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TESIS DOCTORAL

P2X7 inhibition ameliorates the Ubiquitin-proteasome system impairment associated with neurological diseases

La inhibición de P2X7 mejora el deterioro del sistema ubiquitina-proteasoma asociado con enfermedades neurológicas

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Departamento de Bioquímica y Biología Molecular IV

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A te nonno
che mi guardi crescere dall'alto

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Abbreviations

Aβ	Amyloid beta
AD	Alzheimer's disease
ADP	Adenosine-5’-diphosphate
AMP	Adenosine-5’-monosphate
APP	Amyloid Precursor Protein
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
BBB	Blood-brain barrier
BOC	BOC-LSTR-AMC
Bp	base pairs
BSA	Bovine Serum Albumin
BzATP	2’(3’)-O-(4-benzoylbenzoyl)ATP
CaMKII	Ca^{2+}-calmodulin kinase II (
CD39	Ecto-nucleoside triphosphate diphosphohydrolase-1
CD73	Ecto-5’- nucleotidase
CNS	Central nervous system
CT-L	Catalytic activity of the Chymotrypsin-like proteasome
DAPI	4’,6-diamidino-2-phenylindole
DAMP	Damage Associate Molecular Pattern
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTPs	Deoxyribonucleotides
DTT	Dithiothreitol
DUB	Deubiquitinating Enzymes
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-ligase Enzyme
EBSS	Earle’s Balanced Salt Solution
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal Growth Factor

EGFR	EGF receptor
EGTA	Ethylene-bis (oxynitride) -tetra acetic acid
ERKs	Kinases regulated by extracellular signalling
FBS	Fetal bovine serum
FBSi	Inactivated Fetal Bovine Serum
FK2	Antibody to mono- and polyubiquitinated proteins
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Acid fibrillar glial protein
GFP	Green fluorescent protein
GFP-CL1	UPS reporter molecule
GlutamaxTM	L-alanyl-L-glutamine
GPRC	G protein-coupled receptors
GSK-3	Glycogen synthase kinase 3
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase, horseradish peroxidase
Iba-1	Ionized Calcium Binding Adapter Molecule 1
IC50	The half maximal inhibitory concentration
i.c.v	Intracerebroventricular injection
IF	Immunofluorescence
IFN-γ	Interferon-γ
IL	Interleukin
i.p	intraperitoneal
LMP2	Low Molecular-mass polypeptide-2
LMP7	Low Molecular-mass polypeptide-7
MAPK	Mitogens-activated Protein Kinase
MECL-1	Multicatalytic Endopeptidase Complex-like 1; Complex-like endopeptidase multicatalytic-1
MG132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
MTT	3- [4,5-dimethylthiazol-2-yl)] - 2,5, diphenyl tetrazolium
N2a	Mouse neuroblastoma cells 2a
NAP	Z-LLE-β-2-naphthylamine

NeuN	Marker of neuronal nuclei
NLRP3	multi-protein complex nucleotide-binding domain, leucine-rich repeat and pyrin-containing receptor 3
NP40	Nonidet-P40
P1	Purinergic nucleoside receptors
P2	Purinergic nucleotide receptors
P2X7R	Purinergic receptor P2X7
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PFA	paraformaldehyde
PG-L	Catalytic activity of the caspase-like proteasome
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A (cAMP dependent)
PKC	Protein kinase C (Ca2 + / phospholipid dependent)
PLA2c	Phospholipase A2 cytosolic
PLA	Phospholipase A
PLC	Phospholipase C
PNS	Peripheral nervous system
PoliUb	Polyubiquitination
Q-PCR	quantitative PCR
RGD	Arg-Gly-Asp Domain
ROS	Reactive oxygen species
RPM	Revolutions per minute
Rpn	Proteasome subunits with non-ATPase activity
Rpt	Proteasome subunits with ATPase activity
SDS	Sodium dodecyl sulphate
s.e.m	Standard error of the mean
ShRNA	small hairpin RNA, interference RNA
SNP	Single-nucleotide polymorphism
SUCC	SUCC-LLVY-AMC
TBS	Buffer Tris saline
Temed	Tetramethylethylenediamine

Thr	Threonine
T-L	Catalytic activity of the Trypsin-like proteasome
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor α
TRIS	Tris (hydroxymethyl) -aminomethane
U1	Transgenic mouse reporter of UPS activity
Ub	Ubiquitina
Ub^{G76V}-YFP	UFD type UPS reporter molecule
UBLs	Ubiquitin-like proteins
Ub-M-GFP	UFD type UPS reporter molecule
UDP	Uridine-5'-diphosphate
WB	Western Blot; Immunoblot
WT	Wild Type; wild mouse
YFP	Yellow Fluorescent Protein; Fluorescent yellow protein

Summary

The Ubiquitin Proteasome System (UPS) is the primary intracellular pathway leading to the degradation of misfolded, disassembled, or damaged proteins (*Kaushik & Cuervo 2015).* The destruction of a target protein is handled by the 26S proteasome complex following the covalent binding of multiple copies of ubiquitin molecules to a substrate protein via an enzymatic cascade (*Hershko and Ciechanover,1998*). UPS dysfunction has been linked to several neurological disorders, including Alzheimer's disease, Amyotrophic Lateral Sclerosis, Huntington's disease, and epilepsy (*Stefanis and Keller, 2017; Upadhya et al., 2007, Engel et al., 2017*). The impact of the UPS in these diseases may be linked to deficiencies in the clearance of misfolded proteins, which can lead to intracellular protein aggregation, cytotoxicity, and cell death (*Leight et al., 1991; Neumann et al., 2006*).

Alzheimer's disease (AD) is the most common cause of dementia among older adults, characterized by abnormal deposits of proteins from amyloid plaques and tau tangles (*DeTure and Dickson.,2019*). There is evidence that ubiquitin accumulates in both Aβ plaques and neurofibrillary tangles (*Oddo.,2008*). Additionally, these structures have been found to include ubiquitin-B mutant protein (UBB+1), a mutant form of ubiquitin that is responsible for blocking ubiquitin-dependent proteolysis in neuronal cells (*Lindsten et al., 2002*), as well as a mediator of Aβ-induced neurotoxicity (*Song et al., 2003*). The fact that UPS degrades the microtubule-associated protein Tau (*David et al, 2002; Grune et al, 2010*) lends credence to the notion that its dysfunction contributes to intracellular tau accumulation. Moreover, recent research has discovered a link between the accumulation of hyperphosphorylated Tau oligomers in human AD synapses, increased ubiquitinated substrates, and increased proteasome components, which is consistent with UPS failure (*Tai et al., 2012*).

Purinergic signalling, particularly through the P2X7 receptor (P2X7R), has been implicated in numerous neurodegenerative diseases (*Miras-Portugal et al, 2017*), predominantly in AD. Several studies have demonstrated that P2X7R expression is high both in AD patients (*Martin et al, 2019; Martinez-Frailes et al, 2019; McLarnon et al, 2006*) and in a mouse model reproducing AD-associated tauopathy, P301S mice (*Jin et al, 2018; Ruan et al, 2020*). Moreover, in AD, P2X7R has been linked to neuroinflammation (*Martin et al., 2019; Martinez-Frailes et al., 2019*), and neuronal death (*Chen et al, 2013; Irwin et al, 2016*).

Previous works have reported that extracellular nucleotides, through $P2Y_2$ receptor, can modulate the UPS activity in central nervous system cells when the UPS function is compromised, as it occurs in neuropathological conditions such as neuroinflammation (*de Diego-Garcia, L., et al. 2017; de Diego-Garcia, L., et al. 2018*). Considering the aforementioned considerations, the principal aim of this Doctoral Thesis was to determine if the P2X7R is capable of modulating the UPS dysfunction associated with neuroinflammation, particularly in AD. Using a cell model (murine neuroblastoma cell line N2a) transfected with two different UPS reporters, we saw that activation of P2X7R by 2- '(3') - O- (4-benzoylbenzoyl) ATP (BzATP) resulted in a substantial drop of the activity of the UPS. This reduction was due to a significant decrease of two of the three proteasomal peptidase activities, chymotrypsin (CT-L) and postglutamyl (PG-L), caused by a diminished expression of the proteasome constitutive catalytic subunits β5 and β1 both at protein and mRNA levels. We also discovered that intracerebroventricular (i.c.v) injection of BzATP reduced CT-L and PG-L activity, as well as the expression of proteasome constitutive subunits β5 and β1 in the hippocampus of wild-type (WT) mice, but not in P2X7 -/- mice. Furthermore, we were able to identify the intracellular route implicated in this event as Akt/GSK-3/Nrf2.

In a second step, using mice expressing a UPS reporter [mice expressing the UPS reporter ubiquitin^{G76V}-green fluorescent protein (UbGFP mice)], we could determine how the activation of P2X7R plays a key role in the alteration of cellular proteostasis. These studies revealed that the i.c.v. of BzATP promotes Ub^{G76V}-GFP accumulation in neuronal and microglia cells. Consequently, when we examined a tauopathy mouse model, the P301S mice, we observed the same proteasomal disfunctions identified in Alzheimer's patients. Furthermore, we found that inhibiting P2X7R *in vivo* with the selective antagonist GSK 1482160A reversed the proteasomal dysfunction seen in P301S mice, enhancing CT-L and PG-L activities.

Since previous studies have found that another pathological condition, *status epilepticus* (SE), caused by prolonged seizures, is associated with increased P2X7R expression levels (*Engel et al., 2012; Jimenez-Pacheco et al., 2016*) and UPS impairment (*Engel et al., 2017*), we decided to investigate the role of this receptor in the UPS dysregulation that occurs in hippocampal cells after seizures triggered by Kainic Acid (KA). Our results show that, compared to WT mice, P2X7 -/-mice presented a decrease in polyubiquitinated conjugates 8 hours following *status epilepticus.* Likewise, using UbGFP mice, we discovered that cells with UPS disfunction also expressed P2X7R, demonstrating that the receptor plays a critical role in changing the balance

of cellular proteostasis induced by KA administration. All these results strongly suggest that P2X7R is able to modulate UPS activity and may be a novel target for a new therapeutic approach to treat neuroinflammatory diseases associated with UPS impairment, especially AD.

Finally, according to ELISA measurements, we determined that P2X7R plasma levels were significantly higher in Mild Cognitive Impairment (MCI) and AD patients, with the difference being more evident in female MCI individuals. We also observed a negative association between high levels of P2X7R in the blood and the Total Mini-Mental Status Examination (MMSETOT), which is used to assess the risk of dementia progression in MCI patients. These findings suggest that P2X7R measurement could be considered as a diagnostic biomarker to follow the progression of neurodegenerative diseases.

Resumen

El Sistema Ubiquitina-Proteasoma (UPS) es el principal sistema encargado de controlar la proteostasis celular (*Kaushik y Cuervo 2015*). La destrucción de una proteína diana es operada por el complejo de proteasoma 26S después de la unión covalente de múltiples copias de moléculas de ubiquitina a una proteína sustrato a través de una cascada enzimática (*Hershko y Ciechanover, 1998*). La disfunción del UPS se ha relacionado con una variedad de enfermedades neurológicas, incluida la enfermedad de Alzheimer, la Esclerosis Lateral Amiotrófica, la enfermedad de Huntington y la Epilepsia (*Stefanis y Keller, 2007; Upadhya et al., 2007; Engel et al, 2017*). El impacto del UPS en estas enfermedades puede estar relacionado con deficiencias en la eliminación de proteínas mal plegadas, lo que puede conducir a la agregación de proteínas intracelulares, citotoxicidad y muerte celular (*Leight et al., 1991; Neumann et al., 2006*).

La enfermedad de Alzheimer (EA) es la causa más común de demencia entre los adultos mayores, caracterizada por depósitos anormales de proteínas de placas amiloides y ovillos tau (*DeTure y Dickson., 2019*). Existe evidencia de que la ubiquitina se acumula tanto en las placas Aβ como en los ovillos neurofibrilares (*Oddo., 2008*). Además, se ha descubierto que estas estructuras incluyen la proteína mutante ubiquitina-B (UBB + 1), una forma mutante de ubiquitina que es responsable de bloquear la proteólisis dependiente de ubiquitina en las células neuronales (*Lindsten et al., 2002*), así como un mediador de la neurotoxicidad inducida por Aβ (*Song et al., 2003*). El hecho de que el UPS degrade la proteína tau asociada a los microtúbulos (*David et al, 2002; Grune et al, 2010*) da crédito a la noción de que su disfunción contribuye a la acumulación de tau intracelular. Además, una investigación reciente descubrió un vínculo entre la acumulación de oligómeros tau hiperfosforilados en las sinapsis de la EA humana, el aumento de sustratos ubiquitinados y el aumento de los componentes del proteasoma, lo que es consistente con el mal funcionamiento del UPS (*Tai et al., 2012*).

La señalización purinérgica, particularmente a través del receptor P2X7 (P2X7R), se ha implicado en varias enfermedades neurodegenerativas (*Miras-Portugal et al, 2017*), predominantemente en EA. Varios estudios han demostrado que la expresión de P2X7R es alta tanto en pacientes con EA (*Martin et al, 2019; Martinez-Frailes et al, 2019; McLarnon et al, 2006*) como en un modelo de ratón que reproduce tauopatía asociada a EA, ratones P301S (*Jin et al. al, 2018; Ruan et al, 2020*). Además, en la EA, P2X7R se ha relacionado con la

neuroinflamación (*Martin et al., 2019; Martinez-Frailes et al., 2019*) y la muerte neuronal (*Chen et al, 2014; Irwin et al, 2016*).

Trabajos anteriores han demostrado que los nucleótidos extracelulares, a través del receptor $P2Y_2$, pueden modular la actividad del UPS en las células del sistema nervioso central cuando la función del UPS está comprometida, como ocurre en condiciones neuropatológicas como la neuroinflamación (*de Diego-García, L., et al.2017; de Diego-García, L., et al.2018*).

Teniendo en cuenta las consideraciones anteriores, el objetivo principal de esta Tesis Doctoral fue determinar si P2X7R es capaz de modular la disfunción del UPS asociada a la neuroinflamación, particularmente en la EA. Usando un modelo celular (línea celular de neuroblastoma murino N2a) transfectado con dos reporteros diferentes de UPS, vimos que la activación de P2X7R por 2 -'(3') - O- (4-benzoilbenzoil) ATP (BzATP) resultó en una caída sustancial de la actividad del UPS. Esta disminución se debió a una reducción significativa de dos de las tres actividades catalíticas del proteasoma, Quimotripsina-*like* (CT-L) y Postglutamil-*like* (PG-L), causada por una expresión disminuida de las subunidades catalíticas constitutivas del proteasoma β5 y β1 tanto en los niveles de proteína como en el ARNm. También descubrimos que la inyección intracerebroventricular (i.c.v) de BzATP redujo la actividad de CT-L y PG-L, así como la expresión de las subunidades constitutivas del proteasoma β5 y β1 en el hipocampo de ratones de tipo silvestre (WT), pero no en ratones P2X7-/-. Además, pudimos identificar la ruta intracelular implicada en este evento como Akt/ GSK3/Nrf2.

