that J20/VLW mice show a correct GABAergic SH innervation, thereby suggesting that the presence of P-Tau together with amyloid-β accumulation in J20/VLW mice protects against the GABAergic SH denervation associated with amyloid-β and P-Tau separately. Our findings also confirm a permanent effect since no alterations were observed in either 8- or 12-month-old J20/VLW animals. No changes in amyloid-β load or P-Tau accumulation and mislocalization in pyramidal neurons occurred in J20/VLW animals compared to J20 or VLW mice, respectively. Therefore, our findings suggest that the maintenance of correct GABAergic SH innervation in J20/VLW mice is due to the specific pattern of Tau phosphorylation in hippocampal interneurons. In this regard, our data indicate that GABAergic SH innervation is preserved on interneurons accumulating pThr205 Tau in J20/VLW mice, contrary to what is observed in J20 and VLW mice. Conversely, our results regarding pSer262 and pThr231 Tau support the notion that Tau phosphorylated at a single residue in the distinct experimental groups does not have a direct effect on GABAergic SH innervation. This observation would thus support the notion that combined phosphorylation at particular residues of Tau in hippocampal interneurons provides the underlying basis for the maintenance of this pathway.

Tau may participate in the dynamic regulation of $GABA_A$ receptor trafficking at inhibitory synapses through the scaffolding protein gephyrin, which is directly linked to the cytoskeleton.[70] By regulating the clustering of this receptor, gephyrin controls GABAergic synaptic activity and, therefore, inhibitory transmission.[71] Also, it has been described that glycogen synthase kinase 3β (GSK3β), a major Tau kinase activated by amyloid-β, regulates GABAergic synapse formation via the phosphorylation of gephyrin.[72,73]

Further research is required to gain a full understanding of the molecular mechanisms by which a distinct pattern of Tau phosphorylation modulates the function of GABAergic neurons. However, one hypothesis is that GSK3β activation by amyloid-β induces both an increase in P-Tau in the soma of GABAergic interneurons and the phosphorylation of gephyrin, thereby contributing to $GABA_A$ receptor clustering and thus preserving the GABAergic SH synaptic contacts on hippocampal interneurons and stabilizing inhibitory synaptic activity.

Our previous data indicated that impaired GABAergic SH innervation in J20 mice correlates with altered patterns of neuronal hippocampal activity and with internal processes related to operant rewards. Spectral analysis showed a clear decrease in the spectral power of theta and gamma bands in J20 mice, compared to age-matched wild-type animals.[9,28] Furthermore, VLW animals overexpressing mutant hTau display hyperexcitability in the absence of amyloid-β, along with alterations in the GABAergic SH pathway.[30,31] Here we demonstrate a considerable reduction in theta spectral power in VLW animals. In contrast, J20/VLW mice showed only a slight decrease in the spectral power of theta and gamma bands. It has been proposed that the GABAergic SH pathway regulates oscillatory activity, particularly through the recruitment of hippocampal interneurons. The main targets of the GABAergic SH fibres are the axo-axonic and basket PV-positive neurons, which regulate the firing of a large number of pyramidal neurons, hence leading to the generation of oscillatory activity in the range of the theta and gamma frequencies. Overall, our data indicate that, in contrast to the major alterations in theta and gamma rhythms in single transgenic J20 and VLW animals, J20/VLW mice show only minor alterations, pointing to a correlation between preserved GABAergic SH innervation and proper hippocampal rhythmic activity.

The present study explores the relevance of correct GABAergic SH innervation and electrophysiological preservation for the cognitive state of J20/VLW animals. Our results indicate that the simultaneous presence of amyloid-β and P-Tau reverses the cognitive impairment observed in J20 and VLW mice. As described in Alzheimer's disease and some animal models of this pathology,[8,10,16] J20 and VLW animals displayed an imbalance between excitatory and inhibitory circuits associated with hyperexcitability and cognitive deficits. In contrast, J20/VLW animals showed no major alterations in theta and gamma hippocampal rhythms, thereby suggesting a correct excitation/inhibition balance, probably modulated by GABAergic SH fibres and, therefore, by correct hippocampal GABAergic function. Our results reveal no cognitive deficits in J20/VLW animals and point to a correlation between correct GABAergic synaptic function and cognition.

Nevertheless, further studies are needed to confirm the causal role of GABAergic SH pathway preservation in the prevention of cognitive impairment. An interesting experiment to investigate this question would be the intervention to manipulate GABAergic SH pathway activity in J20/VLW mice. This paradigm would allow ascertaining whether either blocking or stimulating the GABAergic SH pathway results in the development of cognitive deficits in our double transgenic model. Similar experiments injecting amyloid-β in the medial septum demonstrated dramatic reductions of hippocampal theta spectral power and significant impairment of memory retention.[74,75] Conversely, the opposite paradigm has also been tested, and it proved that direct optogenetic stimulation of PV-positive GABAergic SH neurons rescues memory impairment in J20 mice.[14] Moreover, indirect manipulation of the medial septum by injecting amyloid-β in the hippocampus, which would be a situation similar to that of J20 mice, demonstrated a significant reduction in the firing rate of GABAergic SH neurons, theta spectral power and recognition memory.[76]

Several research groups have recently described individuals presenting amyloid-β plaques and P-Tau tangles in the absence of cognitive impairment.[32–37] These NDAN participants are thought to have intrinsic mechanisms conferring protection against Alzheimer's disease-associated dementia.

A full understanding of the mechanisms underlying cognitive neuroprotection in the presence of both amyloid-β and P-Tau pathological traits could have a major impact on the design of therapeutic strategies aimed at preventing cognitive decline in patients of Alzheimer's disease. A major factor proposed as responsible for this protection is the preservation of the synaptic machinery that normally degenerates in Alzheimer's disease.[32,36,37] In this regard, we propose that the simultaneous presence of amyloid-β and Tau with a specific phosphorylation pattern in J20/VLW mice confers protection against the synaptotoxic effects of pathogenic oligomers, as seen in NDAN individuals, or triggers a differential pattern of gene expression that protects synaptic structure and function. Our results also point to the preservation of the GABAergic network as a possible factor underlying the recovery of cognitive and physiological deficits in J20/VLW mice. It is worth noting that the studies on NDAN subjects focus on glutamatergic synapses.[32,36,37] Therefore, it would be of interest to analyse the state of GABAergic synapses in NDAN individuals to shed light on potential commonalities shared with J20/VLW mice.

Consistently, post-mortem neuropathological examination and brain imaging studies have revealed cell loss and volume reductions in the nucleus basalis of Meynert and MSDB complex of the basal forebrain in Alzheimer's disease,[77–80] and it is unknown whether NDAN also presents these features. However, to the best of our knowledge, the state of the GABAergic SH pathway in humans has not been studied to date. Our present data, together with the imbalance between excitatory and inhibitory circuits and in the GABAergic network, and oscillatory dysfunctions recurrently associated to Alzheimer's disease,[3–10,16,17] highlight the relevance of analysing the GABAergic SH pathway in humans.

Taken together, our findings suggest that the differential Tau phosphorylation pattern in hippocampal interneurons of J20/VLW mice protects against the loss of GABAergic SH innervation, thereby preventing alterations in LFPs and, subsequently, hindering cognitive deficits. These data support a new role of P-Tau in the maintenance of the GABAergic SH network and hippocampal GABAergic activity and indicate the potential of P-Tau regulation in GABAergic neurons as a therapeutic target in Alzheimer's disease. Finally, we propose the double transgenic mouse line generated herein as a suitable animal model in which to study the cognitive preservation in NDAN participants and to open up new therapeutic strategies to treat Alzheimer's disease-associated dementia.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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Supplementary Material

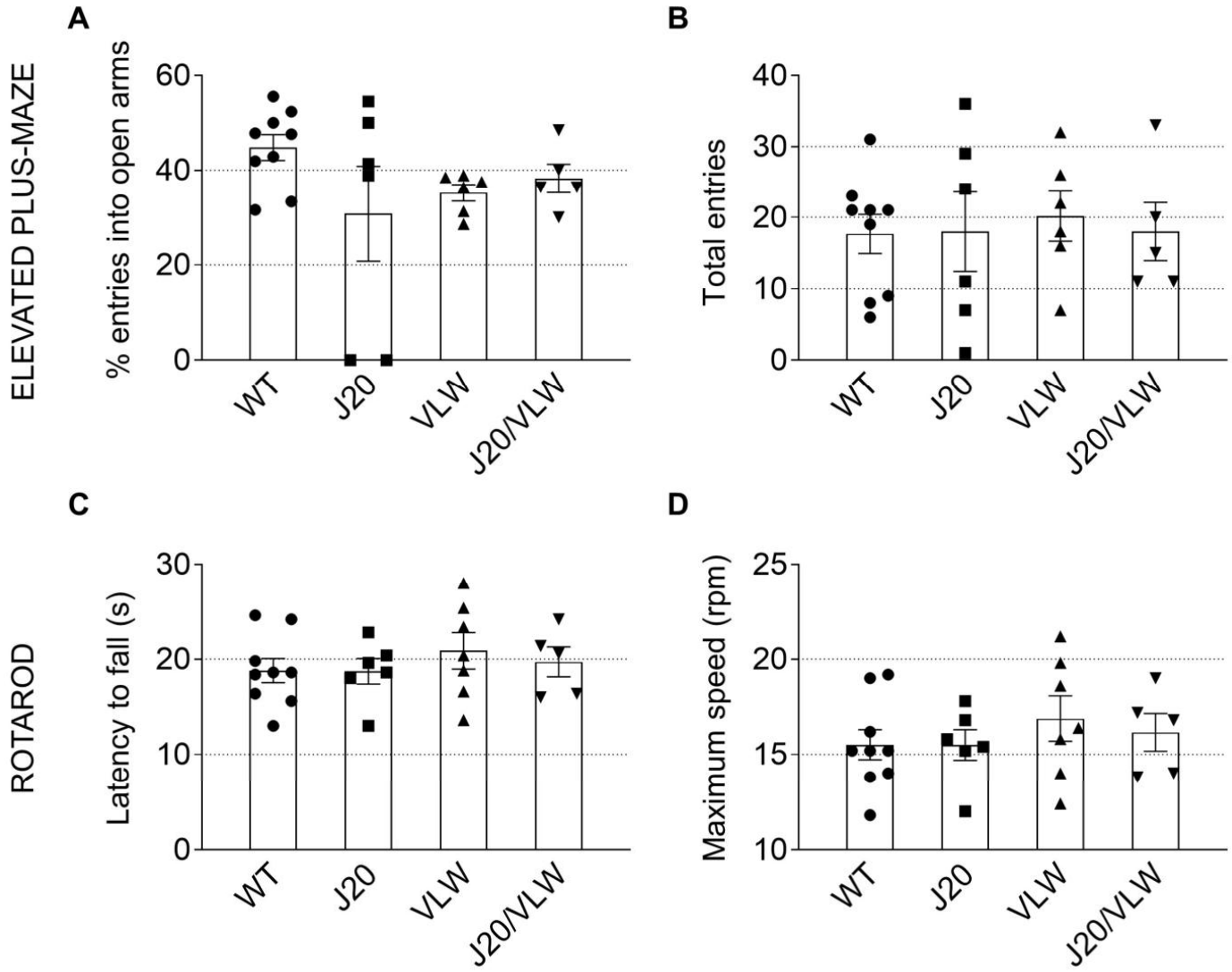
for

Functional protection in J20/VLW mice: a model of Non-Demented with Alzheimer's disease Neuropathology

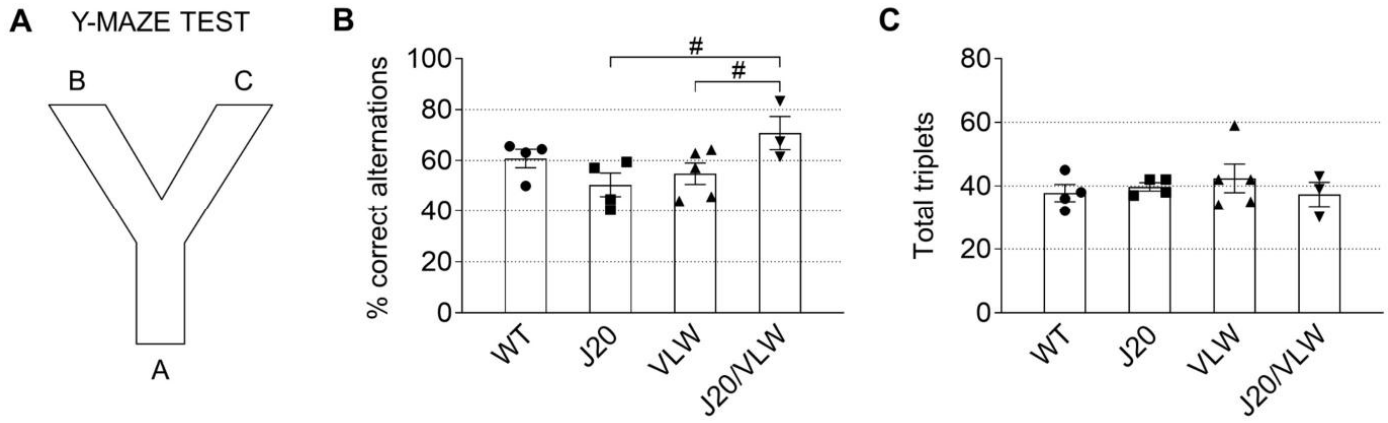
Eva Dávila-Bouziguet et al.