En un segundo paso, utilizando ratones que expresan un reportero UPS [ratones que expresan el reportero UPS ubiquitin^{G76V}-proteína fluorescente verde (ratones UbGFP)], pudimos determinar cómo la activación de P2X7R juega un papel clave en la alteración de la proteostasis celular. Estos estudios revelaron que i.c.v. de BzATP promueve la acumulación de Ub^{G76V}-GFP en células neuronales y de microglia. En consecuencia, cuando examinamos un modelo de ratón con tauopatía, los ratones P301S, observamos las mismas disfunciones proteasómicas identificadas en pacientes con Alzheimer. Además, encontramos que la inhibición de P2X7R *in vivo* con el antagonista selectivo GSK 1482160A revirtió la disfunción proteasomal observada en ratones P301S, mejorando las actividades de CT-L y PG-L.

Dado que estudios previos encontraron que otra condición patológica, el *estado epiléptico* (SE), causado por convulsiones prolongadas, se asocia con un aumento de los niveles de expresión de P2X7R (*Engel et al., 2012; Jimenez et al., 2016*) y un deterioro de UPS (*Engel et*

al.., 2017), decidimos investigar el papel de este receptor en la desregulación del UPS que ocurre en las células del hipocampo después de las convulsiones desencadenadas por el Ácido Kaínico (KA). Nuestros resultados muestran que, en comparación con los ratones WT, los ratones P2X7 - / - presentaron una disminución en los substratos poliubiquitinados 8 horas después del estado epiléptico. Asimismo, utilizando ratones UbGFP, descubrimos que las células con disfunción del UPS también expresaban P2X7R, lo que demuestra que el receptor juega un papel crítico en el cambio del equilibrio de la proteostasis celular inducida por la administración de KA.Todos estos resultados sugieren fuertemente que P2X7R es capaz de modular la actividad del UPS y puede ser un nuevo objetivo para un enfoque terapéutico alternativo para tratar enfermedades neuroinflamatorias asociadas con un deterioro del UPS, especialmente EA.

Finalmente, de acuerdo con las mediciones de ELISA, determinamos que los niveles plasmáticos del P2X7R eran significativamente más altos en pacientes con deterioro cognitivo leve (DCL) y con EA, siendo la diferencia más evidente en las mujeres con DCL. También observamos una asociación negativa entre los niveles altos de P2X7R en la sangre y el Mini Examen del Estado Mental Total (MMSETOT), que se utiliza para evaluar el riesgo de progresión de la demencia en pacientes con DCL. Estos hallazgos sugieren que la medición de P2X7R podría considerarse un biomarcador de diagnóstico para seguir la progresión de las enfermedades neurodegenerativas.

1 Introduction

1.1 Purinergic System and Purinergic Receptors

It is well known that ATP (adenosine 5'-triphosphate) and other nucleotides are involved in many essential biological activities required for appropriate cell functions, including enzyme control, cell metabolism, ionic membrane gradient maintenance, and cell motility. Furthermore, these compounds are the building blocks of nucleic acids (*Khakh & Burnstock 2009*). The first evidence of the action of nucleotides as extracellular messengers was provided by Drury and Szent-Györgyi in 1929 as a result of studies carried out in the cardiovascular system (*Drury & Szent-Gyorgyi 1929*). In 1970, Geoffrey Burnstock discovered that ATP was released by vagal non-adrenergic inhibitory nerves after electrical stimulation, thus attributing to ATP the function of a neurotransmitter (*Burnstock et al., 1970*). It was also Burnstock who introduced, in 1972, the concept of purinergic signaling (*Burnstock, 1972*). After these findings, it was demonstrated that ATP may act either as a co-transmitter or a sole transmitter in most nerves in the peripheral and central nervous systems (*Burnstock, 1976; Burnstock, 2014).* In 1976, Burnstock proposed the existence of specific receptors for nucleosides and nucleotides (*Burnstock, 1976*), which were two years later divided into P1 and P2 receptors for adenosine and ATP/adenosine diphosphate (ADP), respectively (*Burnstock, 1978*). In 1985, P2 receptors were subdivided into P2X and P2Y subtypes based on their pharmacological profiles (*Burnstock & Kennedy, 1985*). In the early 90s, receptors for purines and pyrimidines were cloned and characterized (*Ralevic and Burnstock, 1998*).

The purinergic pathway is involved in numerous systems, including the central nervous, cardiovascular, reproductive, and immune systems (*Burnstock, 2017*). Purines act as widespread extracellular signaling molecules and their physiological effects are mediated through an extended family of purinoceptors. Pyrimidine and purine nucleotides are released from cells through several physiologically relevant mechanisms, including diffusion through membrane hemichannels, activation of membrane transporters, and vesicular exocytosis (*Abbracchio et al., 2009; Burnstock, 2007a; North and Verkhratsky, 2006; Pankratov et al., 2006*). In addition, purines and pyrimidines are released from dying cells: this is an early indicator of cell damage (*Burnstock, 2007a; Burnstock, 2008*). Upon release, ATP (and other nucleotides) are enzymatically degraded within seconds by an extended family of

ectonucleotidases (*Zimmermann, 2006*). This process is physiologically relevant as ATP metabolites are also agonists of different purinergic receptors (**Figure 1**). These purinergic signaling molecules activate two receptor classes: (i) metabotropic P1 receptors, which are activated by adenosine; and (ii) the P2 family of nucleotide receptors, which are subdivided into metabotropic P2Y and ionotropic P2X sub-classes (**Figure 1**). Each component of the system will be explained in detail later. (*Abbracchio et al., 2006; Abbracchio et al., 2009; Burnstock, 1976; Burnstock, 2007b; Burnstock and Kennedy, 1985; Ralevic and Burnstock, 1998)*.

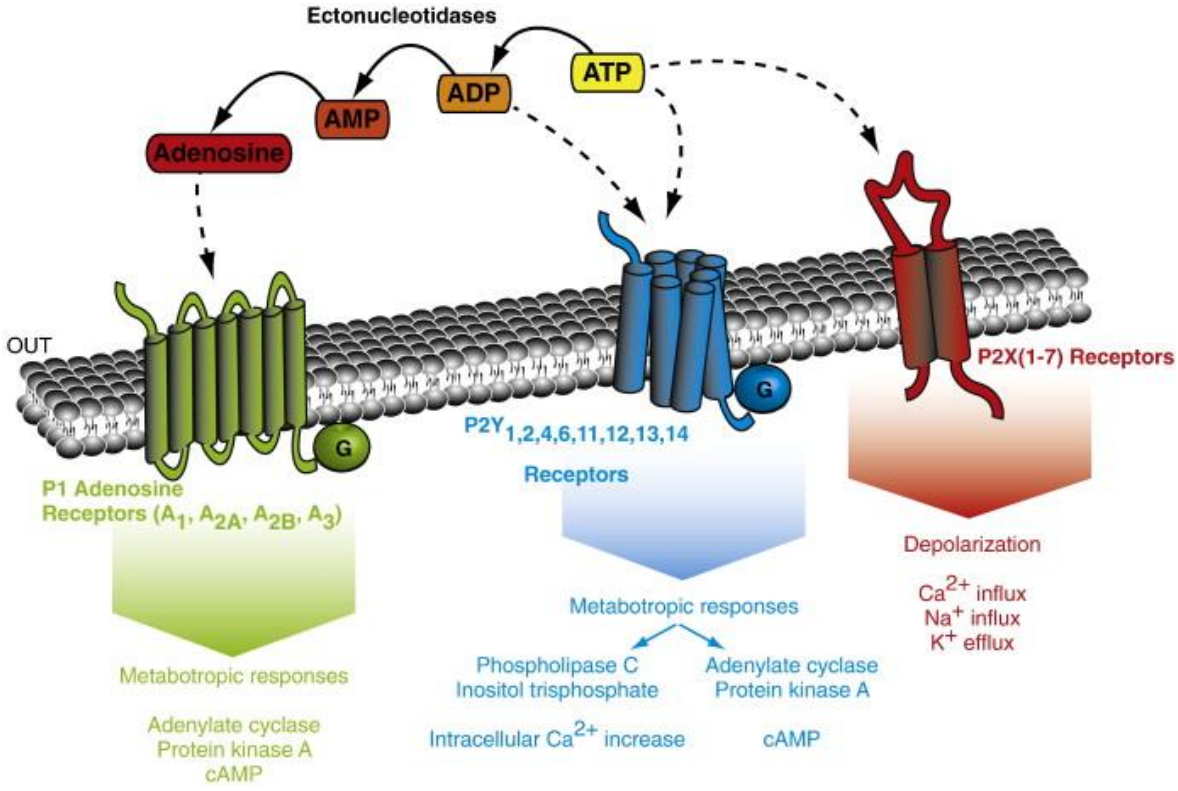

Figure 1: The purinergic signaling pathway. Simplified illustration of extracellular purinergic signaling mediated by ATP and other nucleotides. These molecules activate metabotropic P1 receptors, activated by adenosine, and the P2 family of nucleotide receptors, with the sub-classes of P2Y metabotropic and P2X ionotropic receptors (Adapted from Baroja-Mazo et al., 2013).

1.2 Nucleotide storage and release

The presence of ATP or any other nucleotide in the extracellular environment is required for its function as a neurotransmitter. At first, it was thought that damaged or dead cells were the only source of extracellular ATP. However, it is now generally known that ATP release from healthy cells is a physiological process involving both exocytotic and non-exocytotic mechanisms (*Bodin and Burnstock, 2001; Lazarowski, 2012; Lazarowski et al., 2003*). The common feature is that ATP can be stored in large, dense-core vesicles, together with neurotransmitters. It was found in human blood vessels, smooth muscles, and endothelial cells with noradrenaline (*Donoso et al., 2004*); it was also discovered in adrenal chromaffin cells with serotonin, in astrocytes with neuropeptide Y and glutamate, and in neuron-differentiated cells with dopamine (*Coco et al., 2003; Bankston and Guidotti,1996; Haanes and Novak, 2010*). It has also been shown that ATP is released from cholinergic (*Diaz-Hernandez et al., 2001*), aminergic (*Giraldez et al., 2001*), and GABAergic terminals of the CNS (*Gomez-Villafuertes et al., 2003*) via an exocytotic and Ca2+-dependent release mechanism. Moreover, ATP was found in small synaptic-like vesicles in other cell types in the nervous tissue, notably astrocytes (*Yamboliev et al., 2009*).

The concentration of ATP within these stores varies depending on the cell type. In 2008, Sawada et al. identified the SLC17A9 protein as the vesicular nucleotide transporter (VNUT) (*Sawada et al., 2008*). It is mainly expressed in the brain and adrenal medulla, where it plays a vital role in the storage and release of Cl- dependent nucleotides (*Sawada et al., 2008*).

Besides vesicular release, numerous studies have shown that ATP can be released through non-exocytotic conductive pathways. ATP-binding cassette (ABC) transporters that hydrolyze ATP to support the transmembrane movement of different molecules have been proposed to function as ATP-releasing pathways (Schwiebert, 1999). Mammalian ABC transporters are integral membrane proteins comprised of 12 membrane-spanning domains and two conserved cytoplasmic domains, which bind and hydrolyze ATP (*Hyde et al., 1990*). Over the years, other anion channels have been recognized as ATP-permeable channels, including chloride ion channels (*Nejime et al., 2009*) and volume- and voltage-dependent anion channels (VDAC) (*Okada et al., 2004*). Currently, five families of channels are thought to mediate many forms of physiological and pathophysiological ATP release: connexin hemichannels, pannexin 1 (PANX1), calcium homeostasis modulator 1 (CALHM1), volume-

regulated anion channels (VRACs, also known as volume-sensitive outwardly rectifying (VSOR) anion channels) and maxi-anion channels (MACs) (**Figure 2**).

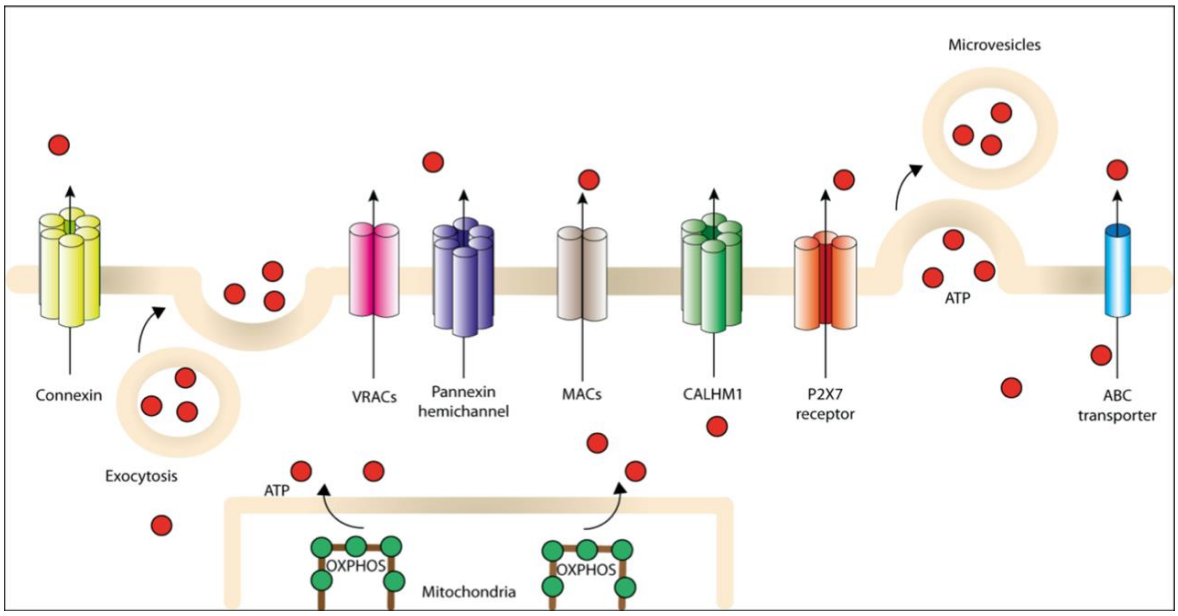

Figure 2: Different pathways implied in the release of ATP. ATP produced within the cell can be actively released through plasma membrane-derived microvesicles, vesicular exocytosis, or various non-exocytotic conductive pathways such as specific ATP-binding cassette (ABC) transporters, the P2X7R, connexin, and pannexin channels, calcium homeostasis modulator 1(CALHM1) channel, volume-regulated ion channels (VRACs) and maxi-anion channels (MACs). *Image is taken from Vultaggio-Poma*, 2020

Connexin and pannexin proteins create hexametric membrane structures termed connexons and pannexons, respectively, that mediate the release of tiny molecules into the extracellular space such as ATP, glutamate, and others with a molecular weight of less than 1–2 kDa *(Bao et al., 2004*). Multiple events, such as intracellular Ca^{2+} increase, redox potential changes, mechanical stress, and P2X7R activation, are involved in the opening and activation of pannexin-1 channels (*Bao et al., 2004*; *Retamal, 2014; Pelegrin and Surprenant, 2009*). The CALHM family of proteins has been proposed to aid ATP release. Only one of the six members of the family (CALHM1-6) has been identified as a functional ATP-permeable ion channel. CALHM1 functions as a pore-forming subunit of a plasma membrane, voltage-gated, non-selective ion channel with a pore wide enough to accommodate ATP (*Siebert et al.,2013*). Cells enable many processes to restore normal cell volume in response to hypotonic cell swelling, the most important of which is the conductive efflux of organic and inorganic osmolytes through anion-selective channels VRACs (also known as VSORs) (*Nilius et al., 1997*). The ATP permeability of VRACs was recently demonstrated by luciferin/luciferase-based

measurements of ATP release from hypotonic Xenopus oocytes (*Gaitan-Penas et al., 2016*). In addition to VRAC channels, cell swelling specifically activates maxi-anion channels (MACs), which are involved in the ATP release process (*Okada et al.,2018*). MACs are voltage-dependent, large-conductance, ATP-permeable anion channels found in all cell types that are activated by several stimuli such as hypoxia, high glucose, and osmotic swelling (*Dutta et al,2004).* An additional pathway for ATP release is the P2X7R. Aside from a cation-selective channel, the P2X7R can generate a large, non-selective pore (macropore) permeable to ATP and other molecules with a molecular weight of up to 900 Da (*Di Virgilio et al., 2018*).