Supplementary Figures 1–8

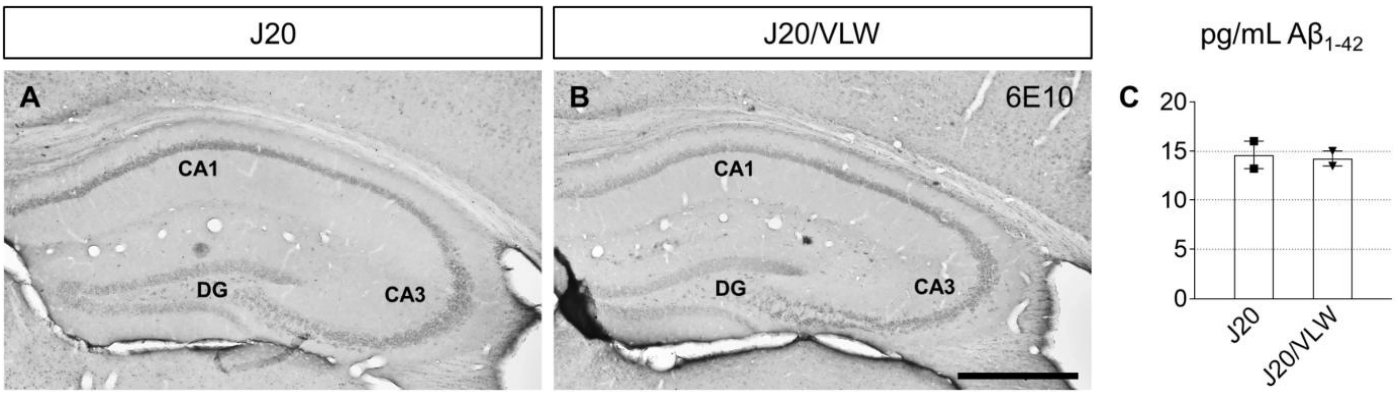
Supplementary Materials and Methods



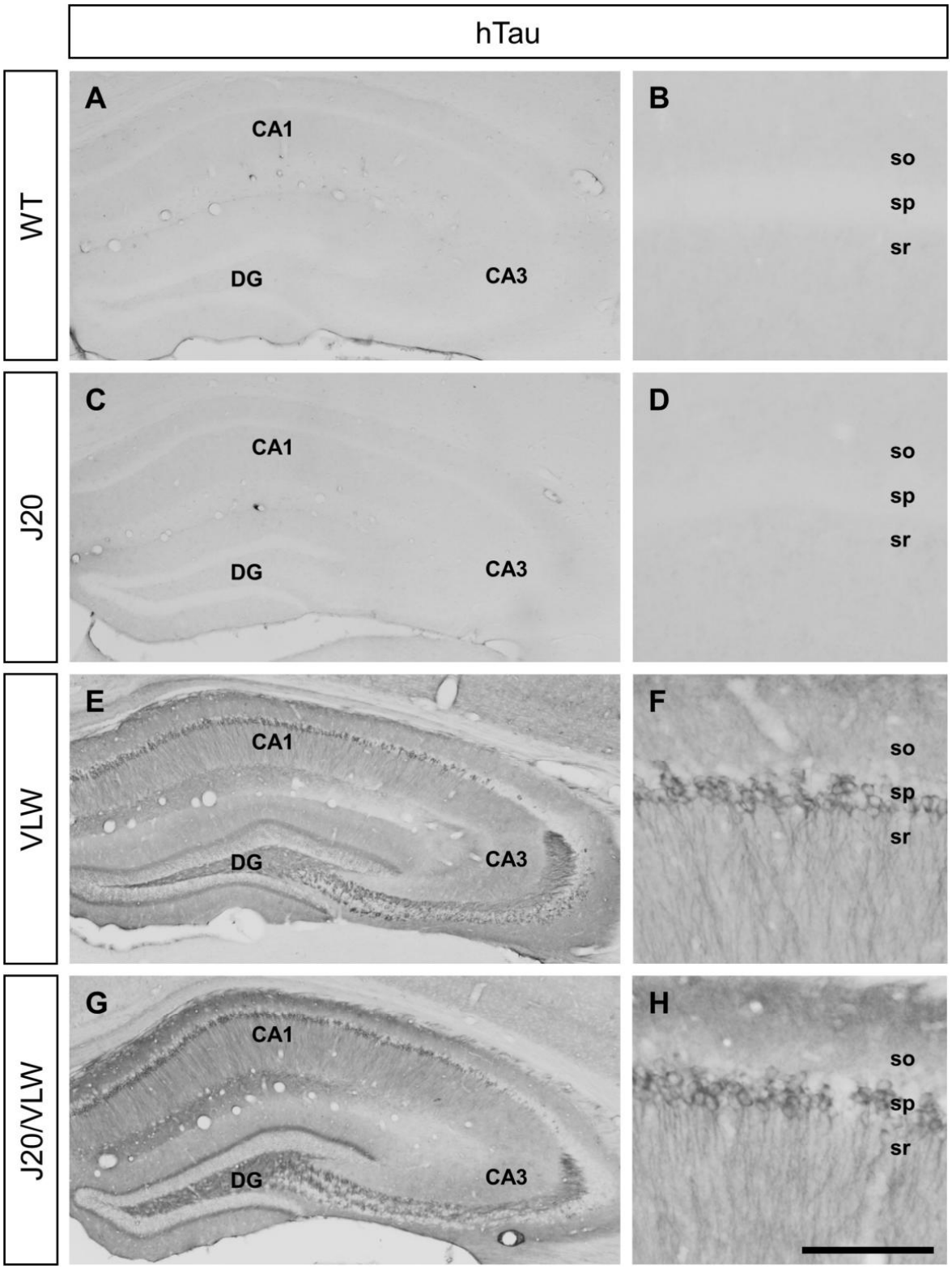
Supplementary Figure 1. No major alterations in anxiety or motor coordination are present in J20/VLW mice. (**A** and **B**) Anxiety-like behavior was assessed using the elevated plus-maze and no significant differences were observed in either the percentage of entries into the open arms (**A**) or the total entries into closed and open arms (**B**) between the four experimental groups. (**C** and **D**) Motor coordination was measured using the accelerating rotarod and no alterations were observed in either the latency to fall (**C**) or the maximum speed (**D**) between the four experimental groups. For (**A**–**D**): one-way ANOVA and Fisher's LSD *post hoc*. 8 mo WT (n = 9), J20 (n = 6), VLW (n = 7), and J20/VLW (n = 5) mice. Each dot represents the mean value per animal. Error bars represent SEM.



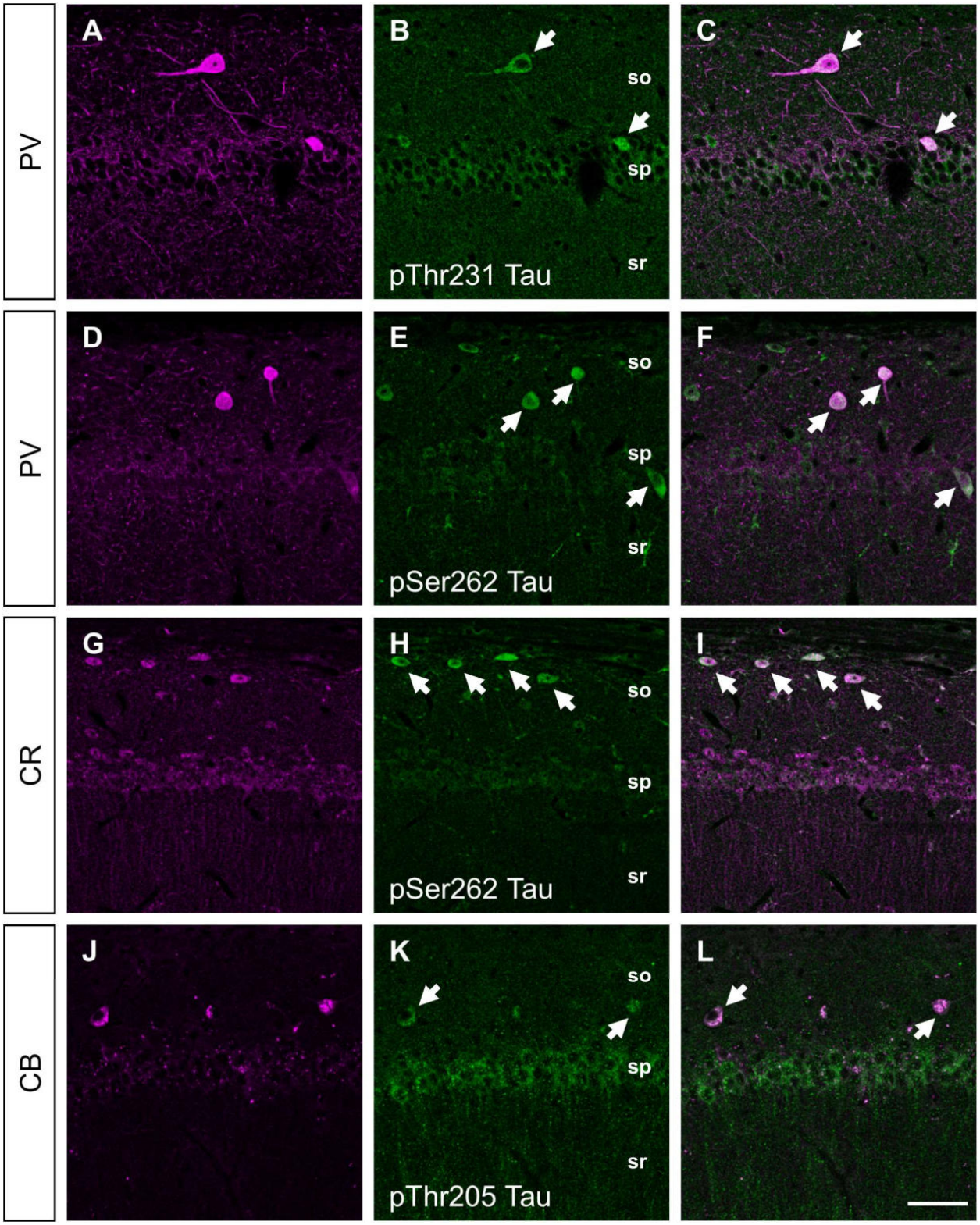
Supplementary Figure 2. No spatial working memory deficits are present in J20/VLW animals. (**A**) Setup for Y-maze test. (**B**) J20/VLW mice present a higher percentage of correct alternations than J20 and VLW animals. (**C**) No significant differences were observed in the number of total triplets between the four experimental groups. For (**B**) and (**C**): one-way ANOVA and Fisher's LSD *post hoc*, $^{\#}P < 0.05$ in *post hoc* when one-way ANOVA is close to significant ($P = 0.067$). 8 mo WT ($n = 4$), J20 ($n = 4$), VLW ($n = 5$), and J20/VLW ($n = 3$) mice. Each dot represents the mean value per animal. Error bars represent SEM.



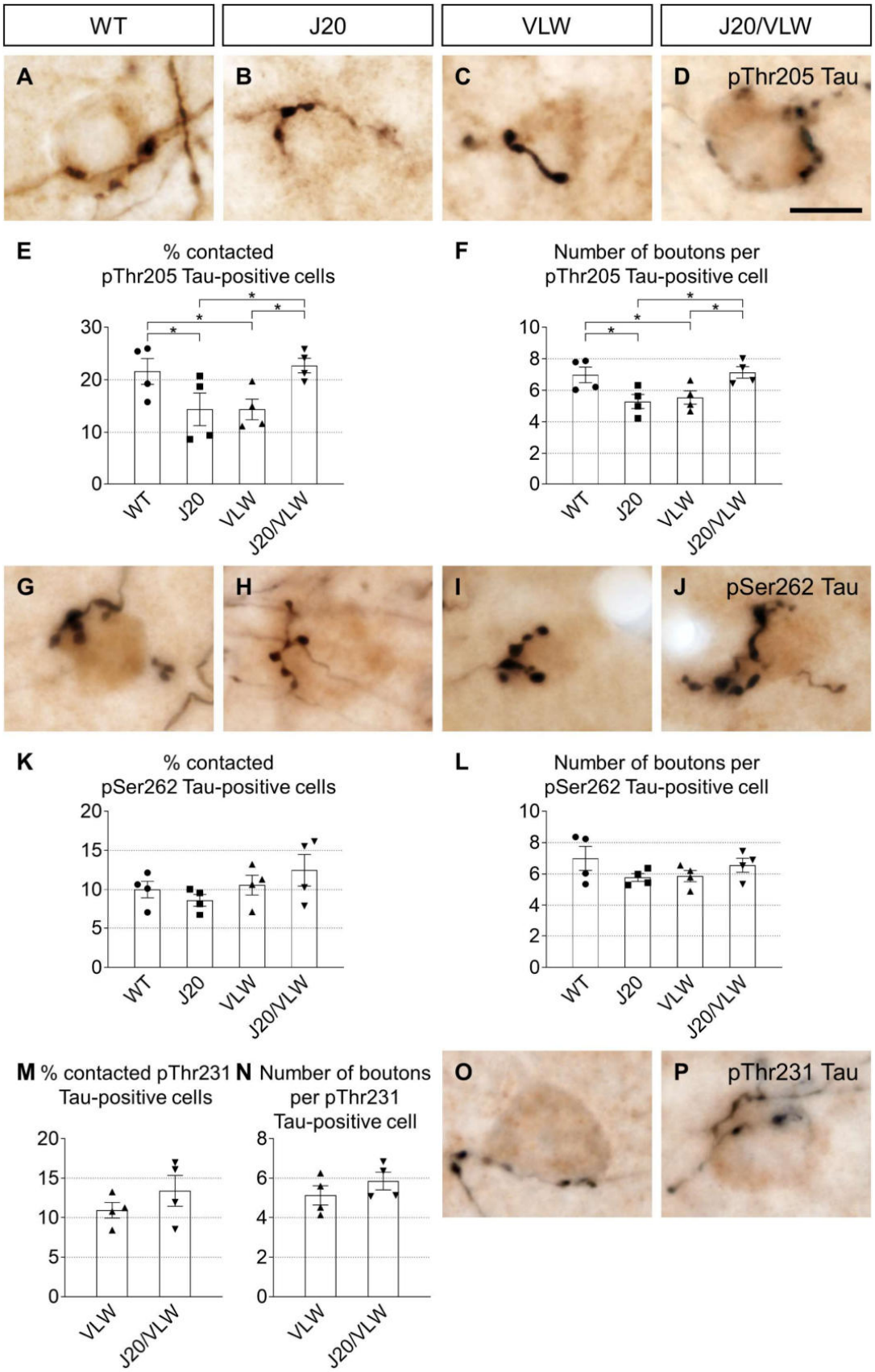
Supplementary Figure 3. Aβ levels of J20/VLW mice are similar to those of J20 animals. Immunodetection and ELISA assay of Aβ. (**A** and **B**) Immunodetection of Aβ and hAPP with 6E10 antibody in hippocampal sections from 8 mo J20 and J20/VLW mice. (**C**) ELISA assay against $A\beta_{1-42}$ in hippocampal and cortical protein extracts from 6 mo J20 and J20/VLW mice. For (**C**): Student's *t*-test. $n = 2$ animals per group. Each dot represents the mean value per animal. Error bars represent SEM. Scale bar: 500 μm.



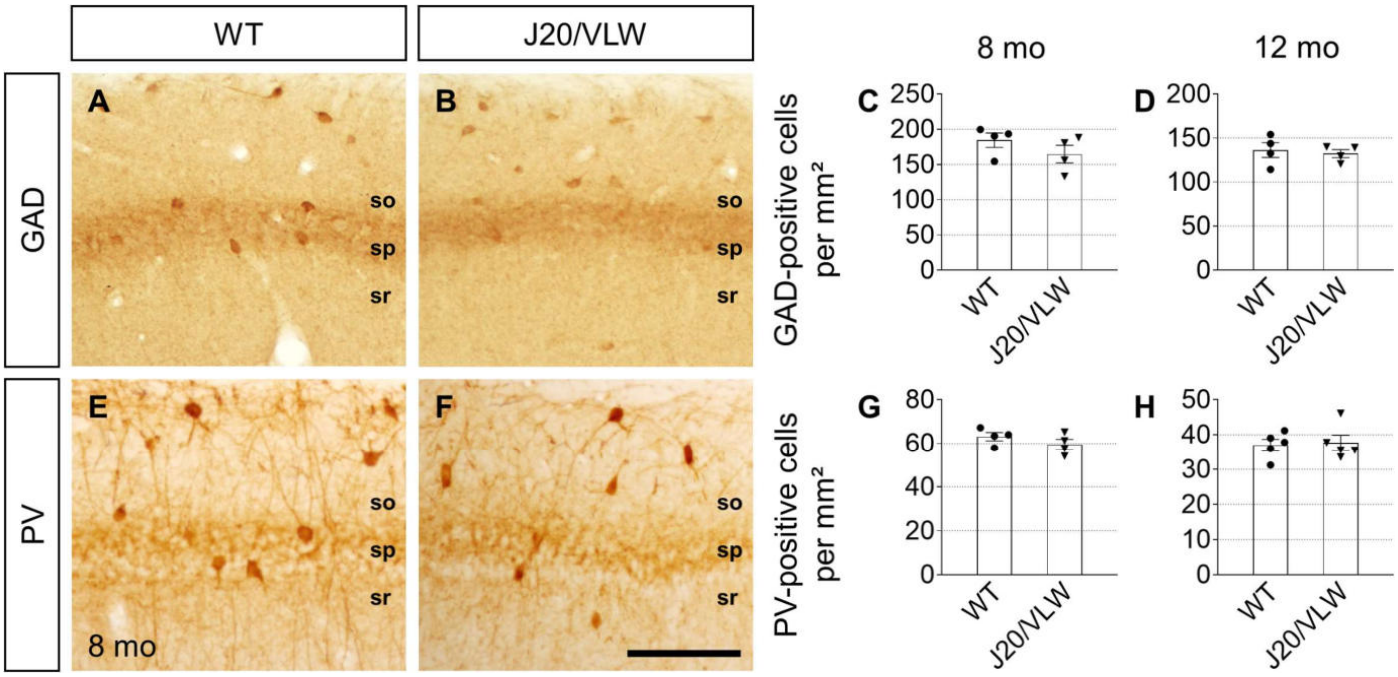
Supplementary Figure 4. hTau is present only in pyramidal neurons of VLW and J20/VLW mice. Immunodetection of hTau with HT7 antibody in hippocampal sections from 8 mo WT, J20, VLW, and J20/VLW animals. WT (**A** and **B**) and J20 (**C** and **D**) mice do not display hTau-positive cells, whereas hippocampal pyramidal cells of VLW (**E** and **F**) and J20/VLW (**G** and **H**) animals present hTau. Abbreviations: so = *stratum oriens*; sp = *stratum pyramidale*; sr = *stratum radiatum*. Scale bar: 500 µm (**A**, **C**, **E**, and **G**), 100 µm (**B**, **D**, **F**, and **H**).



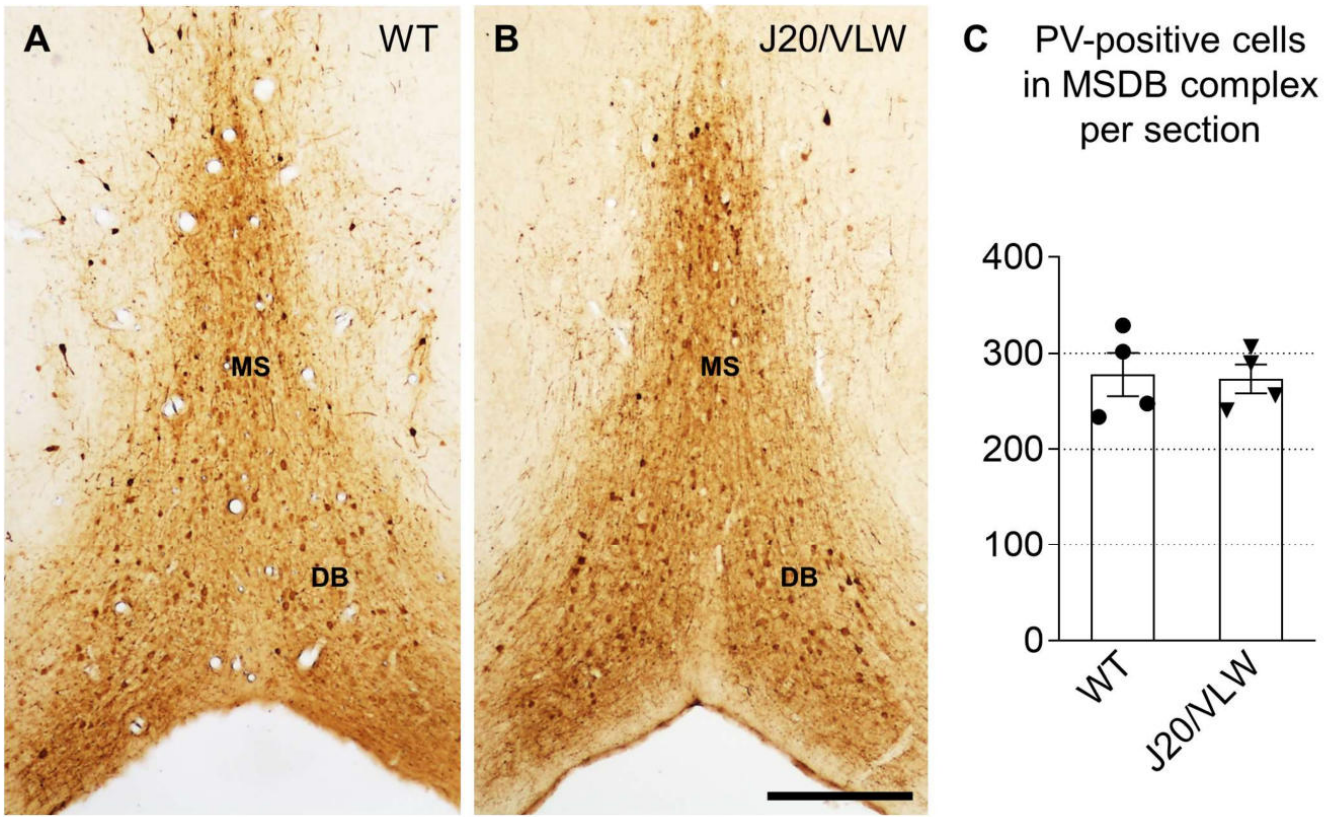
Supplementary Figure 5. pThr231 Tau accumulates in PV-positive interneurons, and pThr205 and pSer262 Tau in PV-, CR-, and CB-positive interneurons in J20/VLW mice. Double immunofluorescent detection of pThr231, pThr205, or pSer262 Tau and interneuron markers PV, CR, or CB in hippocampal sections from 8 mo J20/VLW mice. (**A**–**C**) PV-positive hippocampal interneurons of J20/VLW mice (magenta, **A**) accumulate Tau phosphorylated at Thr231 (green, **B**). Colocalization of pThr231 Tau with PV-positive interneurons (arrows in **C**). (**D**–**I**) PV- and CR-positive hippocampal interneurons of J20/VLW mice (magenta, **D** and **G**) accumulate Tau phosphorylated at Ser262 (green, **E** and **H**). Colocalization of pSer262 Tau with PV- (arrows in **F**) and CR-positive interneurons (arrows in **I**). (**J**–**L**) CB-positive hippocampal interneurons of J20/VLW mice (magenta, **J**) accumulate Tau phosphorylated at Thr205 (green, **K**). Colocalization of pThr205 Tau with CB-positive interneurons (arrows in **L**). Abbreviations: CB = calbindin; CR = calretinin; PV = parvalbumin; so = *stratum oriens*; sp = *stratum pyramidale*; sr = *stratum radiatum*. Scale bar: 50 µm.