1.3 Ecto-enzymatic inactivation of nucleotides

Like all neurotransmitters, once released, nucleotides must be removed from the extracellular environment. For this, there are specific extracellular enzymes called ectonucleotidases. Ectonucleotidases are a family of nucleotide-hydrolyzing cell surface enzymes that play an important role in the purinergic signaling system in the body. They vary in their preference and affinity for the substrate, in the product formed, in the dependence of cations, and their optimum pH. They are widely distributed in all tissues and act consecutively, giving rise to hydrolysis cascades (**Figure 3**). Therefore, ectonucleotidases do not only control the half-life of the original nucleotides but by degrading or modifying them, they give rise to new ligands capable of activating other purinergic receptors. They consist mainly of four families:

a) Ecto-nucleoside triphosphate di-phosphohydrolases (E-NTPDases) also known as ecto-ATPases, ecto-ADPases, ecto-apyrases, or CD39. CD39 is in control of the Ca^{2+} and Mg^{2+}-dependent conversion of extracellular ATP to ADP and subsequently to AMP (*Robson et al., 2006*). The complete structure of CD39 showed that both ATP and ADP are hydrolyzed at the same site, demonstrating CD39's efficient removal of ATP (*Zebisch et al., 2012, Zebisch et al., 2013*).

b) Ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) are part of the superfamily of alkaline phosphatases, with which they share their optimum alkaline pH. The three members of this family of ectoenzymes, NPP1, 2 and 3, hydrolyze 5'-monodiester bonds in nucleotides and their derivatives, releasing nucleosides 5'-monophosphate (*Goding et al.,*

2003). They can also hydrolyze other compounds, such as polyphosphate dinucleotides (*Vollmayer et al., 2003*).

c) Alkaline phosphatases (APPs): Like E-NPPs, they are members of the superfamily of alkaline phosphatases. They form homodimers with three cations at the catalytic site, releasing inorganic phosphate from a wide variety of compounds, and hydrolyze 5'-tri-, -di- and -monophosphate nucleotides, generating the corresponding precursor nucleosides (*Millan, 2006*).

d)Ecto-5'-nucleotidase or CD-73. CD73 is a 71 kDa glycosylphosphatidylinositol-linked protein (*Zimmermann, 1992*). CD73 is responsible for the degradation of AMP to adenosine (*Bin et al.,2018*), which is achieved by exposure of the active site when CD73 changes from the closed to the open state, due to the large flexibility of CD73 homodimers (*Knapp et al., 2012*).

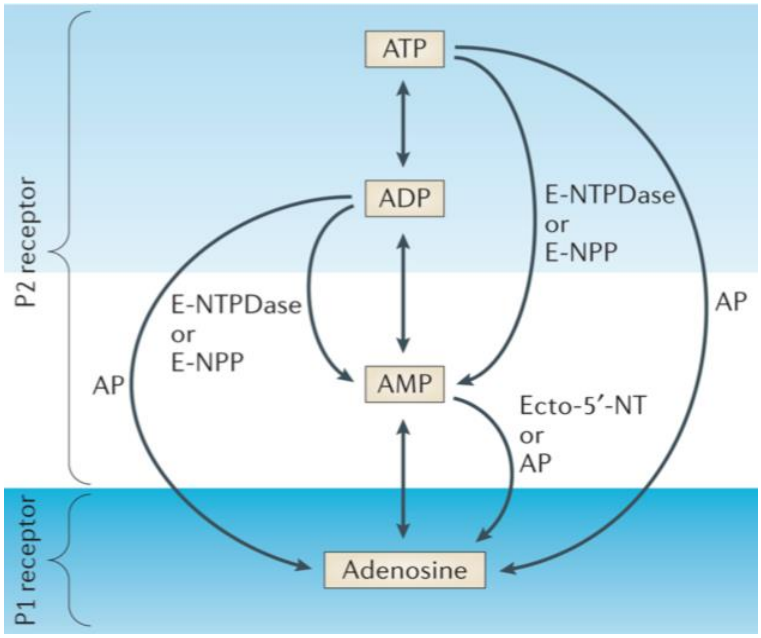

Figure 3: Representative diagram of the action of the different ectonucleotidases on purinergic signaling. Once released into the extracellular space, nucleotides activate P2Y and P2X receptors. Its action is terminated by the action of ectonucleotidase enzymes that degrade nucleotides triphosphate to diphosphate nucleotides, which in turn are agonists of some purinergic receptors (P2). Finally, adenosine is produced and activates its receptors (P1), being finally recaptured by a specific transporter inside the cell for the synthesis of new nucleotides. *Image is taken from Fields et al. 2006*

1.4 P1 Receptors

Also known as P1 receptors, adenosine receptors consist of four subtypes (A_1, A_{2A}, A_{2B}, and A_3) that are all activated by extracellular adenosine, as the name implies, and are coupled with different G proteins (**Table 1**). In common with other G protein-coupled receptors, they contain seven transmembrane (TM) domains of approximately 21 to 28 hydrophobic amino acids composing an α-helix structure. The amino terminus of the protein is present on the extracellular side of the plasma membrane, whilst the protein's carboxy-terminus lies on the cytoplasmic side of the membrane. A pocket for the ligand-binding site is formed by the three-dimensional arrangement of the α- helical TM domains, and the agonist is believed to bind within the upper half of this pore (*Ralevic and Burnstock, 1998*). The TM domains are connected by three extracellular and three cytoplasmic hydrophilic loops (*Ralevic and Burnstock, 1998*). Activation of A2 receptors typically results in the release of cytokines that promote an anti-inflammatory effect under physiological conditions, whereas A_1 and A_3 may have either pro- or anti-inflammatory effects. In the cardiovascular, immune, and nervous systems, these receptors can mediate different physiological effects (*Ledent et al 1997; Sun et al 2001*). The expression of different subtypes of adenosine receptors varies between cell types (*Haskó et al., 2008*). A_{2A} and A_{2B} share 59% sequence homology (*Lucattelli et al., 2011*). However, A_{2A} is better characterized than A_{2B} and is more commonly the target of therapies for diseases, such as Parkinson's and Huntington's disease (*Borea et al., 2018*). There are several specific agonists and antagonists of the different receptor subtypes, which are shown in **Table 1** (*Burnstock 2018*).

Receptor	Main Signalling Pathway	Agonist	Antagonist
A_1	Coupled to G_i proteins	CCPA,S-ENBA, CVT-510 GR79236 2'-MeCCPA, SDZ WAG 994, INO-8875, MRS 5474	DPCPX, N-0840, MRS1754, WRC-0571, PSB36, SLV320, CGS 16943, PQ-69
A_{2A}	Coupled to G_s proteins	HENECA ,CGS 21680 ,CVT-3146,ATL-146e,Regadenoson	KF17837, SCH58261, ZM241385, KW 6002
A_{2B}		Bay60-6583, NECA	PSB603, MRE-2029-F20, MRS1754, PSB0788 MRS1706, PSB1115, Alloxazine, GS-6201
A_3	Coupled to G_i proteins	IBMECA ,MRS5698 , MRS5168 ,DBXRM,VT160,HEMADO	MRS1220, L-268605, MRS1191, MRS1523(rat), VUF8504, VUF5574, MRS1334(human), PSB10

Table 1: Summary of P1 receptors, the main signaling pathway they activate, and the list of the different nucleoside receptor agonists and antagonists. The data in the table was obtained from Burnstock (2018).

1.5 P2 Receptors

Nucleotide receptors, or P2 receptors, are divided into two large families based on their molecular structure and the signal transduction mechanisms associated with their activation. On one hand, there are the P2X ionotropic receptors, which are ligand-activated ion channels, and, on the other hand, the G-protein-coupled P2Y metabotropic receptors (*Abbracchio & Burnstock 1994; Burnstock 2007b; Coddou et al 2011; von Kugelgen & Harden 2011*). In the following subchapters, we will briefly describe the different classes of P2 receptors.

1.5.1 P2Y receptors

P2Y receptors are G protein-coupled receptors (GPCRs). The family currently consists of eight members cloned and characterized in human or mammalian tissues: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (*Abbracchio et al., 2006; Burnstock, 2007b*). They have 7 hydrophobic transmembrane segments linked by three extracellular and three intracellular loops. The N-terminal end is oriented towards the extracellular space, while the C-terminal end has an intracellular orientation (**Figure 4**).

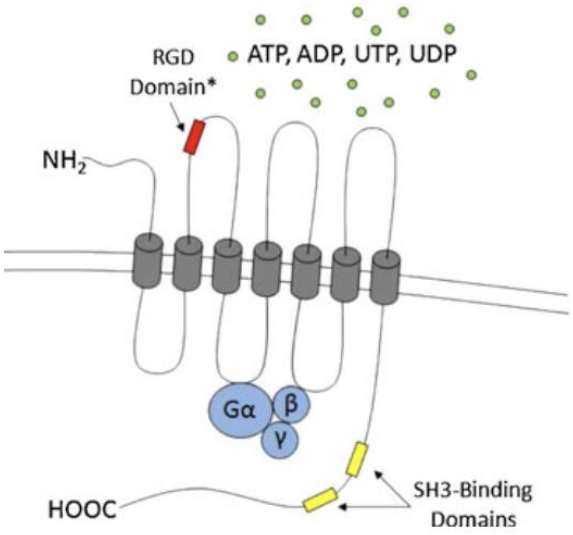

Figure 4: Structural features of P2Y receptors. The P2YRs are classical GPCRs featuring an extracellular N-terminus, seven transmembrane domains, and an intracellular C-terminus that is structurally diverse between P2Y receptor subtypes (*Adapted from Weisman et al., 2012*)

Based on their affinity for adenine and/or uridine nucleotides, P2Y receptors are divided into three groups. The first is made up of the $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{11}$ receptors, which are activated only by adenine nucleotides (ATP/ADP). Another group is made up of receptors activated by both adenine and uracil nucleotides ($P2Y_2$ and $P2Y_4$ receptors); finally, the $P2Y_6$ receptor, which is specific for pyrimidines. A special case is the $P2Y_{14}$ receptor, activated by UDP-glucose and other UDP-sugars. Upon activation, these receptors either activate phospholipase C and release intracellular calcium or affect adenylate cyclase and alter cAMP levels (*Abbracchio et al., 2006; Burnstock, 2007b; von Kugelgen, 2006*). P2Y receptors are present on numerous neurological and immune cells. In the CNS, where the expression of most of the known subtypes has been described, they participate in phenomena of synaptic plasticity, neurotransmitter release, and in the regulation of neurodegeneration (*Burnstock and Knight, 2004, Abbracchio et al., 2006*). **Table 2** shows the main agonists and antagonists identified for P2Y receptors as well as the main signaling pathway they activate.

Receptor	Main Signalling Pathway	Endogenous Agonist	Synthetic Agonist	Antagonist
$P2Y_1$	Coupled to G_q proteins	ADP	2-MeSADP=ADPβS>2-MeSATP, MRS2365	MRS2500 > MRS2279 > MRS2179, PIT, A3P5P
$P2Y_2$		UTP, ADP	UTPγS, INS37217, INS365, Ap4A, INS45973, MRS2768, PSB1114, 2-S-UTP, 6-nitro-UTP, UTPαS	AR-C126313 > Suramin > RB2, PSB-716, MRS2576, PSB-0402, AR-C118925
$P2Y_4$		UTP, ADP	UTPγS, INS37217, 5-Br-UTP, INS365	ATP, RB2, Suramin,PADDS
$P2Y_6$		UDP	UDPβS, MRS2693, PSB0474, INS48823, α, β-MeUDP, 5-Br-UTP	PPADS, Suramin, RB2, MRS2578, MRS2567, MRS2575
$P2Y_{11}$	Coupled to G_q + G_s proteins	ATP	AR-C67085, BzATP, ATPγS, UTP, NF546	Suramin, RB2, NF157, 5'-AMPS, NF340
$P2Y_{12}$	Coupled to G_i proteins	ADP	2-MeSADP, 2-MeSATP	AR-C69931MX, INS49266, PSB0413, ARL66096, 2-MeSAMP
$P2Y_{13}$		ADP	2-MeSADP, 2-MeSATP, ADPβS, BzATP	PPADS, Suramin, RB2, Ap4A, MRS2211, AR-C67085, AR-C69931
$P2Y_{14}$		UDP, UDP-glucose,UDP-galactose	MRS2690	-

Table 2: Table 3. List of the different agonists and antagonists identified for the characterization of P2Y receptors. Adapted from Burnstock, 2018.

1.5.2 $P2Y_2$ receptor

The $P2Y_2$ receptor ($P2Y_2R$), which was previously named as the P_{2U} receptor, was first cloned from mouse neuroblastoma cells (*Lustig et al., 1993*). Subsequently, it has been cloned and pharmacologically characterized in humans (*Parr et al., 1994*) and rat cells or tissues. Equivalent concentrations of ATP and UTP fully activate $P2Y_2$ receptors while ADP and UDP are less effective agonists (*Lustig et al., 1993; Parr et al., 1994; Lazarowski et al., 1995)*.