Supplementary Figure 6. GABAergic SH innervation on P-Tau-positive cells. Double immunodetection of GABAergic SH fibers and P-Tau markers in hippocampal sections from 8 mo WT, J20, VLW, and J20/VLW mice. (**A**–**D**, **G**–**J**, **O** and **P**) GABAergic SH baskets forming synaptic boutons (black) on the soma of pThr205 (**A**–**D**), pSer262 (**G**–**J**), and pThr231 (**O** and **P**) Tau-positive cells (brown). (**E** and **F**, **K** and **L**, **M** and **N**) Quantification of the percentage of pThr205 (**E** and **F**), pSer262 (**K** and **L**), and pThr231 (**M** and **N**) Tau-positive cells contacted by GABAergic SH fibers and the complexity of GABAergic SH contacts. For (**E**), (**F**), (**K**), and (**L**): one-way ANOVA and Fisher's LSD *post hoc*, $*P < 0.05$. For (**M**) and (**N**): Student's *t*-test. $n = 4$ animals per group, 3 sections per animal. Each dot represents the mean value per animal. Error bars represent SEM. Scale bar: 10 µm.



Supplementary Figure 7. No loss of hippocampal GABAergic interneurons is observed in J20/VLW mice. Immunodetection and cell density quantification of the total GABAergic interneuron population (GAD-positive cells) and the PV-positive interneuron subpopulation in hippocampal sections from 8 and 12 mo WT and J20/VLW mice. (**A** and **B**, **E** and **F**) GAD- (**A** and **B**) and PV-positive cells (**E** and **F**) in the CA1 region of the hippocampus of 8 mo WT (**A** and **E**) and J20/VLW (**B** and **F**) mice. (**C** and **D**) Density quantification of GAD-positive cells in the hippocampus of 8 (**C**) and 12 (**D**) mo WT and J20/VLW mice. (**G** and **H**) Density quantification of PV-positive cells in the hippocampus of 8 (**G**) and 12 (**H**) mo WT and J20/VLW mice. For (**C**), (**D**), (**G**), and (**H**): Student's *t*-test. *n* = 4–5 animals per group, 3 sections per animal. Each dot represents the mean value per animal. Error bars represent SEM. Abbreviations: GAD = glutamic acid decarboxylase; PV = parvalbumin; so = *stratum oriens*; sp = *stratum pyramidale*; sr = *stratum radiatum*. Scale bar: 100 µm.



Supplementary Figure 8. No loss of GABAergic SH neurons is observed in J20/VLW mice. Immunodetection of GABAergic SH neurons with PV antibody in septal sections from 8 mo WT and J20/VLW mice. (**A** and **B**) GABAergic SH cells, which express PV, in the MSDB complex of WT (**A**) and J20/VLW mice (**B**). (**C**) Density quantification of PV-positive cells in the MSDB complex of WT and J20/VLW mice. For (**C**): Student's *t*-test. *n* = 4 animals per group, 4 sections per animal. Each dot represents the mean value per animal. Error bars represent SEM. Abbreviations: DB = nucleus of the diagonal band of Broca; MS = medial septal nucleus; PV = parvalbumin. Scale bar: 300 µm.

Supplementary Materials and Methods

ELISA

An ultrasensitive, human-specific Aβ (amino acids 1–42) ELISA kit was obtained (Invitrogen™), and the assay was performed following the manufacturer's protocol. Briefly, samples and detector antibody were incubated in a 96-well plate pre-coated with capture antibody. The plate was then washed and incubated with an HRP-labeled secondary antibody solution. Following successive washes, the plate was incubated with a tetramethylbenzidine substrate solution. The reaction was stopped, and absorbance was read at 450 nm using an Infinite M200 PRO plate reader (TECAN). Human Aβ (amino acids 1–42) concentrations were interpolated from a standard curve. All samples were run in duplicate.

Immunodetection

To determine whether hippocampal interneurons accumulate P-Tau, double fluorescent immunodetections were conducted using AT-180 mouse anti-phosphothreonine 231, T205 rabbit anti-phosphothreonine 205, or S262 rabbit anti-phosphoserine 262 and parvalbumin (PV), calretinin (CR), or calbindin (CB) primary antibodies. Sections were incubated simultaneously with AT-180 (Innogenetics; 1/300), T205 (Invitrogen™; 1/1000), or S262 (Invitrogen™; 1/100) and goat anti-PV (Swant®; 1/3000), goat anti-CR (Swant®; 1/3000), or mouse anti-CB (Swant®; 1/3000) antibodies. They were then incubated with Alexa Fluor 568 donkey anti-goat or anti-mouse IgG against interneuron primary antibodies, whereas P-Tau primary antibodies were targeted with Alexa Fluor 488 donkey anti-rabbit IgG or anti-mouse IgG (Invitrogen™; 1/1000). Sections were mounted onto slides and coverslipped with Mowiol® (Merck).

To detect GABAergic SH projection neurons, sections corresponding to the medial septum were incubated with rabbit anti-PV antibody (Swant®; 1/3000). Primary antibody was visualized by sequential incubation with biotinylated secondary antibody and the ABC complex (Vector Laboratories). Peroxidase activity was developed with hydrogen peroxide and diaminobenzidine. Sections were mounted onto gelatinized slides, dehydrated, and coverslipped with Eukitt® (O. Kindler).

Analysis of histological sections

To estimate the density of GABAergic SH neurons, samples stained with PV were scanned with a NanoZoomer 2.0HT whole slide imager (Hamamatsu Photonics) at 20x. The density of GABAergic SH neurons was quantified in serial MSDB complex sections and was defined as the density of PV-positive cells per section. The cells and the area comprising the MSDB complex were quantified using Fiji software.

Image acquisition

Optical microscopy (Nikon E600, Nikon Corporation) images of the immunohistochemically-stained brain sections were acquired through a digital camera (Olympus DP72, Olympus Corporation) coupled to the microscope and were processed by Cell F^ software (Olympus Corporation).

Confocal microscopy (Leica TCS SP5, Leica Microsystems) images of the immunofluorescence-stained samples were acquired using LAS AF software (Leica Microsystems). To observe possible colocalization between the interneuron and P-Tau markers, images were processed by Fiji software.

Behavioral tests

Y-maze

Spatial working memory was assessed using a Y-shaped maze, composed of three identical arms that intersected at 120° (each arm measuring 30 cm long × 6.5 cm wide × 15 cm high). Animals were left to freely explore the Y-maze for 5 min, starting from the end of the same arm in the maze facing the wall. A visit to the arm was defined as when mice traversed the head and two front paws. The percentage of consecutive visits to the three different arms (correct spontaneous alternation) was calculated considering sequential entries in all three arms, divided by the total number of possible alternations (triplets), calculated as the total number of entries minus two. Animals that performed less than 30 triplets in 5 min were excluded from the analysis to avoid an exploration bias.

Elevated plus-maze

Anxiety-like behavior was evaluated using the elevated plus-maze test. The setup consisted of a black Plexiglas apparatus with four arms (each arm measuring 29 cm long × 5 cm wide)—two open and two closed—set in a cross from a neutral central square (5 × 5 cm) elevated 40 cm above the floor. Light intensity in the open and closed arms was 45 and 5 lux, respectively. Mice were placed in the central square facing one of the open arms and tested for 5 min. The percentage of entries into the open arms was determined as 100 × (entries into open arms) / (entries into open arms + entries into closed arms). Animals that exited the maze during exploration were excluded from the analysis. Total entries into each arm were calculated as a control of exploratory behavior.

Rotarod

Motor coordination was assessed using the accelerating rotarod (5-lane accelerating rotarod; LE 8200, Panlab). On day one, mice were trained to hold onto the rod at a constant speed (4 rpm) for at least 120 s. On day two, mice were trained to hold onto the rod at a constant speed higher than that of the previous day (6 rpm) for at least 120 s. On day three, the test was performed. During the test, the rod accelerated from 4 to 40 rpm within 1 min, and latency to fall and maximum speed were measured in five consecutive trials. Data are expressed as the mean of the five trials.

DISCUSSION

1. Tau phosphorylation pattern in hippocampal interneurons in physiological and pathological conditions

In mature neurons, the primary location of Tau is the axon (Binder et al., 1985). Being an axonal protein able to bind to tubulin and promote microtubule assembly under experimental conditions, Tau is accepted to have its principal function in microtubule stabilization (Drubin & Kirschner, 1986; Weingarten et al., 1975). Nevertheless, this canonical microtubule-binding protein can also be located in the somatodendritic compartment and the nucleus (Brandt et al., 1995; L. M. Ittner et al., 2010; Mondragón-Rodríguez et al., 2012; Sultan et al., 2011; Violet et al., 2014). Numerous studies have reported roles for Tau in dendrites, the nucleus, and even in association with the plasma membrane, including regulation of synaptic plasticity and maintenance of the integrity of genomic DNA and cytoplasmic and nuclear RNA (Camero et al., 2014; Frandemiche et al., 2014; L. M. Ittner et al., 2010; Kimura et al., 2014; Mondragón-Rodríguez et al., 2012; Sultan et al., 2011; Violet et al., 2014).

The regulation of Tau function entails numerous post-translational modifications (Guo et al., 2017; Tapia-Rojas et al., 2019). Among Tau post-translational modifications, phosphorylation has received the most attention due to its profound impact on Tau activity and its involvement in pathological processes like Alzheimer's disease and other tauopathies (Ercan et al., 2017; Guo et al., 2017; Hanger et al., 2007; Morishima-Kawashima et al., 1995; Tapia-Rojas et al., 2019).

By using wild-type (WT) mice and Alzheimer's disease mouse models, as well as samples from human control subjects and Alzheimer's disease patients, we have studied the presence of P-Tau in the hippocampus in physiological and pathological conditions.

1.1 Tau phosphorylation pattern in mice and human subjects in physiological conditions

Several Tau phosphorylation sites have become prominent because they are phosphorylated in Alzheimer's disease (Augustinack et al., 2002; H. Braak et al., 2011; Hasegawa et al., 1992; Paudel et al., 1993). These sites are part of the epitopes recognized by antibodies raised against Alzheimer's disease Tau in a phosphorylation-dependent manner, which are thus used as diagnostic markers (Mandelkow & Mandelkow, 2012; Šimić et al., 2016). Among them, we have analyzed Tau phosphorylated at Thr205 (pThr205 Tau), at Thr231 (pThr231 Tau), and at Ser262 (pSer262 Tau).

Remarkably, our results reveal the presence of pThr205 Tau and pSer262 Tau in the soma of hippocampal interneurons in WT mice. Particularly, some GABAergic interneurons of the three subpopulations studied, PV-, CR-, and CB-positive interneurons, accumulate these species of P-Tau. In contrast, pThr231 Tau is absent from the hippocampus of WT animals. Among PV-positive interneurons, the main subpopulation of GABAergic hippocampal interneurons, more than half of the neurons accumulate pThr205 Tau or pSer262 Tau in WT animals. Analogous to WT mice, our data show that human control subjects present pThr205 Tau and pSer262 Tau in the soma of hippocampal interneurons. Thus, our findings reveal the presence of a specific pattern of phosphorylation and distribution of Tau in hippocampal interneurons in physiological conditions in mice and human subjects, suggesting that Tau exerts distinct, yet unknown, functions in the soma of this neuron population.

Tau phosphorylation at Thr205, Thr231, and Ser262 has been identified in postmortem samples of human subjects with normal cognition (Funk et al., 2014). Likewise, these P-Tau species have been detected in WT mice (Morris et al., 2015). This suggests that the phosphorylation state of endogenous Tau is similar in mice and human subjects in physiological conditions, supporting our present findings.

Phosphorylation is pivotal in the regulation of Tau function, but the number and location of phosphorylation sites determine the specific effects of phosphorylation (Kiris et al., 2011; F. Liu et al., 2007; Mi & Johnson, 2006). The primary role of post-translational modifications, like phosphorylation, is to regulate and increase the functional diversity of proteins by altering their electrostatic properties or structure. The conformational changes that result from phosphorylation control protein-protein interactions, signaling pathways, and protein degradation (Ardito et al., 2017; Ravid & Hochstrasser, 2008). Therefore, phosphorylation is essential for several physiological cellular processes.

In the case of Tau, phosphorylation is developmentally regulated, implying specialized functions of Tau at different developmental stages (Brion et al., 1993, 1994; Yu et al., 2009). In the adult brain, Tau phosphorylation may be critical in regulating its function, localization, and interaction with other proteins (Alquezar et al., 2021; Mueller et al., 2021; Tapia-Rojas et al., 2019). Phosphorylation of Tau in the microtubule-binding domain, including residue Ser262, or the proline-rich domain, like residues Thr205 and Thr231, reduces its affinity for microtubules (Biernat et al., 1993; Cho & Johnson, 2004; Drewes et al., 1995; Schwalbe et al., 2015; Sengupta et al., 1998). As Tau binding modulates the stability of microtubules, Tau phosphorylation regulates neuronal processes such as axonogenesis and neurite outgrowth in the developing brain and in adult hippocampal neurogenesis (Biernat et al., 2002; Biernat & Mandelkow, 1999; Fuster-Matanzo et al., 2012; X.-P. Hong et al., 2010;

Mandell & Banker, 1996a, 1996b). In the axon, phosphorylation of Tau also affects axonal transport. As Tau is involved in motor-driven anterograde fast axonal transport of membrane-bound organelles, including synaptic vesicles and mitochondria, its phosphorylation may facilitate their delivery to the correct destinations (Kanaan et al., 2011, 2012; Morfini et al., 2016).

Moreover, synaptic activity-dependent Tau phosphorylation induces its relocation from the axon into dendrites, and specifically into the postsynaptic compartment of glutamatergic synapses, where it can participate in synaptic plasticity (Frandemiche et al., 2014; L. M. Ittner et al., 2010; C. Li & Götz, 2017a; Mondragón-Rodríguez et al., 2012). In fact, Tau phosphorylation is required for hippocampal LTP and LTD (Ahmed et al., 2014; Frandemiche et al., 2014; Kimura et al., 2014; Mondragón-Rodríguez et al., 2012; Regan et al., 2015). Additionally, phosphorylation at particular residues of Tau differentially affects signaling pathways activated by neurotrophic factors (Leugers et al., 2013; Leugers & Lee, 2010). In physiological conditions, Tau phosphorylation at certain residues is higher in the somatodendritic compartment than in axons, suggesting that these phosphorylated sites modulate the activation of specific signaling pathways at discrete subcellular locations (Mandell & Banker, 1995; Papasozomenos & Binder, 1987).

Beyond the array of precise functions of Tau, accumulating data currently support the role of Tau as a scaffold protein for the regulation of phosphorylation-based signaling pathways, which would encompass most of its described roles (Alquezar et al., 2021; Götz et al., 2013; Morris et al., 2011; Mueller et al., 2021; Sotiropoulos et al., 2017; Trushina et al., 2019). Several features of Tau reinforce this notion. First, its extensive distribution allows the control and localization of signaling proteins to distinct subcellular compartments (Binder et al., 1985; Brandt et al., 1995; C. Liu & Götz, 2013; Loomis et al., 1990). Second, the numerous signaling proteins that interact with Tau provide it with a broad interactome that enables the coordination of many signaling pathways (Morris et al., 2011; Sinsky et al., 2020). Third, the highly dynamic conformational flexibility presented by Tau due to its nature as an intrinsically disordered protein facilitates the interaction with multiple binding partners (Brandt et al., 2020; Trushina et al., 2019; Uversky, 2015). Thus, its widespread subcellular distribution, broad interactome, and dynamic structural properties point to Tau as a scaffold protein or signaling hub (Alquezar et al., 2021; Götz et al., 2013; Morris et al., 2011; Mueller et al., 2021; Sotiropoulos et al., 2017; Trushina et al., 2019).

In physiological conditions, Tau phosphorylation at the sites we have observed in WT mice and human control subjects could be regulating essential cellular processes. In this context, these sites would only be phosphorylated in response to pertinent stimuli in physiologically relevant signaling pathways. In contrast, phosphorylation at these sites in

pathological conditions could be due to deviant stimuli causing an imbalance between kinase and phosphatase activities. Deregulation of Tau phosphorylation in these circumstances would explain Tau toxicity owing to its loss of physiological functions, aside from its commonly acknowledged gain of toxic functions. Moreover, we have found these P-Tau species specifically in the soma of GABAergic hippocampal interneurons, a neuron population often overlooked in Alzheimer's disease studies despite its crucial influence on neural circuits. In addition to the growing list of physiological Tau roles, considering the features of Tau mentioned above, it cannot be discarded that P-Tau exerts so far unidentified functions in the somatodendritic compartment of GABAergic neurons, which diverge in many aspects from glutamatergic neurons.

In the search for disease-modifying treatments for Alzheimer's disease, various therapeutic strategies have attempted to target Tau through anti-Tau antibodies or modulators of phosphorylation or aggregation (Cummings et al., 2019; M. Medina, 2018; VandeVrede et al., 2020; Y. Xia et al., 2021). Multiple pharmacological treatments currently under development or undergoing clinical trials, including kinase inhibitors, phosphatase activators, or immunotherapy, aim to decrease Tau phosphorylation to prevent the toxicity that results from the toxic gain of function of P-Tau, like mediating Aβ-induced toxicity (VandeVrede et al., 2020; Y. Xia et al., 2021). Our data point to a physiological role of pThr205 Tau and pSer262 Tau in the soma of hippocampal interneurons. Thus, caution must be taken when modulating Tau phosphorylation pharmacologically, as it could affect not only the toxic effects but also the vast repertoire of crucial activities dependent on it. Indeed, several clinical trials involving strategies to reduce Tau phosphorylation by targeting kinase or phosphatase activities have failed (VandeVrede et al., 2020; Y. Xia et al., 2021). A deeper understanding of the dynamics of Tau phosphorylation in different hippocampal neuron types under physiological and pathological conditions could contribute to the design of new therapeutic approaches for Alzheimer's disease and other tauopathies. Furthermore, broadening the current knowledge of Tau function and dysfunction may help determine the potential of interneurons as therapeutic targets to prevent the cognitive impairment associated with Alzheimer's disease.

1.2 Tau phosphorylation pattern in Alzheimer's disease mouse models and human patients

The main component of neurofibrillary tangles, one of the canonical hallmarks of Alzheimer's disease, is P-Tau. Hence, Tau phosphorylation has been considered a decisive factor in the pathogenesis of Alzheimer's disease. Nevertheless, as discussed above, Tau can be phosphorylated in a physiologically relevant manner.

We have found that Tau residues that are phosphorylated in Alzheimer's disease and are relevant to its diagnosis, Thr205 and Ser262, are phosphorylated in physiological conditions in mice and human subjects. Our results indicate that J20 and VLW animals, Alzheimer's disease mouse models expressing Aβ and mutant human Tau, respectively, also present pThr205 Tau and pSer262 Tau in the soma of hippocampal interneurons. Analogous to WT mice, some PV-, CR-, and CB-positive interneurons accumulate these P-Tau species. In the case of pThr231 Tau, it is absent from the hippocampus of J20 animals. Conversely, pThr231 Tau accumulates in the somatodendritic compartment of pyramidal neurons, in mossy cells of the dentate gyrus, and in the soma of hippocampal interneurons in VLW mice, as our group previously described (Soler et al., 2017). Regarding hippocampal interneurons, only the subpopulation of PV-positive interneurons accumulates pThr231 Tau, as previously reported by our group (Soler et al., 2017). Moreover, our results reveal the presence of pThr205 Tau and pSer262 Tau in the soma of hippocampal interneurons in Alzheimer's disease patients, similar to human control subjects.

It has been established that Aβ can induce Tau hyperphosphorylation (Bolmont et al., 2007; Busciglio et al., 1995; Götz et al., 2001; Jin et al., 2011; Nisbet et al., 2015; Pérez et al., 2005). Likewise, missense mutations in the *MAPT* gene cause Tau to be more prone to hyperphosphorylation (A. del C. Alonso et al., 2004; Han et al., 2009; Spillantini et al., 1998).

Our results show that the density of hippocampal interneurons accumulating pSer262 Tau does not differ in J20 and VLW mice compared to WT animals. Thus, phosphorylation of Tau at Ser262 does not seem to be influenced by the presence of Aβ and mutant human Tau in J20 and VLW mice, respectively. This suggests that pSer262 Tau has specific physiological functions in the soma of hippocampal interneurons independent of Alzheimer's disease pathology, despite being considered a pathological P-Tau species (Augustinack et al., 2002; H. Braak et al., 2011; Hasegawa et al., 1992; Mandelkow & Mandelkow, 2012; Šimić et al., 2016).

In contrast to pSer262 Tau, the density of hippocampal interneurons accumulating pThr205 Tau is decreased in VLW mice, compared to WT animals. It has been acknowledged that phosphorylation at specific residues can influence, by either inducing or repressing, the phosphorylation at other sites of Tau (Ando et al., 2016; Bertrand et al., 2010; Kimura et al., 2016; S. J. Liu et al., 2004; J.-Z. Wang et al., 1998; Zheng-Fischhofer et al., 1998). As our group previously described, we have found that PV-positive interneurons accumulate pThr231 Tau in VLW mice (Soler et al., 2017). Nevertheless, our results show that pThr231 Tau and pThr205 Tau can coexist in the same interneuron, suggesting that the former does not repress the latter.