Activation of the G-coupled $P2Y_2R$ by ATP or UTP is linked to the stimulation of phospholipase C (PLC), leading to an increase in the production of inositol 1,4,5 trisphosphate (IP3) and diacylglycerol that elevates the intracellular Ca^{2+} concentration and activates

protein kinase C (PKC), respectively (*Abbracchio et al.,2006*). $P2Y_2R$ activation also increases the synthesis and/or release of arachidonic acid (AA), prostaglandins, and NO (*Lustig et al., 1996; Welch et al., 2003; Xu et al., 2003*). Furthermore, $P2Y_2R$ activation stimulates epidermal growth factor receptor (EGFR) phosphorylation and significantly enhances the activities of the MAP kinases ERK1/2 and related adhesion focal tyrosine kinase (RAFTK) via a mechanism involving Src (*Seye et al 2004; Soltoff et al 1998*). It has also been shown that human $P2Y_2R$ may transactivate the EGFR or platelet-derived growth factor (PDGF) receptor through two SH3-binding domains in the intracellular carboxyl-terminal tail of the receptor (*Liu et al., 2004*). $P2Y_2Rs$ are expressed in glial cells (i.e., astrocytes and microglia), neurons, and endothelium, primary cell types comprising the CNS (*Weisman et al.,2012*). In neurons, upregulation of $P2Y_2Rs$ by IL-1β promotes the nucleotide-induced non-amyloidogenic processing of Amyloid Precursor Protein (APP) and the phosphorylation of cofilin, responses that are neuroprotective *(Kong et al., 2009; Leon-Otegui et al.,2011*). The neuroprotective role associated with $P2Y_2R$ is also linked to its capability to modulate the Ubiquitin-Proteasome System (UPS) (*de Diego-García et al., 2017; de Diego-García et al., 2018*), a role that will be discussed in further detail later in this thesis.

1.5.3 P2X receptors

P2X receptors are ion channels inserted into the plasma membrane, which, upon stimulation by nucleotides and extracellular dinucleotides, allow the flux of cations, including $Na^{+,}$ K^+, and Ca^{2+} (*Khakh and North, 2012; North, 2002*). To date, seven different P2X subunits, P2X1 through P2X7, have been cloned and characterized (*Abbracchio et al., 2009; North, 2002; Surprenant and North, 2009)*. They play a prominent role in rapid synaptic transmission between neurons and in neuromuscular transmission in smooth muscle, where ATP acts as the main neurotransmitter (*Edwards et al., 1992; Sneddon et al., 2000*). Within the community of ligand-activated ion channels, the P2X receptors constitute a structurally well-differentiated family (*North, 1996*). They have two transmembrane regions (TM1 and TM2) and cytosolic N- and C-termini, with protein kinase binding motifs. TM1 is involved in the opening of the channel and TM2 in its formation (**Figure 5**). An extracellular loop located between the two transmembrane segments constitutes between 50 and 70 percent of the structure of these subunits.

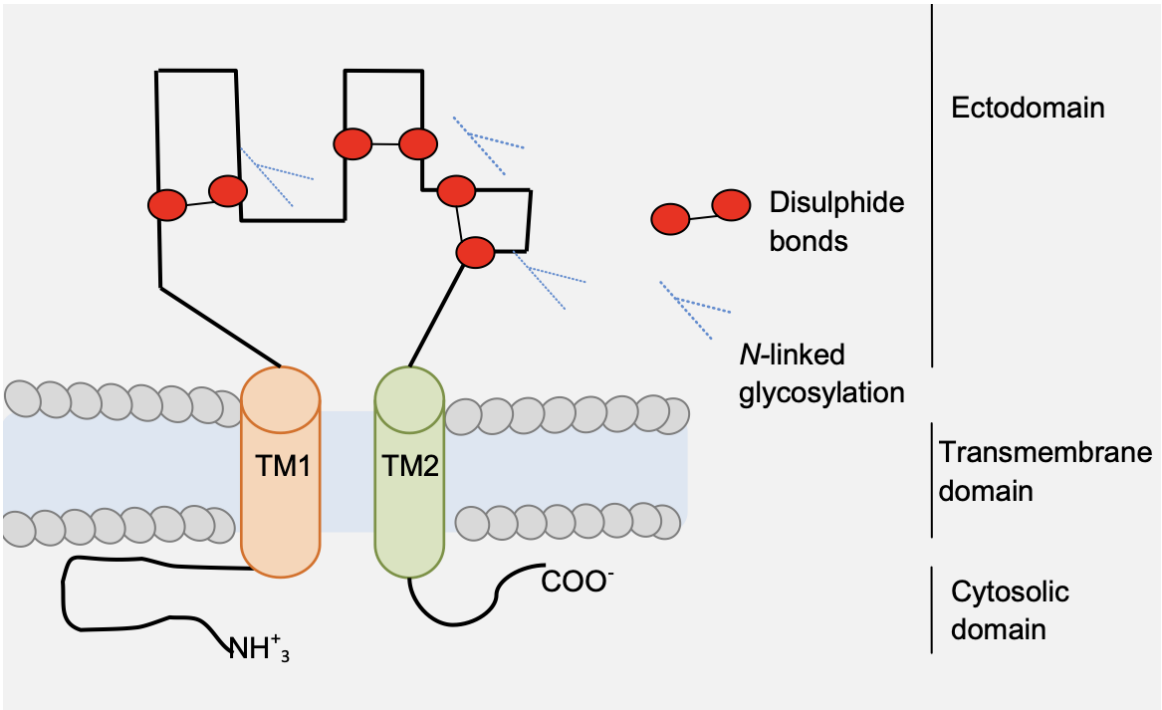

Figure 5: Structural features of P2X receptors. The diagram shows important internal interactions (formation of disulphide bridge and glycosylation sites) in the ectodomain domain needed for proper receptor function. *Adapted from Khakh and North,2006*

In all subunits (24-31 aa), the amino end is uniformly short in size and participates directly in the control of ionic conductivity through these channels (*Boue-Grabot et al., 2000; Ennion and Evans, 2002; Liu et al., 2003*). On the other hand, the sequence and scale of the carboxyl terminus are highly variable: very short for the P2X1, 3, 4, and 6 receptors, intermediate for the P2X2 and 5 receptors, and unusually wide for the P2X7 subunit, with more than 200 aa. This fact suggests that the carboxy-terminal end could confer specific properties to each receptor. The C-terminal region presents in all isoforms a conserved YxxxK motif close to TM2 that seems to be associated with the correct transport of the receptor to the membrane and the stabilization of its expression on the cell surface (*Bobanovic et al., 2002, Chaumont et al., 2004*). This domain also has numerous glycosylation sites involved in ligand affinity (*Hu et al., 2002*). The two transmembrane segments (TM) of the P2X subunits are not capable of forming an ionic pore on their own, which is why they need to interact to each other's as homo- or heterotrimers to form a functional channel (*Nicke et al., 1998; Aschrafi et al., 2004; Nagaya et al., 2005*). The assembly does not occur randomly between any P2X subunit. To date, the following heteromeric receptor combinations have been described: P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6, and P2X2/5 (*Abbracchio et al.,*

2009; Compan et al., 2012; Roberts et al., 2006). The P2X7 subunit heteromerization capability is not exactly understood. First, it was thought to be part of a homomeric channel. It was later suggested that the P2X4 subunit could constitute functional heteromers (*Guo et al., 2007),* but more recent studies have disproved this hypothesis because the P2X4/7 heteromeric receptors are not very stable and do not represent a significant percentage in the models studied (*Nicke 2008*).

1.5.4 Functional characteristics and regulation of P2X receptors

The functional and pharmacological properties of the P2X receptors depend on the subunits that form the channel. The different P2X receptors can be classified according to their response profile, their affinity for agonists and antagonists, and their sensitivity to calcium, magnesium, protons, or zinc. In certain cases, the pharmacological tools available to differentiate between the various P2X receptors are very small, and some agonists and antagonists can function on P2X and P2Y receptors at the same concentrations. In general, two broad classes can be differentiated within the P2X receptor family according to strict pharmacological criteria (**Table 3**): on one hand, the group of P2X1 and P2X3 receptors, which are characterized by their high affinity for ATP and which can be activated by the synthetic analogue α,β-meATP. The second group, comprising the receptors P2X2, P2X4, P2X5, P2X6, and P2X7, is characterized by receptors that display lower ATP affinity and are insensitive to α,β-meATP (*Coddou et al.,2011*). As previously discussed, the lack of selectivity between the various subtypes of purinergic receptors is not limited to agonists, since most P2X receptors are inhibited by the most frequently used antagonists (Suramin and PPADS) and even several P2Y in the case of Suramin.

Receptor	Agonist	Antagonist
P2X1	BzATP > ATP = 2-MeSATP ⩾ α,β-meATP = L-β,γ-meATP (rapid desensitisation); PAPET-ATP	NF864 > NF449 > IP5I ⩾ TNP-ATP > RO 0437626 > NF279, NF023, RO1, MRS2159
P2X2	ATP ⩾ ATPγS⩾2-MeSATP >> α,β-meATP (pH + zinc sensitive); β,γ-CF_2ATP	PSB-1011 > RB2, isoPPADS > PPADS > Suramin, NF770, NF778, Aminoglycoside
P2X3	2-MeSATP ⩾ ATP ⩾ Ap4A ⩾ α,β-meATP (rapid desensitisation); PAPET-ATP; BzATP	TNP-ATP, AF353, A317491, RO3, isoPPADS > NF110 > PPADS, Ip_5I, phenol red, RN-1838, Spinorphin
P2X4	ATP>> α,β-meATP, CTP	5-BDBD >> TNP-ATP, PPADS > BBG, Paroxetine, phenolphthalein, CO donor (CORM 2), 5MPTP
P2X5	ATP = 2-MeSATP = ATPγS >> α,β-meATP > AP4A	BBG > PPADS, Suramin
P2X6	Only functions as a heteromultimer	-
P2X7	BzATP > ATP ⩾ 2-MeSATP >> α,β-meATP (clemastine potentiates)	KN62, BBG, KN04, MRS2427, O-ATP, RN-6189, Perazine, AZ10606120, A740003, A-438079, A-804598, GSK-1370319, Comp 31 (GSK), AZD-9056, CE-224,535, JNJ-47965567, JNJ-42253432 (penetrates BBB), decavanadate, AZ11657312

Table 3: List of the different agonists and antagonists used for the characterization of the P2X receptors. The data reflected in the table has been obtained from (*Burnstock, 2007b; Burstock 2018; Coddou et al., 2011; Donnelly-Roberts et al., 2009; Illes and Alexandre Ribeiro, 2004; Norenberg and Illes, 2000; North, 2002*).

As a result of the diverse P2X receptors (P2XRs) expression within mammalian cell microenvironments, receptor expression must be strictly regulated to ensure that receptor-mediated responses occur in a coordinated manner (*Olteanu et al., 2007)*. **Table 4** provides a list of the various tissues that express P2X channels. The constitutive cycling of ionotropic P2XRs is one of the mechanisms by which these receptor-mediated responses can be controlled to maintain homeostasis (*Murrell-Lagnado and Qureshi., 2008*). In general, P2XRs tend to act as "cellular sensors" within the numerous microenvironments where they have been observed (*Burnstock.,2007a*), including changes in the microenvironment, including changes in the levels of proton ions (H^+), trace metal ions (Zn^{2+}, Cu^{2+}), calcium ions, and, of

course, ATP. Depending on the P2X subunit involved, relative permeability varies for each of these ions, but it can also be changed depending on its control (**Table 4**).

Receptor	Modulation through ions	Principle Tissue Distribution
P2X1	$H^+\downarrow$, $Zn^{2+}\downarrow$, $Cd^{2+}\downarrow$	Brain, spinal cord, smooth muscle, platelets, sympathetic ganglia
P2X2	$H^+\uparrow$, $Zn^{2+}\uparrow$, $Cu^{2+}\uparrow$, $Ca^{2+}\downarrow$	Brain, spinal cord, smooth muscle, retina, sympathetic ganglia, chromaffin cells
P2X3	$Zn^{2+}\uparrow$, $H^+\downarrow$, $Ca^{2+}\downarrow$	Brain, spinal cord, sympathetic ganglia, sensory neurons
P2X4	$H^+\downarrow$, $Zn^{2+}\uparrow$	Brain, spinal cord, sympathetic ganglia, testicles, colon
P2X5	$Zn^{2+}\uparrow$, $H^+\downarrow$, $Ca^{2+}\downarrow$	Brain, spinal cord, skin cells, heart, thymus,
P2X6	$Zn^{2+}\uparrow$, $H^+\downarrow$, $Ca^{2+}\downarrow$	Brain, skeletal muscle, pituitary gland
P2X7	$H^+\downarrow$, $Mg^{2+}\downarrow$, $Cu^{2+}\downarrow$, $Zn^{2+}\downarrow$, $Ca^{2+}\downarrow$	Retina and cochlea ganglia, immune cells, brain, spinal cord, pancreas, skin

Table 4: Modulation and physiological expression of P2X receptors in mammalian systems. The data reflected in the table corresponds to mammalian P2X receptors and has been obtained from (*Burnstock, 2007b; Burstock 2018; Coddou et al., 2011; Donnelly-Roberts et al., 2009; Illes and Alexandre Ribeiro, 2004; Norenberg and Illes, 2000; North, 2002*).

The P2X1 and P2X3 receptors desensitise the fastest after agonist stimulation, with their responses dropping by 90% in 1-2 seconds. The other P2XRs responses are much more sustained, with the P2X7R sustaining its response for minutes (*North, 2016*). Depolarization caused by the entry of calcium via the P2XRs in neuronal cells enables the opening of voltage-gated calcium channels, causing a further increase in free cytosolic calcium levels. This increase may subsequently trigger various signaling cascades, depending on the neuronal form, which may involve the regulation of protein kinases such as Ca^{2+}-calmodulin kinase II (CaMKII) (*Leon et al., 2006*), the mitogen-activated protein kinase (MAPK), protein kinase C (PKC) (*Erb et al., 2006*) or glycogen synthase kinase 3 (GSK-3) (*Diaz-Hernandez et al., 2008; Ortega et al., 2009*); as well as the release of neurotransmitters such as acetylcholine (*Diaz-Hernandez et al., 2002*), glutamate (*Gualix et al., 2003*) or γ-aminobutyric acid (GABA) (*Gomez-Villafuertes et al., 2001*).