Multiple kinases phosphorylate the same sites of Tau (Hanger et al., 2009; J.-Z. Wang et al., 2012). Although Tau contains phosphorylatable tyrosine residues, the vast majority are serine and threonine residues, and a large proportion of these are targeted by proline-directed protein kinases that phosphorylate serine or threonine residues in Ser/Thr-Pro sequences (Alquezar et al., 2021; Hanger et al., 2009). Glycogen synthase kinase 3β (GSK3β) is a proline-directed protein kinase phosphorylating Thr205 that requires the previous phosphorylation at other sites, which is referred to as priming (Kimura et al., 2018; Sutherland, 2011). Particularly, phosphorylation at Thr205 by GSK3β can be primed by protein kinase A and by cyclin-dependent kinase 5 (Cdk5) (Kimura et al., 2018; T. Li et al., 2006; S. J. Liu et al., 2004; Plattner et al., 2006; J.-Z. Wang et al., 1998). Cdk5 can phosphorylate Thr205 as well, but it is unable to if Ser202 has already been phosphorylated (Kimura et al., 2013, 2016, 2018).

As VLW mice express mutant human Tau and exhibit high levels of P-Tau, they may present altered priming patterns of Tau phosphorylation. Moreover, there is a crosstalk between different types of post-translational modifications, which can be through either competition or cooperation (Alquezar et al., 2021; S. Park et al., 2018). If any of these circumstances occur in VLW animals, phosphorylation at Thr205 could be hindered, leading to the observed reduction in the density of hippocampal interneurons accumulating pThr205 Tau.

Contrary to VLW mice, the density of hippocampal interneurons presenting pThr205 Tau is increased in J20 mice, compared to WT animals. It has been demonstrated that Aβ can induce Tau hyperphosphorylation (Bolmont et al., 2007; Busciglio et al., 1995; Götz et al., 2001; Jin et al., 2011; Nisbet et al., 2015; Pérez et al., 2005). Our data indicate that the regions that show an increase in the density of pThr205 Tau-positive interneurons correlate with those exhibiting a higher Aβ plaque burden. This suggests that the increase in the density of interneurons accumulating pThr205 Tau is caused by an inductive effect of Aβ on Tau phosphorylation at this residue. It has been described that phosphorylation of Tau at Thr205 inhibits Aβ-induced toxicity by disrupting the assembly of Tau/Fyn/PSD-95/NMDA receptor complexes required to mediate excitotoxicity, contrasting with the classical view that Tau phosphorylation downstream of Aβ has pathological effects (A. Ittner et al., 2016; L. M. Ittner et al., 2010). GABAergic hippocampal interneurons, and the GABAergic system in general, appear to be resistant to neurotoxicity in Alzheimer's disease (Canas et al., 2014; Mitew et al., 2013; Reinikainen et al., 1988; Rissman et al., 2007). Thus, our results indicating an inductive effect of Aβ on Tau phosphorylation at Thr205 could reflect a protective mechanism of hippocampal interneurons against neurotoxicity.

Altogether, our data reveal the presence of a particular Tau phosphorylation signature in hippocampal interneurons in pathological conditions in J20 and VLW mice. Phosphorylation at Ser262 could fulfill a physiological role, as it is indistinguishable from that of WT animals. Conversely, phosphorylation at Thr205 is influenced by mutant human Tau in VLW animals and by Aβ in J20 mice, the latter supporting the notion that pThr205 Tau protects hippocampal interneurons against Aβ-induced toxicity in Alzheimer's disease.

Because phosphorylation regulates the physiological roles of Tau, abnormal phosphorylation patterns are likely to contribute to the pathogenesis of Alzheimer's disease and other tauopathies both through the loss of physiological roles and the gain of toxic functions. It has been proposed that the conversion of functional into pathological Tau is not due to a single post-translational modification but to the junction of intrinsic structural alterations, resulting from a combination of extensive post-translational modifications, and adverse extrinsic cellular conditions (Alquezar et al., 2021). Moreover, the crosstalk between different post-translational modifications, through either competition or cooperativity, may have complex outcomes critical for Tau function, aggregation, and degradation (Alquezar et al., 2021; S. Park et al., 2018). Our study could not cover the numerous phosphorylation sites or the vast repertoire of post-translational modifications of Tau. Thus, the presence of other post-translational modifications that favor the preservation of physiological roles or the acquisition of toxic functions cannot be discarded.

1.3 Induction of murine Tau phosphorylation in interneurons by mutant human Tau present in pyramidal neurons in the hippocampus

Previous studies from our group revealed that pThr231 Tau is present in pyramidal neurons and PV-positive interneurons in the hippocampus of VLW mice (Soler et al., 2017). As previously reported, our data shows that mutant human Tau is only expressed in pyramidal neurons in this mouse model (Lim et al., 2001). Moreover, our results indicate that the presence of pThr231 Tau is exclusive of VLW mice and is absent in WT and J20 animals. This suggests that accumulation of pThr231 Tau in pyramidal neurons, possibly resulting from the expression of mutant human Tau in these neurons, leads to phosphorylation of endogenous murine Tau in hippocampal interneurons in VLW mice.

It has been demonstrated that Tau pathology spreads in a prion-like manner. Pathological Tau is transmitted intercellularly through anatomically connected networks and seeds, or induces, hyperphosphorylation and aggregation of other Tau molecules, further propagating Tau pathology (Brettschneider et al., 2015; Clavaguera et al., 2009, 2013; De Calignon et al., 2012; DeVos et al., 2018; Frost et al., 2009; L. Liu et al., 2012). Pathological

Tau seeds consist of oligomeric or fibrillar Tau and do not necessarily have to be phosphorylated, but seeded Tau always becomes hyperphosphorylated (Falcon et al., 2015; Goedert & Spillantini, 2017; W. Hu et al., 2016; Miao et al., 2019; Takeda et al., 2015; Usenovic et al., 2015; Y. Wang et al., 2017). However, phosphorylation at specific sites, including Thr231, can enhance Tau seeding efficiency, whereas dephosphorylation of Tau seeds reduces their seeding capacity (A. del C. Alonso et al., 1996; Dujardin et al., 2020; W. Hu et al., 2016; Rosenqvist et al., 2018). Moreover, it has been established that pathological human Tau from either transgene expression or experimental injection can seed endogenous murine Tau, coaggregate with it, and spread intercellularly (Clavaguera et al., 2009, 2013; De Calignon et al., 2012; Lasagna-Reeves, Castillo-Carranza, Sengupta, Guerrero-Muñoz, et al., 2012; L. Liu et al., 2012; J. W. Wu et al., 2013).

On the whole, our data reveal that expression of mutant human Tau in pyramidal neurons leads to induction of murine Tau phosphorylation at Thr231 in PV-positive interneurons in the hippocampus *in vivo*.

2. Characterization of a new animal model of Alzheimer's disease: J20/VLW mice

In the framework of the amyloid cascade hypothesis of Alzheimer's disease pathogenesis, it has been considered that Aβ precipitates the disease process, downstream of which Tau pathology arises. Both pathologies have been conceived as temporally related but acting independently. However, current experimental and clinical data support a synergistic interaction between Aβ and Tau that drives Alzheimer's disease progression.

By crossing J20 and VLW mice, we have generated a new double transgenic mouse model simultaneously presenting Aβ and Tau pathologies: the J20/VLW mouse line.

2.1 Amyloid-β pathology in the hippocampus in J20/VLW animals

Tau mediates the toxicity induced by Aβ (Frandemiche et al., 2014; L. M. Ittner et al., 2010; Jin et al., 2011; Rapoport et al., 2002; Roberson et al., 2007, 2011; Zempel et al., 2013). However, data regarding whether Tau modulates Aβ burden are conflicting. Double transgenic Tg2576/JNPL3 and APP23/B6-P301L mice, expressing $hAPP^{Sw}$ and $hTau^{P301L}$, present an Aβ load comparable to that of animals expressing only $hAPP^{Sw}$ at 9–11 and 24 months, respectively (Bolmont et al., 2007; J. Lewis et al., 2001). In contrast, Tg2576/VLW and APP/PSEN1/rTgTauEC mice display enhanced Aβ deposition at 16 months compared to animals lacking $hTau^{P301L}$ expression (Pooler et al., 2015; Ribé et al., 2005). However, APP/PSEN1/Thy-Tau22 and APP/PSEN1/rTg4510 mice show decreased Aβ burden in comparison with APP/PSEN1 animals at 7 and 12 months, respectively (R. E. Bennett et al., 2017; Chen et al., 2016).

Our results show no changes in the levels of soluble Aβ or Aβ plaque burden in J20/VLW mice compared to J20 animals at 8 and 12 months. This indicates that expression of mutant human Tau does not induce an increase in Aβ pathology in J20/VLW mice in comparison with J20 animals.

2.2 Tau phosphorylation in the hippocampus in J20/VLW mice

Multiple lines of evidence demonstrate that Aβ induces Tau hyperphosphorylation (Bolmont et al., 2007; Busche & Hyman, 2020; Busciglio et al., 1995; Götz et al., 2001; Jin et al., 2011; Nisbet et al., 2015; Pérez et al., 2005).

Our data reveal no major differences in the levels of pThr231 Tau between WT, J20, VLW, and J20/VLW animals. Conversely, there is an upwards trend in the levels of pThr205 Tau in J20 and J20/VLW mice compared to WT and VLW animals. This suggests an inductive

effect of Aβ on Tau phosphorylation at Thr205, as we have found in hippocampal interneurons in J20 mice. In J20/VLW mice, P-Tau is localized to the somatodendritic compartment of pyramidal neurons, analogous to our current and previous findings in VLW animals (Soler et al., 2017). We have detected no changes in pThr231 Tau or pThr205 Tau levels in pyramidal neurons between VLW and J20/VLW animals.

Besides pyramidal neurons, we have found that the somas of hippocampal interneurons accumulate P-Tau in physiological and pathological conditions (Figure 26). Our results show that the presence of Aβ enhances Tau phosphorylation at Ser262 in hippocampal interneurons in J20/VLW mice compared to VLW animals, as previously described in Tg2576/VLW mice expressing hAPP^{Sw} and aged rhesus monkeys following Aβ injection (Ando et al., 2016; Geula et al., 1998; Pérez et al., 2005; Zempel et al., 2010). Furthermore, it has been reported that induction of Tau phosphorylation at Thr231 by Aβ depends on Ser262 phosphorylation (Ando et al., 2016). Hence, the upwards trend shown by our data in the density of pThr231 Tau-positive interneurons in J20/VLW mice may be due to the increased pSer262 Tau-positive interneuron density in this mouse model. Conversely, our results reveal that the density of pThr205 Tau-positive interneurons in J20/VLW mice is higher than in VLW animals, lower than in J20 mice, and similar to that of WT animals. Therefore, the increase in the density of pThr205 Tau-positive interneurons induced by Aβ in J20 mice and the decrease observed in VLW animals are abolished in J20/VLW mice.