1.5.5 P2X7

P2X7R is the principal receptor studied in this thesis. P2X7R is encoded by the *P2RX7* gene (*Rassendren et al., 1997*) situated on chr 12 (12q24) in humans (*Buell et al., 1998*). The *P2RX7* gene is strongly polymorphic and can present a variety of single nucleotide polymorphisms (SNP) (*Sluyter, 2017*). P2X7R was originally classified as the "P2Z" receptor, but following the cloning of this receptor, it was renamed to P2X7, becoming the seventh member of the P2X receptor family (*Surprenant et al., 1996*). Each full-length P2X7 subunit has a total molecular weight of 68 kDa (*Nicke, 2008*) or 75-82 kDa after N-glycosylation (*Adinolfi et al., 2010*). P2X7R is considered an atypical receptor because it requires higher ATP levels for activation than the rest of the P2X subunits (*North, 2002; Surprenant et al., 1996*). The first computational structure of P2X7 (*Jiang et al., 2013*) was based on the crystal structure of the zebrafish P2X4 receptor (*Kawate et al., 2009*), while the first crystal structure of the panda P2X7R has been described by Karasawa and Kawate (*Karasawa and Kawate, 2016*). Each P2X7 subunit is comprised of two transmembrane domains, with the three ATP binding sites present on the extracellular loop, while the carboxyl and amino termini are found intracellularly (*Jiang et al., 2013, Karasawa and Kawate, 2016*). The structure of the P2X7 receptor has a "dolphin-like" shape: the large extracellular domain constitutes the main body, consisting of the head, upper body, lower body, dorsal fin, left flipper and right flipper, and the two α-helical TM domains form the fluke anchoring P2X7 to the plasma membrane (*Karasawa et al., 2017*) (**Figure 6**).

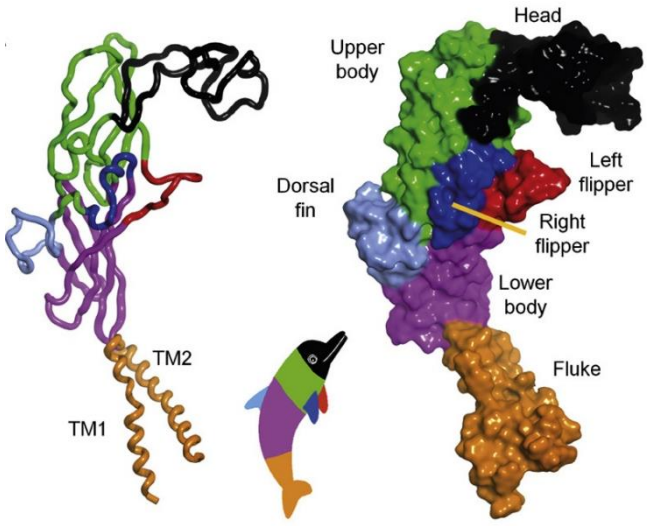

Figure 6: Structure of P2X7R. The P2X7 monomer is represented by both secondary structure (left) and molecular surface (right) styles. The shape of the monomer is compared to a dolphin with the subdivision of the monomer regions. *Image is taken from di Virgilio et al., 2017.*

P2X7R is documented to be expressed in a considerably wide range of organs and tissues and is associated with multiple functions. It was initially cloned in macrophages (*Buisman et al., 1988; Di Virgilio, 1995*) and was found in a broad range of immune system cells, including lymphocytes B and T, monocytes, erythrocytes, macrophages, and also in osteoclasts (*Burnstock and Knight, 2004*). There is a rapid and reversible opening of the P2X7R after a short exposure to the agonist (less than 10 seconds) that activates a series of cellular responses such as the formation of reactive oxygen species (*Moreira-Souza et al., 2017*), the shedding of surface molecules such as CD23 (*Pupovac et al., 2015*), CD62L (*Sluyter and Wiley, 2002*) and TNF-α (*Barberà-Cremades et al., 2017*), and up-regulation of CD80 and CD86 (*Lioi et al., 2015*). P2X7R activation also triggers the activation of the multi-protein complex nucleotide-binding domain, leucine-rich repeat and pyrin-containing receptor 3 (NLRP3) inflammasome and the release of Interleukin 1 beta (IL-1β) and IL-18 (*Buell et al., 1998, Piccini et al., 2008*), IL-2 (*Yip et al., 2009*), IL-6 (*Caporali et al., 2008*), IL-17 (*Ghiringhelli et al., 2009*), IL-18 (*Piccini et al., 2008*), and prostaglandin E2 (*Barbera`-Cremades et al., 2012*). Activation of the P2X7R, and the consequent entry of calcium into the cell interior, has also been associated with other signaling pathways involving calcium calmodulin kinases II (*Diaz-Hernandez et al., 2008*), phospholipase D (PLD) (*Humphreys and Dubyak, 1996*), NF-kB (*Ferrari et al., 1997*), GSK-3 (*Diaz-Hernandez et al., 2008*) and MAPK (*Aga et al., 2002*). Furthermore, P2X7R activation can also promote cell death, through apoptosis (*Zheng et al., 1991*) and necrosis (*Dagvadorj et al., 2015*). On the other hand, the activation of the P2X7R is also capable of inducing the activation of other proteins in a calcium-independent process, such as the activation of PKD in cultured rat astrocytes (*Carrasquero et al., 2010*). P2X7R has been related to several physiological events including neuronal differentiation (*Glaser et al., 2014; Messemer et al., 2013; Tsao et al., 2013*), axonal growth and branching (*Diaz-Hernandez et al., 2008*), and neurotransmitter release (*Leon et al., 2006; Miras-Portugal et al., 2003; Sperlagh et al., 2002*).

Quantitative investigations of P2X7 expression and immunohistochemical examination in the brain revealed that the receptor is highly expressed on microglia and macrophages, followed by astrocytes, oligodendrocytes, dendritic cells, monocytes, and natural killer cells (*Deuchars et al., 2001; Kukley et al., 2001; Gu et al., 2000; Gu et al., 2004; Xiang and Burnstock, 2005*). The existence of the receptor in neurons has long been debated; earlier investigations showed a lack of P2X7 mRNA in these cells, and the apparent non-

specificity of the antibodies employed to detect P2X7R prompted additional concerns. Later investigations, however, utilizing novel pharmacological and biomolecular techniques, revealed evidence for the existence of functional P2X7Rs in neurons, as demonstrated by microfluorimetry, electrophysiology, or immunohistochemistry techniques (*Deuchars et al., 2001; Armstrong et al., 2002; Sperlágh et al., 2002; Miras-Portugal et al., 2003; Atkinson et al., 2004*). In 2008, Yu et al., using in situ hybridization analysis, suggested the presence of P2X7 mRNA in neurons (*Yu et al., 2008*). Simultaneously, using pure cultures of hippocampus neurons, our research group revealed that the P2X7 receptor is involved in the control of axonal development and branching (*Diaz-Hernández et al., 2008*). Over the years, novel mouse models for studying P2X7R *in vivo* have been developed. One of them is a knock-in mouse that expresses a humanized P2X7 under the control of endogenous murine regulatory elements and is sensitive to Cre recombinase-mediated activation (*Metzger et al., 2017*); analysis of this model confirmed the presence of the receptor in glutamatergic pyramidal neurons of the CA3 hippocampal areas (*Metzger et al., 2017*). Another model used in various studies is a mouse that expresses EGFP directly downstream of the P2X7 mouse promoter (^{P2X7}EGFP mice); selective agonists and antagonists have been used to demonstrate the presence of functional P2X7 receptors in EGFP-hippocampal neurons (*Engel et al., 2012; Sebastián-Serrano et al., 2016*). Sebastián- Serrano *et al* demonstrated that granule neurons in the hippocampal dentate gyrus of postnatal P2X7 hypomorphic mice displayed altered synaptogenesis and dendrite branching, implying that this receptor plays a role in the development of somatodendritic compartments (*Sebastián-Serrano et al., 2016*). Furthermore, another study from our research group, demonstrated a significant reduction of P2X7R transcription in neuronal cells at the early and advanced stages of Alzheimer's Disease, using a specific amyloid mouse model (*Martínez-Frailes et al., 2019*). Despite the aforementioned considerations, the existence of P2X7R in neurons remains a source of debate (*Illes et al.,2017; Miras-Portugal et al.,2017*).

1.5.5.1 Pharmacological properties of P2X7 receptor

Like other P2X receptors, P2X7 is a trimeric channel stimulated by the binding of ATP to its extracellular domain to promote subunit rearrangement and opening of a cation channel (*Di Virgilio et al., 2018*). However, when compared to other P2X receptors, the P2X7R

has a low affinity for the physiological agonist ATP, requiring >100 µM for activation (*Khakh and North, 2006*). As a result, it can detect high concentrations of extracellular ATP released during cellular stress and damage. The synthetic analog 2'(3')-O-(4-benzoyl benzoyl) -ATP (BzATP) that binds to the receptor with an affinity 10 to 30 times higher than ATP is the most potent non-physiological agonist available to date (*Gever et al., 2006*). Stimulation of P2X7 with agonists for a short period time (< 10 seconds) results in a rapid influx of Na^+ and Ca^{2+} and an efflux of K+ (*Surprenant et al., 1996, Rassendren et al., 1997, Egan and Khakh, 2004*). P2X7 has been proposed as the pore-forming molecule (*Di Virgilio et al., 2018*), but other molecules such as pannexin-1 may also be involved (*Pelegrin and Surprenant, 2006*). However, their relative contribution remains to be resolved. Suramin and PPADS, the most used P2XR antagonists, also block the P2X7R (*Jacobson et al., 2002*). Brilliant Blue G (BBG) is a stronger and more selective P2X7R antagonist that binds to the receptor through a different binding site than ATP and has a slow dissociation rate (*Michel et al., 2007*). This compound, however, has low selectivity against P2X7Rs, even blocking P2X4, P2X5, and P2Y1 and P2Y2 rats, while potentiating human P2Y1 at concentrations of 1-3 µM (*Jacobson et al., 2002; Jacobson, 2006*). Oxidized ATP (oATP) is an irreversible inhibitor of P2X7Rs that requires incubations of 1 to 2 hours to inhibit channel activation (*Di Virgilio, 2003*), however, it has other pharmacological actions such as P2X1 and P2X2 receptors blockage (*Evans et al., 1995*) or pro-inflammatory signaling reduction (*Beigi et al., 2003*). Other blockers, such as KN-62 (known as a type II calcium/calmodulin kinase inhibitor, CaMKII), exert a non-competitive blocking effect on the human P2X7R, but not on the rat receptor (*Gargett and Wiley, 1997*). Two new selective P2X7 antagonists were developed by Abbott Laboratories in 2006: A-438079, a tetrazole derivative that competitively and reversibly blocks P2X7Rs, and A-740003, a cyanoguanidine compound that competitively acts on receptors in the high nanomolar range, both in rats and humans (*Honore et al., 2006; Nelson et al., 2006*). GlaxoSmithKline has also produced a variety of compounds with outstanding physicochemical properties, such as the BBB-permeable GSK-1482160 in rats (*Abdi et al., 2010*). Nowadays, JNJ-47965567 is one of the last P2X7R antagonists produced by Janssen Pharmaceuticals. Unfortunately, its low oral bioavailability has stopped its clinical development, as well as the lack of deeper details on its time in the brain (*Bhattacharya et al., 2013*). Nevertheless, new dihydro- and tetrahydro-triazolopyridine series have been discovered and widely optimized. JNJ-54175446 and JNJ-55308942 have ideal PK properties, such as solubility, metabolism profile,

bioavailability, and half-life, as well as good permeability and tolerability of BBB (*Letavic et al., 2017; Chrovian et al., 2018*).

1.5.5.2 The role of P2X7 receptor in neuroinflammation

Although P2X7R plays an important role in both neuronal differentiation and neuronal physiology by regulating axonal elongation and synaptic functionality, respectively, its role changes radically in pathological conditions. It is commonly assumed that during neuroinflammation, nucleotides, along with other pro-inflammatory chemicals, are released from the brain parenchyma, activating their unique receptors in various neuronal lineages (*Burnstock, 2007*). Large amounts of ATP are released into the extracellular space as a result of cell stress or tissue damage, which activates the low-affinity target P2X7R, the key sensor for ATP during inflammation (*Di Virgilio,2015*). This receptor is also the main trigger of the protective/regenerative immune response, consisting of the maturation and release of several interleukins, mainly IL-1β (*Adinolfi et al., 2018*). The role of P2X7Rs in neuroinflammation is supported by the fact that they are expressed in various cells of the CNS where they mediate inflammasome signaling (*Franceschini et al., 2015*). For instance, it is recognized that P2X7Rs are critically involved in microglial activation, thus contributing to the microglial role in inflammatory responses to CNS insults (*Boumechache et al., 2009; Monif et al., 2010*). It is well known that activation of P2X7R induces the assembly and activation of NLRP3 inflammasome to activate caspase-1 in microglial cells and peripheral immune cells, which in turn transforms pro-interleukin (IL)-1β into mature IL-1β, the master pro-inflammatory cytokine (**Figure 7**). The P2X7-NLRP3-IL1β signal is activated when ATP is present as a danger signal in high concentrations in extracellular space, triggering the microglial neuroinflammatory cascade (*Di Virgilio,2007*; *Sutterwala et al., 2006; Bhattacharya et al., 2016*). P2X7R is also essential for the generation of IL-6 pro-inflammatory mediators and tumour necrosis factor α (TNF- α) induced by ATP (*Iwata et al., 2016*). Interestingly, P2X7R activation has been shown to promote additional inflammatory processes such as the translocation of nuclear factor kappa-light-chain-enhancer of activated B cells or nuclear factor of activated T-cells (*Ferrari et al., 1997*) and the release of endothelial growth factor (*Adinolfi et al., 2012*).

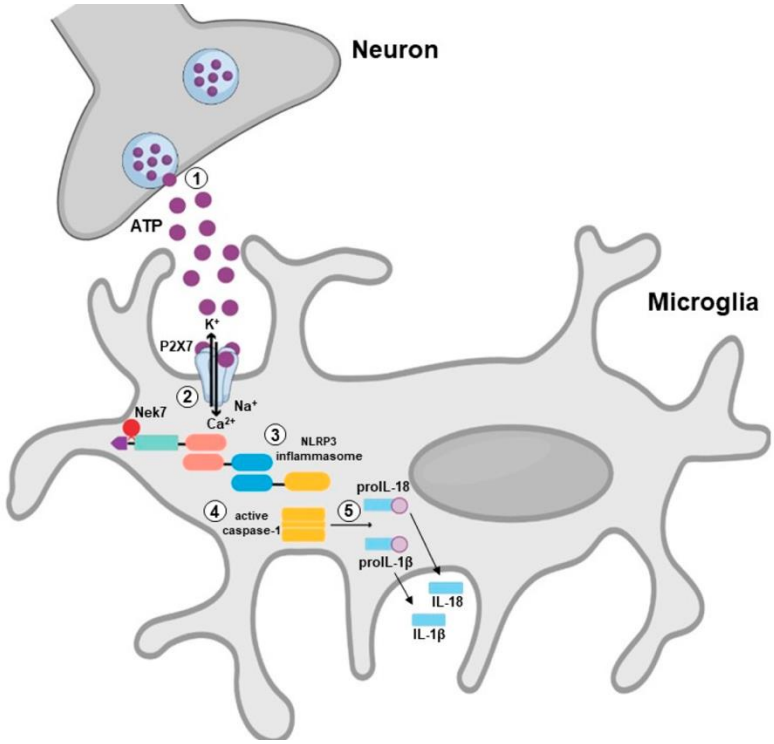

Figure 7: P2X7R-mediated NLPR3 inflammasome activation. To begin, large amounts of ATP produced by neurons or astrocytes activate P2X7R-positive microglia. Second, P2X7R stimulation causes K+ efflux, which can lead to inflammasome formation and NLRP3 activation. Following that, caspase-1 activation initiates the NLRP3 inflammasome, which stimulates the maturation of interleukins by cleaving pro-IL-1 and pro-IL-18 into IL-1 and IL-18, respectively. Finally, cytokines develop and are released (*Image adapted from Ribeiro et al., 2019)*

1.5.5.3 Involvement of P2X7R in Alzheimer's Disease

Over the last two decades, the involvement of P2X7R within the neuroinflammation processes associated with Alzheimer's Disease (AD) has been sustained by several *in vitro* and *in vivo* studies, as well as postmortem studies in the brain of AD patients (*Parvathenani et al., 2003; McLarnon et al., 2006; Ryu and McLarnon, 2008; Sanz et al., 2009; Delarasse et al., 2011; Diaz-Hernandez et al., 2012; Sanz et al., 2014; Martin et al., 2019; Martinez-Frailes et al., 2019*). Several studies support the participation of P2X7R in different mechanisms associated with AD pathogenesis, such as: the amyloid precursor protein (APP) amyloidogenic processing (*Delarasse et al., 2011; Diaz-Hernandez et al., 2012*), synaptic dysfunction (*Lee et al., 2011; Saez-Orellana et al., 2016, 2018; Goncalves et al., 2019*), oxidative stress (*Parvathenani et al., 2003; Lee et al., 2011; Zhang et al., 2015*), and neuroinflammation (*Kim et al., 2007; Sanz et al., 2009; Chiozzi et al., 2019; Martin et al., 2019; Martinez-Frailes et al., 2019*) (**Figure 8**).

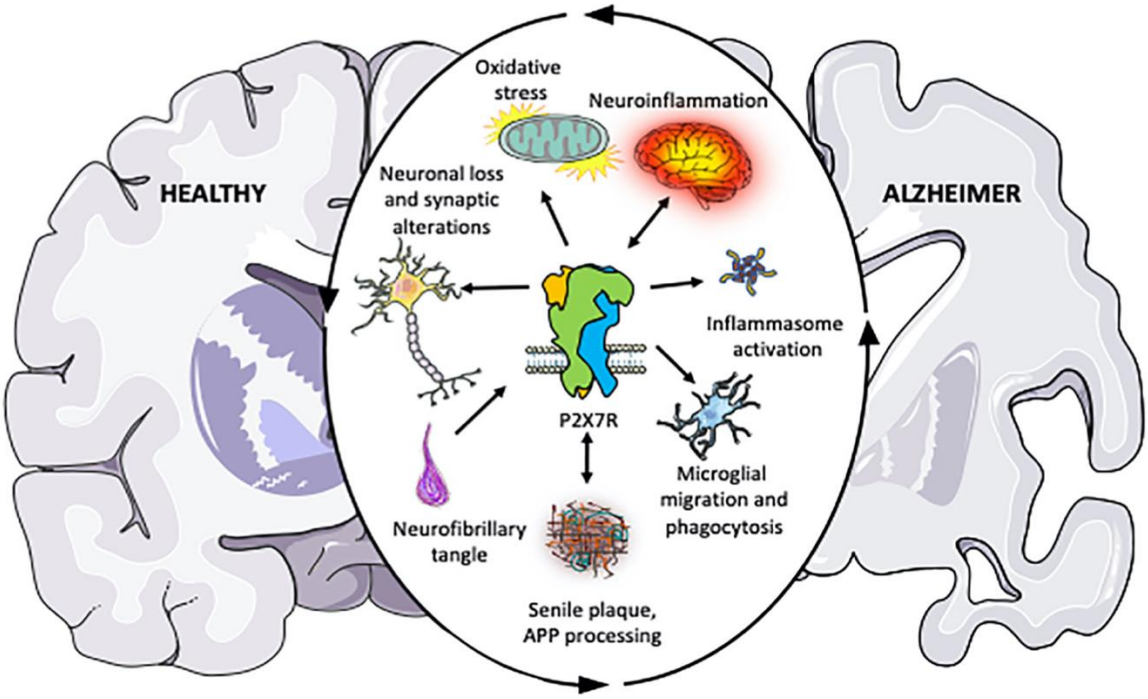

Figure 8: Schematic representation of the different physiopathological processes associated with Alzheimer's disease in which P2X7R could be involved. P2X7R tends to be active in modulating the processing of amyloid APPs. P2X7R upregulation in AD patients and mouse models of AD is also evidence of the involvement of this receptor in the AD-associated neuroinflammation phase. In addition, P2X7R also leads to neuronal loss and synaptic changes, oxidative stress, inflammatory assembly, and altered microglial activity, all of which contribute to AD progression. (*Image taken from Francistiová et al., 2020*).

The most common cause of senile dementia is AD, affecting more than twenty million individuals worldwide. AD can be divided into two forms: familiar AD and sporadic AD. Three main genes (amyloid precursor protein (APP) gene, presenilin1 (PSEN1) gene, and presenilin 2 (PSEN2) gene) are responsible for the familiar form (*Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995*). The sporadic type is more complex and most likely results from a mixture of genetic and environmental factors. Clinical symptoms of AD include gradual memory loss, diminished executive function, and problems carrying out normal activities. Early symptoms of AD include changes in thinking or unconscious actions, memory failure regarding new knowledge, and dysfunctional changes in language and speech (*Tarawneh and Holtzman, 2012*). Furthermore, 20 to 30 % of early AD patients exhibit major depressive symptoms and mood changes (*Zubenko et al.,2003*). Patients in the advanced stages of AD have extreme memory loss, hallucinations, disorientation, and a lack of self-sufficiency, and some die as a result of a respiratory syndrome (*Kalia, 2003; Lauter 1968*), infection, or fasting (*Tarawneh and Holtzman, 2012; Lauter 1968).* The pathological hallmarks of AD are

neurofibrillary tangles (NFT) formed by the intraneuronal accumulation of paired helical filament (PHF) tau and extracellular plaques, which are extracellular deposits of amyloid peptide (Aβ), derived from the metabolic processing of amyloid precursor protein (APP) (*Camden et al., 2005; Leon-Otegui et al., 2011; Morawe et al., 2012*).

One of the principal proofs supporting a potential association of P2X7R in AD was the increased P2X7R expression found in microglial cells surrounding amyloid plaques both in AD patients and in distinctive AD mouse models (*Parvathenani et al., 2003; McLarnon et al., 2006*). Later investigations, utilizing two distinct mouse models of AD, confirmed that P2X7R was upregulated in activated microglial in parallel with AD progression (*Lee et al., 2011; Martinez-Frailes et al., 2019*). Besides, an analogous assumption was recently reported using a tauopathy mouse model, P301S mice, where P2X7Rs were mainly expressed by astrocytes (*Jin et al., 2018*). Additionally, different studies using both *in vitro* and *in vivo* approaches have proposed that P2X7R might be one of the factors governing APP processing (*Delarasse et al., 2011; Leon-Otegui et al., 2011; Darmellah et al., 2012; Diaz-Hernandez et al., 2012*). *In vivo* inhibition of P2X7Rs with BBG in an AD mouse model (J20) induced a significant decrease in hippocampal amyloid plaques that was associated with a decrease in GSK3 activity, increasing the proteolytic processing of APP through increased α-secretase activity (*Diaz-Hernandez et al., 2012*). Another study affirmed that genetic depletion of P2X7R prompts a significant reduction in the number of senile plaques in 10-months-old APP/PS1 mice deficient in P2X7 (*Jankowsky et al., 2004*). This decrease was joined by a drastic decrease in Aβ peptide levels and a rescue of the cognitive deficit developed by APP/PS1 mice (*Martin et al., 2019*).

Other than the roles already described, P2X7R has an impact on activating the NLRP3 inflammasome, which is known to contribute to the pathogenic inflammatory response that happens during AD. For example, in an AD transgenic mouse model, NLRP3 inflammasome deficiency overturned the pro-inflammatory activation of microglia and improved disease progression (*Heneka et al., 2013*). In agreement with this, it has been described that pharmacological blockade or knocking out the P2X7R in different AD mouse models has beneficial outcomes by decreasing neuroinflammation (*Ryu and McLarnon, 2008; Chen et al., 2018; Martin et al., 2019*).

P2X7R activation not only promotes NLRP3 inflammasome assembling but also stimulates microglial migration. Indeed, using *in vitro* and *in vivo* approaches, a recent study confirmed that ATP-induced P2X7R activation promotes microglial migration (*Martin-Frailes*

et al., 2019). Other microglia feature that has been related to purinergic signaling is its phagocytic capacity. Martinez-Frailes et al., 2019 found that GSK 1482160A, a selective P2X7R inhibitor, significantly increased the phagocytosis by microglial cells expressing P2X7R. Additionally, *in vitro* studies using cultured primary human microglial cells confirmed that P2X7R activation-induced BzATP decreased the phagocytic capacity of microglial cells (*Janks et al., 2018*).

It is known that the production and the presence of high levels of reactive oxygen species (ROS) is a common component detected in AD brains (*Albers and Beal, 2000; Sebastian-Serrano et al., 2019*) and might favour the progression of the disease by promoting oxidative stress and mitochondrial dysfunction (*Dias et al., 2013*). There is proof that P2X7R might be the essential receptor engaged in the generation of hydrogen peroxide (H_2O_2) by activating microglial cells (*Nuttle and Dubyak, 1994*). An early investigation showed that stimulation of isolated microglial cells from rodent brains with ATP or BzATP, improved Ca^{2+} and H_2O_2 production in rat microglia. These *in vitro* effects were impeded by three P2X7R antagonists, oATP, BBG, and PPADS (*Parvathenani et al., 2003*). Like-minded to the antioxidative effect of P2X7R antagonists, *in vivo* administration of selective P2X7R antagonist, A438073, avoided ROS production and oxidative DNA damage induced by P2X7R activation in spinal cord dorsal horn neurons (*Munoz et al., 2017*).

P2X7R is, by all accounts, likewise associated with synaptic dysfunction and cellular death in AD. It was established that Aβ deposits contribute to the synaptic loss being more evident in the nearness of the senile plaques (*Lanz et al., 2003*). A later report presented that P2X7R overexpression induced by Aβ oligomers, enlarged synaptic failure and neuronal dyshomeostasis in a cellular model of AD (*Sáez-Orellana et al., 2018*).

Considering the aforementioned data and due to the pivotal role of P2X7R in neuroinflammation, it has been postulated that P2X7R represents a promising therapeutic target for the treatment of some neurodegenerative diseases (*Chessell et al., 2005; Mehta et al., 2014; Takenouchi et al., 2010; Bhattacharya & Biber, 2016*). One of the main common features of neurodegenerative diseases is the accumulation of intracellular misfolded proteins, related to a malfunction of the Ubiquitin-Proteasome System (UPS) (*Dantuma & Lindsten 2010*). The UPS is the main system in charge of controlling cellular proteostasis (*Kaushik & Cuervo 2015*). Dysfunction of the UPS is associated with many neurological diseases, including AD, Amyotrophic Lateral Sclerosis (ALS), Parkinson's disease (PD),

Transmissible Spongiform Encephalopathies (TSE), and Huntington's disease (HD) (*Leigh et al., 1991; Neumann et al., 2006*).

1.6 The Ubiquitin-Proteasome System

1.6.1 Ubiquitination and Regulation

The majority of protein degradation in eukaryotic cells occurs via the Ubiquitin-Proteasome System (UPS). The degradation of a target protein in this pathway is triggered by the covalent binding of several copies of ubiquitin molecules to a substrate protein by the action of an enzymatic cascade (*Hershko and Ciechanover,1998*). The poly-ubiquitin chain then acts as a signal that shuttles the target protein through the proteasome, where the polypeptide chain is cleaved into short amino acids (*Bhattacharyya et al.,2014*) (**Figure 9**). This enzymatic mechanism is involved in regulating numerous and varied cellular processes, such as proteasomal-dependent protein degradation, antigen processing, apoptosis, biogenesis of organelles, cell cycle and division, DNA transcription and repair, differentiation and development, neural and muscular degeneration, morphogenesis of neural networks, modulation of cell surface receptors, the secretory pathway, response to stress and extracellular modulators, ribosome biogenesis or viral infection (*Deshaies & Joazeiro, 2009; Raiborg & Stenmark, 2009; Ulrich & Walden, 2010; Zinngrebe et al, 2013*).