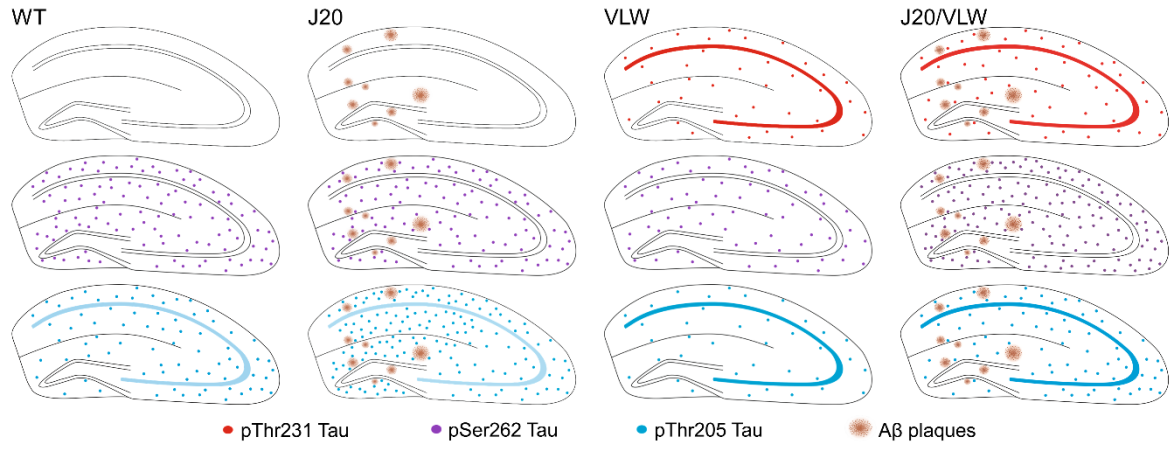


Figure 26. Tau phosphorylation pattern in hippocampal interneurons in control animals and Alzheimer's disease mouse models.
Representation of the density of hippocampal interneurons accumulating pThr231, pSer262, or pThr205 Tau in the soma in WT, J20, VLW, and J20/VLW mice. The presence of pThr231 Tau or pThr205 Tau in pyramidal neurons in VLW and J20/VLW animals and of Aβ plaques in J20 and J20/VLW mice are shown. *Adapted from Dávila-Bouziguet et al., 2019.*

Tau phosphorylation at Ser262 in the microtubule-binding domain, or at Thr205 or Thr231 in the proline-rich domain, reduces its affinity for microtubules (Biernat et al., 1993; Cho & Johnson, 2004; Drewes et al., 1995; Schwalbe et al., 2015; Sengupta et al., 1998).

Moreover, it has been recognized that Tau localization to different subcellular compartments can be modulated by phosphorylation (Kanaan et al., 2012; Mandell & Banker, 1996b; Mondragón-Rodríguez et al., 2012; Pooler et al., 2012; Sultan et al., 2011).

Altogether, our results suggest that Tau phosphorylation at Thr205, Thr231, and Ser262 facilitates its somatodendritic localization and favors novel physiological roles of Tau in subcellular compartments other than the axon in hippocampal interneurons in J20/VLW mice.

2.3 GABAergic septohippocampal innervation in J20/VLW mice

In glutamatergic and GABAergic synapses, Aβ species cause synaptic dysfunction and loss (Palop & Mucke, 2010, 2016; Spires-Jones & Hyman, 2014). Our group previously described that accumulation of Aβ in J20 mice and presence of P-Tau in VLW animals lead to impaired GABAergic septohippocampal innervation (Rubio et al., 2012; Soler et al., 2017). Moreover, Aβ oligomers induce dysfunction of hippocampal PV-positive interneurons and impair the GABAergic synapses they establish on pyramidal neurons, leading to reduced IPSPs in pyramidal neurons (Hijazi et al., 2019, 2020; Hollnagel et al., 2019; K. Park et al., 2020). Likewise, GABAergic synapses are altered in mice expressing $hAPP^{Sw}$ and there is a loss perisomatic GABAergic synapses established on pyramidal neurons adjacent to Aβ plaques in Alzheimer's disease patients and APP/PSEN1 mice (Bell et al., 2006; Garcia-Marin et al., 2009; Petrache et al., 2019). Furthermore, hippocampal GABAergic synapses are disrupted and IPSPs in pyramidal neurons are reduced in mice expressing $hTau^{P301L}$ or treated with Tau oligomers derived from the brain of Alzheimer's disease patients, both models accumulating P-Tau (Ruan et al., 2021; Shimojo et al., 2020).

Our current results indicate that GABAergic septohippocampal innervation is preserved in J20/VLW mice. This protection from denervation is permanent, as innervation is maintained at 8 and 12 months. We have detected no changes in Aβ burden or P-Tau accumulation in pyramidal neurons in J20/VLW animals compared to J20 or VLW mice, respectively. Thus, our findings suggest that preservation of GABAergic septohippocampal innervation is due to the presence of a particular Tau phosphorylation pattern in hippocampal interneurons in J20/VLW mice. Our data indicate that GABAergic septohippocampal innervation is preserved on interneurons accumulating pThr205 Tau in J20/VLW mice, contrary to J20 and VLW animals. Conversely, our results regarding innervation of interneurons presenting pThr231 Tau or pSer262 Tau reveal no differences between the double and the single transgenic mouse models. This further supports the notion that combined phosphorylation at specific Tau residues in hippocampal interneurons underlies the maintenance of the GABAergic septohippocampal pathway.

The potential physiological roles of Tau and its phosphorylation in GABAergic synapses are scarcely characterized compared to glutamatergic synapses. Several data indicate that phosphorylation at Thr205, Thr231, or Ser262 is associated with localization of Tau at glutamatergic synapses, where Tau plays a physiological role in synaptic plasticity (L. M. Ittner et al., 2010; Jin et al., 2011; Morris et al., 2015; D. Xia et al., 2015; Zempel et al., 2010). In WT mice, pThr205 Tau, pThr231 Tau, and pSer262 Tau have been detected in the postsynaptic compartment of hippocampal and cortical synapses (Morris et al., 2015). Phosphorylation in the proline-rich domain of Tau, especially at Thr205, may contribute to its dendritic localization (Jin et al., 2011; Zempel et al., 2010). Likewise, phosphorylation of Tau at Thr231 or Ser262 markedly enhances its targeting to dendritic spines (D. Xia et al., 2015). During synaptic activity mimicking LTP, Tau is phosphorylated at Thr205 and is translocated from dendrites into the postsynaptic compartment (Frandemiche et al., 2014). Moreover, under NMDA receptor-mediated synaptic activity mimicking LTD, Tau phosphorylation at Thr231 increases in dendrites and in the postsynaptic compartment (Mondragón-Rodríguez et al., 2012). In addition, phosphorylation modulates the interaction of Tau with synaptic proteins, including PSD-95 and Fyn kinase (Bhaskar et al., 2005; A. Ittner et al., 2016; Mondragón-Rodríguez et al., 2012; Regan et al., 2015).

Most data on this subject regarding GABAergic synapses focus on the structural and functional alterations in GABAergic synapses due to P-Tau in Alzheimer's disease. However, it has been described that under $GABA_A$ receptor-mediated synaptic activity, Tau is phosphorylated at Thr205 (Nykänen et al., 2012).

Whereas Tau phosphorylation in glutamatergic synapses as a result of NMDA receptor activation is reversible, allowing it to switch between a phosphorylated and an unphosphorylated state, Aβ-induced Tau phosphorylation is irreversible (Mondragón-Rodríguez et al., 2012). This dichotomy suggests that Tau phosphorylation accomplishes a physiological role at synapses, and its deregulation by Aβ or other factors contributes to the pathological process in Alzheimer's disease. The existence of these dynamics of Tau phosphorylation in the context of glutamatergic synapses allows for the possibility that a similar process occurs in GABAergic synapses.

In GABAergic synapses, Tau may participate in the regulation of $GABA_A$ receptor trafficking through the scaffolding protein gephyrin, which is directly linked to the cytoskeleton (Essrich et al., 1998). Gephyrin regulates the clustering of $GABA_A$ receptors, thus controlling the activity of GABAergic synapses and inhibitory neurotransmission (Maric et al., 2017). Moreover, GSK3β is both one of the main kinases phosphorylating Tau upon activation by Aβ and a regulator of GABAergic synapses through phosphorylation of gephyrin (Tyagarajan et al., 2011; Tyagarajan & Fritschy, 2014). Thus, it is possible that

GSK3β activation by Aβ causes an increase in P-Tau in the somatodendritic compartment of GABAergic interneurons and, simultaneously, the phosphorylation of gephyrin. This would lead to $GABA_A$ receptor clustering and preservation of GABAergic septohippocampal synapses, resulting in the stabilization of inhibitory neurotransmission in the hippocampus.

Overall, data suggest that synaptic activity leads to phosphorylation at different Tau sites, which modulate the interactions of Tau and its binding partners. Therefore, the interaction of Tau with synaptic proteins appears to be regulated by phosphorylation. Nevertheless, a wider knowledge of Tau phosphorylation signatures is necessary to thoroughly understand its physiological roles at synaptic compartments and, especially, at GABAergic synapses.

2.4 Hippocampal oscillatory activity in J20/VLW animals

Previous data from our group indicate that impaired GABAergic septohippocampal innervation correlates with altered hippocampal oscillatory activity in J20 mice (Rubio et al., 2012; Vega-Flores et al., 2014). Compared to WT animals, J20 mice present a clear decrease in the spectral power of the theta and gamma bands. Moreover, VLW mice display altered GABAergic septohippocampal innervation and hyperexcitability (García-Cabrero et al., 2013; Soler et al., 2017). Our current results show a considerable reduction in the spectral power of the theta band in VLW animals compared to WT mice. In contrast, J20/VLW animals present only a minor decrease in the spectral power of the theta and gamma bands.

Multiple data indicate that the GABAergic septohippocampal pathway regulates hippocampal oscillatory activity in the theta and gamma bands. It has been demonstrated that GABAergic septohippocampal innervation directly controls hippocampal theta oscillations (Buzsáki, 2002; Gangadharan et al., 2016; Hangya et al., 2009; Varga et al., 2008; Villette et al., 2010; Xu et al., 2004). The most abundant targets of GABAergic septohippocampal axons are basket and axo-axonic PV-positive interneurons, which govern the firing of a large number of pyramidal neurons (Freund & Antal, 1988; Freund & Buzsáki, 1996; Gulyás et al., 1990). Concurrently, it has been proved that fast-spiking PV-positive interneurons directly modulate hippocampal gamma oscillations (Bartos et al., 2007; Buzsáki & Wang, 2012; Chung et al., 2020; H. Hu et al., 2014; Korotkova et al., 2010; K. Park et al., 2020). Moreover, through cross-frequency coupling, theta oscillations modulate oscillatory activity in the gamma band (Belluscio et al., 2012; Bragin et al., 1995; J. L. Butler et al., 2016; Chrobak & Buzsáki, 1998; Csicsvari et al., 2003).

Thus, through the recruitment of hippocampal interneurons and, especially, of PV-positive interneurons, the GABAergic septohippocampal pathway controls hippocampal

theta and gamma oscillations. Our data show that J20/VLW mice display minor alterations in theta and gamma oscillations, in contrast to the major impairment present in J20 and VLW animals, thus pointing to a correlation between preserved GABAergic septohippocampal innervation and conserved hippocampal oscillatory activity.

2.5 Cognitive function in J20/VLW mice

Several studies manifest the presence of cognitive impairment in the J20 and VLW mouse models, primarily affecting learning and memory (Cheng et al., 2007; Cissé et al., 2011; Navarro et al., 2008; Palop et al., 2003; Rossi et al., 2020; Wright et al., 2013). Our current results show that the cognitive deficits observed in J20 and VLW animals are absent in J20/VLW mice. The present study examines the relationship between correct GABAergic septohippocampal innervation, preserved hippocampal oscillatory activity, and cognitive function in J20/VLW animals. Alzheimer's disease patients and animal models, including J20 and VLW mice, display an imbalance between excitation and inhibition that results in network abnormalities and that could underlie cognitive deficits (Ambrad Giovannetti & Fuhrmann, 2019; Lauterborn et al., 2021; Palop et al., 2007; Palop & Mucke, 2016; Quiroz et al., 2010; Stoiljkovic et al., 2019; Verret et al., 2012; Vico Varela et al., 2019; Zott et al., 2018). Conversely, J20/VLW mice present no significant alterations in hippocampal theta and gamma oscillations, suggesting a correct balance between excitation and inhibition, presumably modulated by GABAergic septohippocampal innervation and, thus, by the GABAergic system. Therefore, our data point to a correlation between the proper function of the GABAergic system and cognitive function.

Although the GABAergic septohippocampal pathway and its main targets, PV-positive interneurons, are promising candidates to underlie the preservation of hippocampal oscillatory activity and cognitive function, further studies are needed to confirm their causal role. One approach to explore this issue could be manipulating the GABAergic septohippocampal pathway in J20/VLW animals and single transgenic mice. This paradigm would allow ascertaining whether blocking or stimulating the GABAergic septohippocampal pathway results in the impairment of hippocampal oscillations and cognitive function in J20/VLW mice or in their rescue in J20 and VLW animals, respectively.

Multiple studies have employed different strategies to examine this question. It has been demonstrated that Aβ injection in the medial septum in rats leads to a reduction in the spectral power of the theta band in the hippocampus and to memory deficits (Colom et al., 2010; Özdemir et al., 2013). Indirect manipulation of the medial septum through Aβ injection in the hippocampus, which mimics the circumstances of J20 mice, causes a reduction in the rhythmic activity of GABAergic septohippocampal neurons, decreased spectral power of

the hippocampal theta band, and memory deficits in rats (Villette et al., 2010). Similarly, specific pharmacological inhibition of GABAergic septohippocampal neurons in rats impairs memory (Krebs-Kraft et al., 2007). Moreover, optogenetic silencing of GABAergic septohippocampal neurons during REM sleep abolishes hippocampal theta oscillations and disturbs memory consolidation in mice (Boyce et al., 2016). Conversely, direct optogenetic stimulation of PV-positive GABAergic septohippocampal neurons in J20 mice rescues the spectral power of the gamma band and memory (Etter et al., 2019). In addition, chemogenetic silencing of GABAergic septohippocampal neurons or, specifically, of the synaptic contacts they establish on hippocampal interneurons located in the CA1 region, induces deficits in memory retrieval in mice (Sans-Dublanc et al., 2020). The same study demonstrated that optogenetic modulation of PV-positive interneurons in the CA1 region, the targets of GABAergic septohippocampal innervation, mimics the effects of manipulating the latter, thus validating the involvement of both in memory retrieval.

2.6 J20/VLW mice as a new animal model of cognitive resilience to Alzheimer's disease

The existence of individuals presenting Aβ and Tau pathologies in the absence of cognitive impairment has been repeatedly reported (Aizenstein et al., 2008; Beker et al., 2021; D. A. Bennett et al., 2006; Jack et al., 2017; W. J. Jansen et al., 2015; Knopman et al., 2003; Rowe et al., 2010; Snitz et al., 2020). These individuals, CRAD subjects, are believed to possess intrinsic mechanisms conferring protection against the cognitive impairment associated with Alzheimer's disease. A key factor proposed to underlie this cognitive resilience is the preservation of the essential components of the synaptic machinery that are dysfunctional in Alzheimer's disease (Arnold et al., 2013; Bjorklund et al., 2012; Perez-Nievas et al., 2013; A. J. Silva et al., 1998; Singh et al., 2020; Walker et al., 2022; Zolochevska et al., 2018).

Our data indicate that a particular Tau phosphorylation signature in hippocampal interneurons, stemming from the simultaneous presence of Aβ and mutant human Tau, may confer protection against the synaptotoxic effects of pathological oligomers or trigger a differential gene expression pattern that protects synaptic structure and function in J20/VLW mice, analogous to CRAD individuals. Indeed, Tau has been involved in the maintenance of correct theta and gamma oscillations, which may be related to the reduction of hyperexcitability owing to its phosphorylation in or near the microtubule-binding domain in sites such as Thr205, Thr231, or Ser262 (Cantero et al., 2011; Hatch et al., 2017; A. Ittner et al., 2016; L. M. Ittner et al., 2010; Mondragón-Rodríguez et al., 2012, 2018). Furthermore, our results point to the preservation of the GABAergic system as a crucial

factor underlying the rescue of hippocampal oscillatory activity and cognitive function in J20/VLW animals.

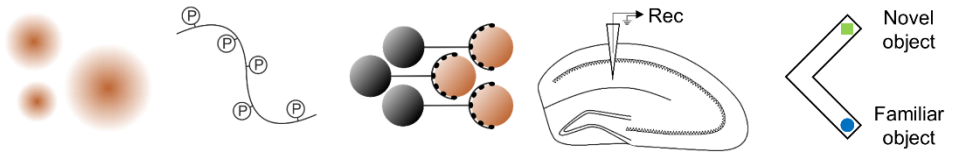
In light of the features presented by the J20/VLW mouse line generated herein, it would be interesting to examine the similarities between this animal model and CRAD subjects. For instance, a significant matter to study would be the characteristics of synapses in J20/VLW mice. To date, studies on CRAD subjects have focused on glutamatergic synapses. The present findings lay the groundwork for replicating the studies in GABAergic synapses and extensively characterizing the GABAergic system in physiological and pathological conditions. In order to expand our knowledge of the state of the GABAergic system in Alzheimer's disease and its role in cognitive resilience, it could be useful to analyze GABAergic neurons and synapses in J20/VLW animals compared to single transgenic mice or WT animals. This may allow identifying the factors underlying cognitive resilience, which could be subsequently validated in CRAD subjects.

Post-mortem examination of neuropathological features and *in vivo* imaging studies have revealed cell loss and volume reductions in the nucleus basalis of Meynert and MSDB complex in Alzheimer's disease (Cantero et al., 2020; Cullen et al., 1997; Grothe et al., 2012; Kerbler, Fripp, et al., 2015; Kilimann et al., 2014; Vogels et al., 1990). In addition, the volume of septal nuclei in human subjects correlates with memory and other cognitive functions that depend on the hippocampus, reinforcing the contribution of the septohippocampal pathway to memory in humans (T. Butler et al., 2012; Grothe et al., 2010, 2016; Kerbler, Nedelska, et al., 2015). Moreover, a generalized decrease in the spectral power of the gamma band has been described in the brain of Alzheimer's disease patients (Koenig et al., 2005; Stam et al., 2002). However, the state of the GABAergic septohippocampal pathway has not been studied in human subjects. Our data, along with the imbalance between excitatory and inhibitory circuits and aberrant network activity associated with Alzheimer's disease, emphasize the need to analyze the GABAergic septohippocampal pathway and hippocampal oscillations in humans.

Despite the lack of human data regarding the GABAergic septohippocampal pathway, it is a promising target for therapeutic intervention in Alzheimer's disease to prevent cognitive impairment. Deep brain stimulation of the fornix, the main fiber tract through which septohippocampal axons travel, has been demonstrated to slow the rate of cognitive decline and to improve memory in Alzheimer's disease patients in clinical trials (Fontaine et al., 2013; Laxton et al., 2010; Leoutsakos et al., 2018; Lozano et al., 2016). Moreover, gamma frequency optogenetic stimulation of PV-positive GABAergic septohippocampal neurons and chemogenetic inhibition of hyperactive hippocampal PV-positive interneurons have been shown to rescue learning and memory impairments in

Alzheimer's disease mouse models (Etter et al., 2019; Hijazi et al., 2019). Another therapeutic approach aimed at reinforcing the GABAergic system is GABAergic neuron grafting in the hippocampus, which has been shown to improve learning and memory in mouse models of Alzheimer's disease (Y. Liu et al., 2013; Lu et al., 2020; Martinez-Losa et al., 2018; Tong et al., 2014).

Altogether, our data suggest that a differential Tau phosphorylation pattern in hippocampal interneurons protects against the loss of GABAergic septohippocampal innervation, thereby avoiding alterations in hippocampal oscillatory activity and, thus, preventing cognitive impairment in J20/VLW mice (Figure 27). These findings support a new role for Tau phosphorylation in preserving the GABAergic septohippocampal pathway and the function of the GABAergic system in the hippocampus and indicate the potential of regulating Tau phosphorylation in GABAergic neurons as a therapeutic approach to Alzheimer's disease.



	Amyloid-β	P-Tau	GABAergic septohippocampal pathway	Theta and gamma oscillations	Cognitive function
WT mice	–	–	+++	+++	+++
Control subjects	–	–	+++	+++	+++
J20 mice	+	–	+	+	+
VLW mice	–	+	+	+	++
Alzheimer's disease patients	+	+	Synaptic dysfunction Aberrant network activity		+
J20/VLW mice	+	+	+++	++	+++
Cognitive resilience to Alzheimer's disease subjects	+	+	Synaptic integrity		+++

Figure 27. Summary of neuropathological, synaptic, electrophysiological, and cognitive features in control, Alzheimer's disease, and CRAD conditions in mouse models and human subjects.
Comparison of WT mice and human control subjects, J20 and VLW animals and Alzheimer's disease patients, and J20/VLW mice and CRAD subjects. J20/VLW mice present Aβ and P-Tau, yet display preserved GABAergic septohippocampal synapses, hippocampal oscillations, and cognitive function, similar to CRAD subjects. *Adapted from Dávila-Bouziguet et al., 2022.*

The etiology and pathogenesis of Alzheimer's disease are exceptionally complex. Hence, translating data from animal models to a clinical context must be done cautiously. Like most biological systems, Alzheimer's disease should be intrinsically non-linear (Busche & Hyman, 2020). Therefore, in order to properly understand it and treat it, our attention has to shift away from the linear view that Aβ and P-Tau are always pathological and take part in an invariable pathophysiological cascade. Moreover, animal models that accurately represent the evolution of Aβ and Tau pathologies, as well as the state of synapses, network activity, and cognitive function, are necessary. In addition, with the identification of CRAD subjects, individuals presenting Aβ and Tau pathologies but lacking clinical symptoms of dementia, there is an imperative need for animal models that replicate these features to characterize and identify the mechanisms driving cognitive resilience. We propose the double transgenic mouse line generated in this thesis as a suitable animal model to analyze the cognitive resilience of CRAD subjects and explore new therapeutic strategies to treat the cognitive impairment associated with Alzheimer's disease. By recapitulating the two central pathologies of Alzheimer's disease but presenting synaptic, electrophysiological, and cognitive preservation, J20/VLW mice could provide a new outlook in studying this devastating disease and interpreting the resilience against dementia of CRAD subjects.

CONCLUSIONS

1. In physiological conditions, pThr205 and pSer262 Tau accumulate in the soma of hippocampal interneurons in mice and human subjects.

2. In pathological conditions, pThr205 and pSer262 Tau accumulate in the soma of hippocampal interneurons in Alzheimer's disease mouse models and human patients.

3. Accumulation of pThr205 Tau correlates with the presence of Aβ plaques in the hippocampus in J20 mice. Conversely, accumulation of pSer262 Tau is independent of the presence of Aβ and mutant human Tau in the hippocampus in J20 and VLW mice, respectively.

4. Mutant human Tau present in hippocampal pyramidal neurons induces the phosphorylation of endogenous murine Tau at Thr231 in PV-positive hippocampal interneurons in VLW mice.

5. Aβ levels and plaque burden of J20/VLW mice are similar to those of J20 animals.

6. The levels of pThr231 and pThr205 Tau of J20/VLW mice are comparable to those of VLW animals.

7. Compared to VLW animals, J20/VLW mice present an increase in the density of interneurons accumulating pSer262 Tau and an upwards trend in the density of those accumulating pThr231 Tau. The pThr205 Tau-positive interneuron density in J20/VLW mice is comparable to that of WT animals.

8. GABAergic septohippocampal innervation is preserved in J20/VLW mice, in contrast to J20 and VLW animals.

9. Hippocampal oscillations, markedly impaired in J20 and VLW animals, are partially conserved in J20/VLW mice.

10. Contrary to J20 and VLW animals, J20/VLW mice exhibit a preserved cognitive function.

11. The J20/VLW mouse line could be a suitable animal model to study the cognitive resilience of CRAD subjects.

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