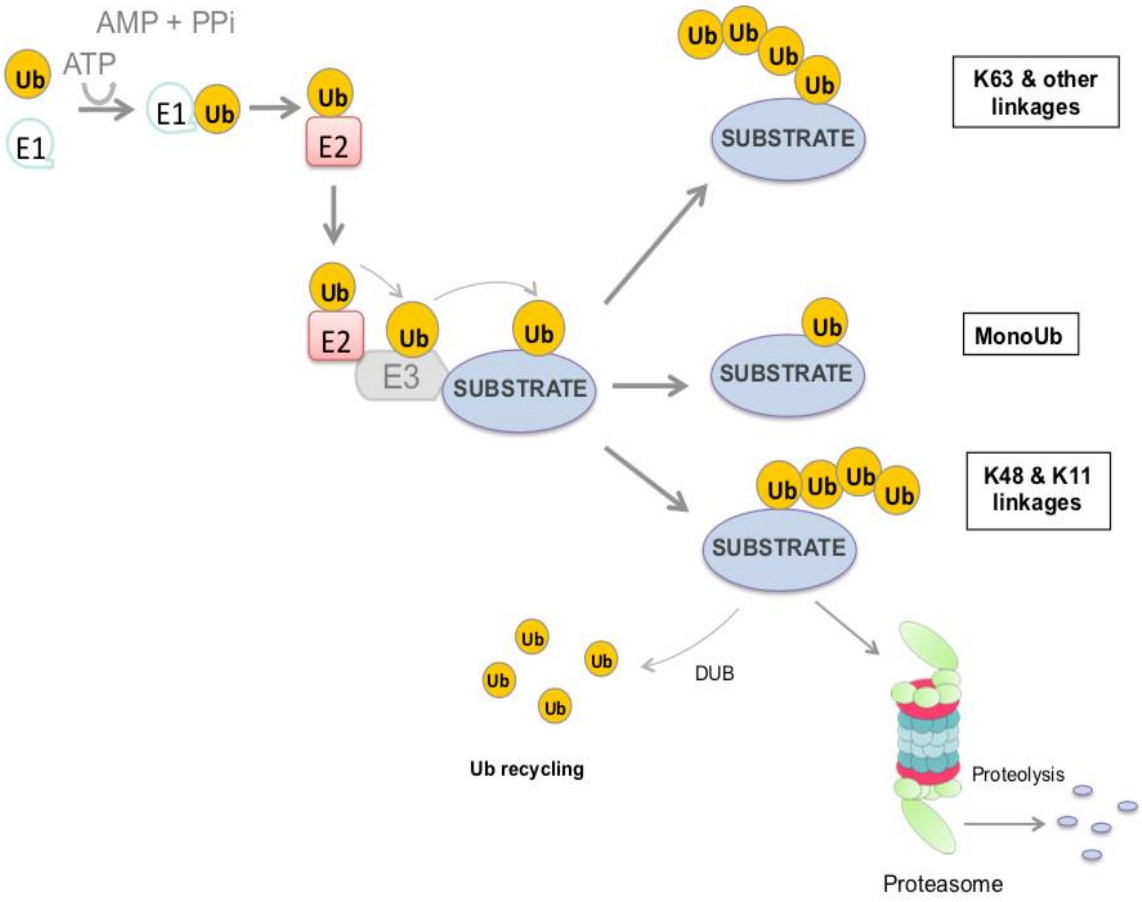

Figure 9: Scheme of the Ubiquitin-Proteasome System consisting of E1, E2, and E3 enzymes that work in sequence to link single ubiquitin moieties to substrate proteins in an ATP-dependent manner. An ATP-dependent reaction involving the formation of a thiol ester bond between cysteine at the active site of the E1-activating enzyme and G76 in Ubiquitin initiates the ubiquitylation cascade. Ubiquitin is then moved to the active cysteine of an enzyme that is E2-conjugating and interacts with an E3-ligase. Finally, the 26S proteasome is shuttled to ubiquitylated substrates and Ubiquitin is recycled for another round of reactions. DUBs oppose the degradation of the substrate by reversing the ubiquitylation process.

Ubiquitin (Ub) is a small regulatory protein (8.6 kDA) that belongs to the family of Ubiquitin-like proteins (UBLs) and was first identified in 1975 (*Goldstein et al., 1975*). A post-translational modification by covalent attachment through an isopeptide bond between the C-terminal glycine 76 of Ub and the amino group of a protein substrate's lysine residue, called ubiquitination, is the fundamental feature of Ub. The localization, stability, and properties of its target protein can be changed by Ubiquitin. An analysis of proteomics found that Ub modifies around a thousand different proteins (*Peng et al, 2003*). Ub can also bind to unique surfaces, such as domains that bind to Ubiquitin, and can also form non-covalent interactions with either Ubiquitin moieties or Ubiquitin chains (*Dikic et al., 2009*).

Ubiquitination requires a multi-enzymatic cascade involving the action of three distinct forms of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and

ubiquitin-ligase enzyme (E3). First, by an ATP-dependent reaction, the E1 enzyme binds the free Ub and activates its C-terminal glycine residue (G76) (*Haas & Rose.,1982*). Second, through its catalytic cysteine, E1 attacks the activated form of Ub, forming a thioester bond between E1 and Ub. (*Ciechanover et al., 1982*). The C-terminus of the activated Ub is then moved by the E1 to a catalytic cysteine residue on the E2 (*Haas et a.l, 1982; Pickart & Rose., 1985*). Ub is eventually transferred from there to an E3 enzyme, which will catalyse the binding of the Ub G76 residue through an amide isopeptide binding to the ε-amino group of lysine on the target protein (*Hershko & Ciechanover 1998*).

Only one E1 enzyme can activate Ub and transfer it to a greater number of E2s (*Hershko and Ciechanover, 1998; Pickart, 2001*). Each of these E2s interacts with many E3s, and through their recognition motifs, a much greater number of E3s recognize a set of substrates. Ubiquitination plays an important role by regulating degradation, activating and inactivating proteins, modulating protein-protein integrations, and organizing the cellular localization of the protein (*Glickman and Ciechanover,2002; Mukhopadhyay and Riezman, 2007; Schnell and Hicke, 2003*). In its sequence, Ub comprises 7 lysines (K6, K11, K27, K29, K33, K48, and K63) (*Peng et al., 2003*). All these lysine residues can form ubiquitin-ubiquitin isopeptide linkage in vivo and the type of linkage strongly influences the consequences of ubiquitination and the fate of target proteins. Furthermore, a target protein can be modified at one or more Lysine residues by a single ubiquitin molecule (monoubiquitination), by Lysine-linked chains of ubiquitin (polyubiquitination), or by a combination of both (*Komander, 2009*). The Lys-48 (K48) residue can be processively conjugated by another ubiquitin moiety to form a polyubiquitin chain of at least four ubiquitins. The most famous fate of proteins labeled with this polyubiquitin chain is the proteasome 26S-mediated degradation, which is also the best-characterized function of ubiquitination (*Hershko and Ciechanover, 1998; Koegl et al., 1999; ; Pickart, 2001; Kuhlbrodt et al., 2005*). Similarly, K11-linked polyubiquitination of proteins can also result in their proteasomal degradation (*Grice et al.,2015*). However, K63-linked poly-Ub chains lead to non-degradative cellular processes, including kinase activation, DNA damage tolerance, signal transduction, and endocytosis (*Hochrainer & Lipp 2007*).

1.6.2 The 26S Proteasome

The 26S proteasome is a large and complex multimeric structure consisting of two different structures: the catalytic core particle of the 20S and one or two similar regulatory particles of 19S that mediate the recognition, deubiquitylation, and translocation of the substrate protein within the complex 20S complex (*Pickart,2001*). Once in the core particle, in an ATP-dependent manner, the proteolytic active site degrades the proteins into small peptides. A large multimer is the central particle containing the proteasome's catalytic activity, consisting of 28 subunits arranged into four stacked subunit rings. (**Figure 10**).

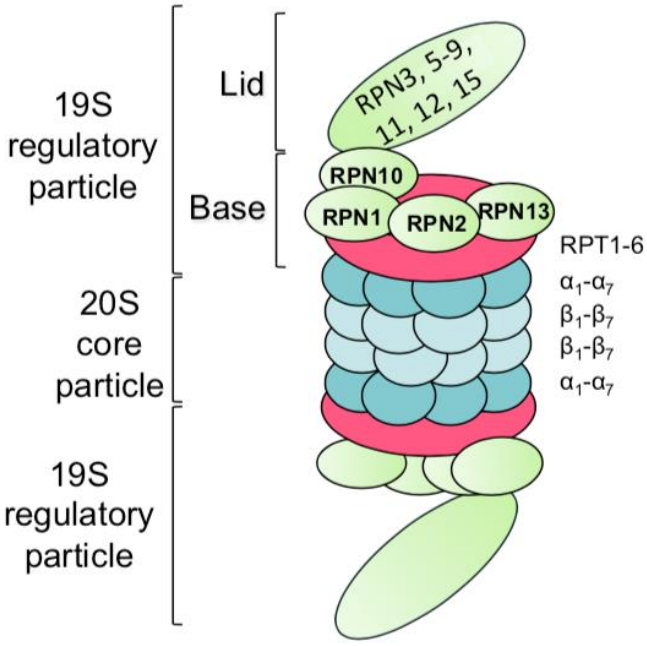

Figure 10: Schematic representation of the 26S proteasome. The 26S proteasome consists of the 20S proteasome catalytic and a regulatory particle, which can be subdivided into the complex of the Base and the Lid. A 700-kDa barrel containing four rings arranged in a stack is the 20S proteasome (20S CP). It comprises 28 subunits, which are the products of two homologous gene classes (α and β). The two external rings consist of only α-subunits, whereas the two internal rings are made up of β-subunits.

There are 7 different subunits in each ring that are arranged in a hollow (*Groll el al., 1997*). Each heptameric ring is made up of either α or β subunits, forming a structure of α1-7-β1-7-β1-7-α1-7. Only 3 of the 7 β subunits are responsible for the proteolytic activity: β1[proteasome subunit beta type-1(*psmb6*)] has caspase-like activity and cleaves peptide bonds after acidic amino acids, β2 [proteasome subunit beta type-2 (*psmb7*)] has trypsin-like activity and cleaves after basic residues and β5 [proteasome subunit beta type-5(*psmb5*)] is

chymotrypsin-like activity and cleaves after hydrophobic residues (*DeMartino & Slaughter 1999;Groll et al 1997*).

The α-ring controls the access of substrates into the inner chamber. The position of the 7 subunits forms the α-ring blocks or allows the entrance of the substrate. This gate exists in a continual alternation between open and closed states (*Osmulski et al, 2009*). When the N-termini of the 7 subunits faces the interior of the ring, the chamber remains closed (*Groll et al, 2000*). To open the gate, additional factors, such as subunits of the regulatory particles, need to bind the outer ring of the core particle, which allows a shift of the N-termini α subunits (*Stadtmueller & Hill, 2011*). The 20S alone is not capable of degrading the substrates. To be effective, it needs to bind to the 19S regulatory particles. The 19S complex can be located at one or both ends of the 20S complex and is responsible for the opening of the α-ring and the deubiquitylation, unfolding, and translocation of the molecules into the catalytic compartment. The 19S has at least 19 subunits and can be divided into two sub-structures, referred to as "base" and "lid" respectively (*Pickart & Cohen, 2004*). While the base is necessary for 20S proteolytic activity to be activated, the lid is required for ubiquitinated substrates to degrade. The base is composed of 6 ATPase subunits (Rpt1-6) and 4 non-ATPase subunits (Rpn1, 2, 10, and 13). When they touch each other, the ATPase ring and the 7 α subunits of the 20S core form a complex. In an ATP-dependent step, the ATPases unfold the substrate and then translocate the substrate into the proteolytic chamber (*Aubin-Tam et al, 2011*). The lid consists of 9 non-ATPase subunits (Rpn3,5-9,11,12,15) that recognize the degradation of ubiquitinated substrates (*Glickman et al, 1998*). The lid and the base are connected by the Rpn10 subunit and are responsible for stabilizing the complex.

1.6.3 Proteasomal degradation of substrates

Ubiquitination alone is not enough to target a substrate for degradation. The target protein must also have an unstructured region, in addition to carrying a poly-ubiquitin chain, which will serve as the starting point for degradation. First, there is an association between the ubiquitinated substrate (Ub-S) and the Ub-binding receptor (Rpn10, Rpn13, or Rpn15) in the proteasome or in the shuttle receptors with a particular domain (*Lander et al, 2012*) (**Figure 11**). Once the substrate has bound to the proteasome, the Ub-S chain must be deubiquitinated thanks to the action of the DUBs of the 19S subunit: Ubp6 (Usp14 in humans),

Uch37, or the Rpn11 subunit of the lid (*Kawakami et al 1999*). Rpn11 cleaves Ub chains *en bloc* by cleavage at proximal Ub (*Verma et al, 2002*). Ubp6 is a base-associated DUB that cleaves from its distal end within polyubiquitin chains or trims them. Uch37 is a DUB associated with proteasomes that destroy Ubs one at a time (*Lam et al, 1997*).

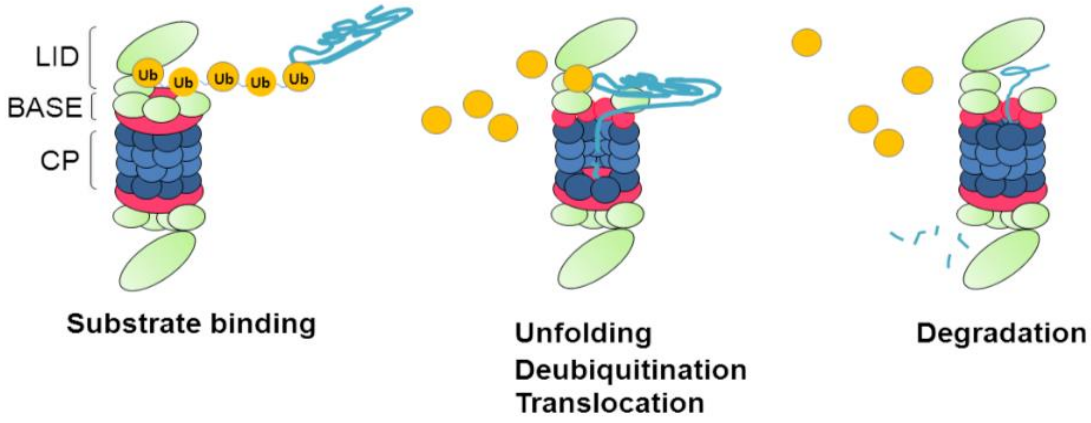

Figure 11: Steps of proteasomal degradation. Through an ATP-dependent process involving three enzymes, Ub is conjugated to proteins that are intended for degradation. Ubiquitination of a protein helps the 19S regulatory particle to identify it. In the 20S proteasome, the hexametric ATPase complex opens a gate and translocates the protein through its central pore, allowing upstream regions to unfold. The ubiquitin chain is extracted by DUBs on the 19S, and the ATPases complete the substrate injection into the 20S where it is degraded into small peptides.

1.6.4 Immunoproteasome

In mammals, in response to inflammatory cytokines such as interferon- γ (IFN-γ) and tumor necrosis factor-α (TNF-α), the constitutively expressed catalytic β-subunits are replaced by inducible β-counterparts known as immunosubunits, to generate immunoproteasomes (iPs) (*Groettrup et al.,2010*) (**Figure 12**). The iP is expressed predominantly in immune system cells such as T cells, B cells, monocytes, macrophages, dendritic or medullary epithelial thymic cells (*Kniepert and Groettrup 2014*). Immunosubunits β1i [also known as low molecular mass peptide 2 (LMP2); proteasome subunit beta 9 (*psmb9*)], β2i [also known as multicatalytic endopeptidase complex-like 1 (MECL-1); *psmb10*] and β5i [also known as LMP7; *psmb8*] are preferentially incorporated during proteasome assembly to form the iP upon IFN- α induction (*Ebstein et al. 2012*). Although the iP shares between 60-70% homology with the constitutive proteasome (*Ferrington & Gregerson 2012*), both vary in many ways, such as the assembly time needed for their biosynthesis or the

complex's half-life. β1i, β2i, and β5i exhibit preferential substrate cleavage after acidic, basic, and hydrophobic amino acid residues, respectively (*Groettrup et al. 2001)*. In addition, the iP has increased chymotrypsin-and trypsin-like activities but decreased caspase-like activity (*Rock & Goldberg 1999, Tanaka & Kasahara 1998*). Proteasomes containing both constitutive and immunosubunits, which are called mixed-type proteasomes, can be produced depending on the development and availability of the immunosubunits (*Dahlmann 2016*). In addition, two tissue-specific variants of the 20S proteasome have been discovered to date, apart from the iPs. Thymoproteasomes are expressed primarily in thymus cortical epithelial cells, where the positive selection of T lymphocytes plays a crucial function. Spermatoproteasomes are located only in the testes where they are required during spermatogenesis (*Kniepert and Groettrup 2014*).

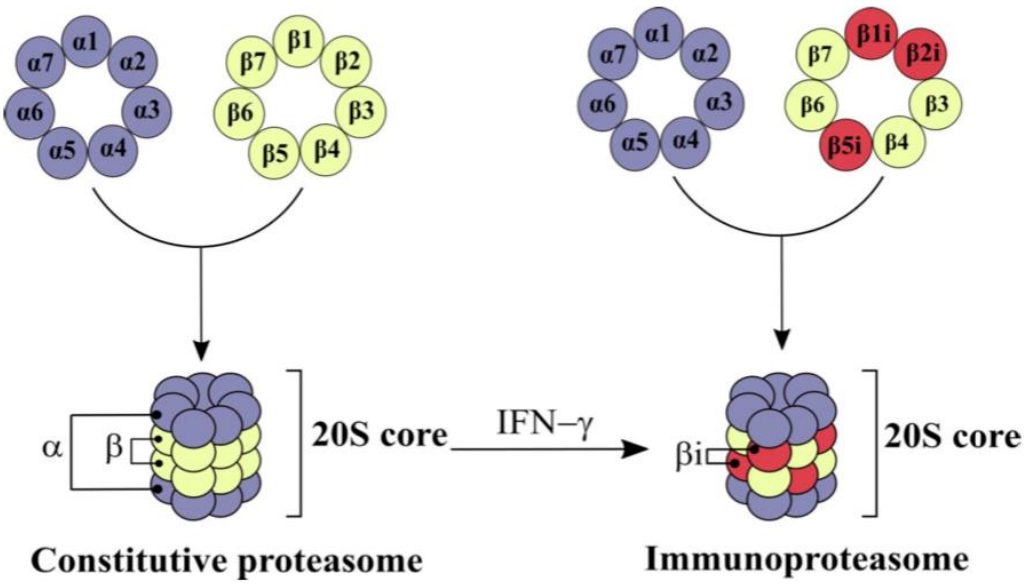

Figure 12: Structure of the constitutive proteasome and the immunoproteasome. The 20S proteasome's catalytic core is made up of two outer seven α-rings and two inner seven β-rings that assemble to form the constitutive proteasome. In the presence of pro-inflammatory cytokines such as IFN-γ, the synthesis of three catalytic β "immunosubunits" occurs, which are incorporated into newly formed proteasomes instead of their constitutive catalytic equivalents to form the 20S immunoproteasome. *Adapted from Vachharajani thesis, 2016*

1.6.5 AD pathogenesis and the Ubiquitin-Proteasome System

UPS dysfunction is associated with many neurological disorders, including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, and epilepsy (*Upadhya and Hegde,2007; Hegde and Upadhya 2007; Engel et al,2017*). Over the last few years, evidence has accumulated that supports the premise that the UPS system plays a role in AD. The presence of ubiquitin and ubiquitinated proteins in the AD brain were the initial clues suggesting that the UPS was involved in the pathogenesis of AD. Moreover, it is known that proteasome activity is lower in AD brains than in age-matched controls (*Keller et al., 2000; Lopez Salon et al., 2000*). In addition, high levels of ubiquitin have been found in AD patients' brain homogenates and cerebrospinal fluid samples (both lumbar and postmortem punctures) (*Kudo et al., 1994*). Also, there is evidence that in AD brains, ubiquitin accumulates in both Aβ plaques and neurofibrillary tangles (*Oddo, 2008*). Under normal conditions, the UPS system controls Aβ accumulation in neuronal cells by either decreasing Aβ production or encouraging its proteolytic degradation (*Hong et al., 2014*). As a result, any disruption in the UPS can result in Aβ accumulation in the cytoplasm of neurons, facilitating the formation of Aβ plaques and, consequently, cell death. Furthermore, the accumulation of Aβ deposits may inhibit proteasomal activity, resulting in a malfunction of the multivesicular bodies sorting pathway, which further inhibits Aβ degradation, creating a vicious cycle that contributes to the formation of pathogenic plaques (*Hong et al., 2014; Tramutola et al., 2016*). Moreover, there are still other mechanisms that might explain the association between the UPS and Aβ, like the fact that these structures contain ubiquitin-B mutant protein (UBB+1), a mutant form of ubiquitin carrying a 19-amino acid C-terminal extension generated by a transcriptional dinucleotide deletion (*van Leeuwen et al., 1998*). Notably, UBB+1 has been shown to block ubiquitin-dependent proteolysis in neuronal cells (*Lindsten et al., 2002*), to cause neuritic beading of mitochondria in associating with neuronal differentiation (*Tan et al.,2007*), and to be a mediator of Aβ-induced neurotoxicity (*Song et al.,2003*).

1.6.5 Tau aggregation and the Ubiquitin-Proteasome System

One of the key players in AD pathogenesis is the microtubule- associated protein tau, an axonal protein mainly expressed in the central and peripheral nervous system. The tau protein controls the stability and assembly of microtubules, a function that is finely regulated by the phosphorylation system (*Wang and Mandelkow,2012*). In many neurodegenerative disorders, Tau aggregates are present and correlate with the degree of memory loss in AD. The accumulation of hyperphosphorylated Tau, with the consequent aggregation into paired helical filaments (PHFs) and formation of neurofibrillary tangles (NFTs), has been described as a characteristic feature of a family of neurodegenerative diseases collectively known as Tauopathies (*Xu et al.,2014*).

The UPS is involved in tau clearance, with a particular enzymatic cascade involved in tau ubiquitination and degradation. Any dysregulation of the UPS, like Aβ formation, may halt tau protein degradation and, in combination with hyperphosphorylation, promote the formation of tau tangles (*Lee et al., 2013*). The evidence that UPS degrades tau (*David et al., 2002; Grune et al.,2010*) supports the idea that its impairment would contribute to intracellular tau accumulation. Interestingly, tauopathies are characterized by UBB+1 accumulation (*Fischer et al.,2003*). Proteasomal activity deficiency within susceptible neurons can also facilitate tau aggregation, whose dysfunction can be due to age-related insufficiency or the proteotoxic effect of tau aggregates (*Lee et al., 2013; Myeku et al., 2016*). Other mechanisms that might also accelerate the accumulation of tau protein are the dysfunction of the tau-specific E2 enzyme UBCH5B (ubiquitin-conjugating enzyme E2 D2, alias UBE2D2), and E3 ligase CHIP (carboxyl-terminus of Hsc70 interacting protein) that was found to serve as a Ub ligase for phosphorylated tau (*Saidi et al., 2015; Shimura et al.,2004*). A recent study found a link between the accumulation in human AD synapses of hyperphosphorylated Tau oligomers, increased ubiquitinated substrates, and increased proteasome components, consistent with UPS dysfunction (*Tai et al., 2012*). In addition, a commonly accepted indicator of a UPS-toxic Tau relationship is that not only are the Tau aggregates found in degenerating neurons phosphorylated but they are also ubiquitinated (*de Vrij et al., 2004*).

1.6.6 Epilepsy and Ubiquitin Proteasome System

Epilepsy is a brain disorder characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, physiological, and social consequences of this condition (*Fisher et al., 2005*). An epileptic seizure is a temporary event of signs and/or symptoms due to irregular repetitive or synchronous neuronal activity in the brain, according to the definitions of the International League against Epilepsy (ILAE) 2005 (*Fisher et al. 2005*). Loss of consciousness, excessive twitching of muscles, short periods of amnesia, sleep disturbances, as well as other visual, cognitive, psychic, or autonomic disturbances can be clinical symptoms of seizures. An abnormal discharge in a population of hyper-excitable neurons results in epileptic seizures. Typically, seizures are generated in the cortical and hippocampal structures. A seizure's clinical manifestation depends on its origin location, time course, discharge outbreak (*Avanzini and Franceschetti 2003*). Interestingly, seizures are a co-morbidity of many neurodegenerative diseases, including AD and Huntington's disease (*Vossel et al., 2013; Cepeda-Prado et al., 2012*).

During epileptogenesis, that is, the process of converting a nonepileptic brain into one capable of generating spontaneous, recurrent seizures, a plethora of structural and cellular mechanisms occur, fostering persistently increased neuronal excitability and abnormal plasticity (*McNamara ,1999; Thijs et al.,2019*). Those changes that happen in the brain may include neurodegeneration, gliosis, axonal injury, damage to the blood-brain barrier, recruitment of inflammatory cells into brain tissue, and reorganization of individual neuronal cells' molecular architecture (*Pitkänen and Lukasiuk, 2011*).

Due to UPS's critical functions, disruptions in protein ubiquitination or UPS in neurons often lead to impaired synaptic transmission and related disease phenotypes in neurological disorders. Because ion channel homeostasis is critical in the control of neuronal excitability, impaired ubiquitination or UPS in neurons can also cause seizures and epilepsy (*Macdonald and Kang, 2012, Hall et al., 2017, Widagdo et al., 2017*). Indeed, alterations in the ubiquitination of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) or voltage-gated potassium channels, have been related to increased seizure intensity or susceptibility in epilepsy (*Ekberg et al., 2007;Kim et al., 2018*). Also, Lafora disease (LD), a rare neurodegenerative disorder with severe forms of progressive myoclonus epilepsy, is caused by recessive mutations in the gene encoding the E3 ubiquitin ligase Malin (*Gentry et al., 2005*).

A study found that Lafora bodies, glycogen-like intracellular inclusions found in LD, play a role in the sequestration of chaperons and the 20S proteasome subunit (*Rao et al.,2010*). Moreover, mutations in the E3 ligase Ube3a have been linked to Angelman syndrome, a rare neurological condition associated with intractable seizures (*Margoli et al.,2015*). Previous research has shown that in experimental epilepsy models and in patients with temporal lobe epilepsy, immunoproteasome expression is upregulated (*Mishto , et al. 2011*). More recently, after repeated seizures (*status epilepticus*) and subsequent accumulation of polyubiquitinated substrates in the brain, UPS function is impaired (*Engel et al., 2017).* In addition, the inhibition of UPS undergoes spatiotemporal changes over time and depends on the type of cell and affected brain area, becoming more noticeable in brain regions less prone to seizures, including neurons and astrocytes (*Engel et al., 2017*).

1.6.7 Purinergic Signaling in Ubiquitin Proteasome System dysfunction

Recent works have reported that extracellular nucleotide may regulate proteasome activity (*de Diego-Garcia et al.,* 2017) and the UPS impairment associated with neuroinflammation (*de Diego Garcia et al., 2018*). It was found that $P2Y_2R$ activation, in particular, can boost CT-L and PG-L proteasome activity by increasing the expression of their catalytic proteasome subunits β5 and β1, respectively. The activation of Src, PI3K, and ERK is the intracellular signaling mechanism underlying these changes (*de Diego-Garcia et al., 2017*). Furthermore, it was demonstrated that *in vivo* injection of a specific $P2Y_2R$ agonist significantly improves UPS activity in neural cells (*de Diego Garcia et al., 2018*). The neuroprotective role associated with $P2Y_2R$ has been mainly observed under pathological conditions like neuroinflammation, oxidative stress, and neurodegenerative diseases (*Weisman et al.,2012*) where UPS impairment takes place (*Dantuma and Lindsten., 2010*).

The selective loss of $P2Y_2R$ has been linked to AD neuropathology (*Lai et al., 2008*). Furthermore, another study found that the absence of the receptor increases plaque development and Aβ levels in the hippocampus in an amyloid mouse model (*Ajit et al 2014*). While $P2Y_2R$ has been related to neuroprotective benefits, P2X7R appears to have the opposite impact. As previously stated, P2X7R is elevated in Alzheimer's disease patients, particularly in microglial cells surrounding senile plaques (*Parvathenani et al., 2003;*

McLarnon et al., 2006), and inhibition or genetic depletion of the receptor has been shown to reduce the quantity and size of amyloid plaques by lowering Aβ levels (*Diaz-Hernandez et al., 2012; Jankowsky et al.,2004; Martin et al., 2019*). Proteasomal dysfunction in the AD brain has been linked to the inhibitory binding of filamentous tau to proteasomes (*Keck et al., 2003*) and recent research has discovered tau-driven proteasome impairment in a mouse model of tauopathy (*Myeku et al., 2016*). Furthermore, Aβ inhibits the proteasome and enhances amyloid and tau accumulation (*Tseng et al., 2008*).

Considering that neuroinflammation associated with AD can affect UPS activity (*de Diego Garcia et al., 2018; Pintado, et al., 2012*) and cause P2X7R upregulation (*Martinez-Frailes et al., 2019*), and given that UPS dysfunction is one of the key molecular mechanisms underlying the development of several neurodegenerative disorders, including AD (*Gadhave et al., 2016*), it is rational to speculate that activation of P2X7R might contribute to the progression of AD by causing UPS impairment.

Objectives

Previous research has demonstrated that extracellular nucleotides through the activation of a specific receptor can modulate the Ubiquitin Proteasome System (UPS). As mentioned in the Introduction, UPS impairment is one of the common mechanisms underlying several neurological disorders, including Alzheimer's Disease and epilepsy, and it has been widely demonstrated that the purinergic receptor P2X7 (P2X7R) plays an important role in disease progression by contributing to neuroinflammation and APP processing.

Therefore, in this doctoral thesis, we proposed the following objectives:

1. Investigate the implication of P2X7R in the modulation of the UPS and determine the molecular pathway involved.
2. Examine the role of P2X7R in the UPS impairment associated with Alzheimer's Disease and Epilepsy.
3. Analyze the potential benefits of blocking P2X7R as a new therapeutic approach to rescue the UPS impairment associated with neurological diseases.
4. Evaluate P2X7R as a potential blood biomarker for Alzheimer's Disease.

Objetivos

Investigaciones anteriores han demostrado que los nucleótidos extracelulares pueden modular el Sistema Ubiquitina-Proteasoma (UPS) a través de la activación de un receptor específico. Como se ha mencionado en la Introducción, el deterioro del UPS es uno de los mecanismos comunes que subyacen a varios trastornos neurológicos, incluida la enfermedad de Alzheimer y Epilepsia, y se ha demostrado ampliamente que el receptor purinérgico P2X7 (P2X7R) juega un papel importante en la progresión de la enfermedad al contribuir a la neuroinflamación y al procesamiento de la proteína precursora del amiloide (APP).

Este hecho nos llevó al planteamiento de los siguientes objetivos:

1. Investigar la implicación de P2X7R en la modulación del UPS y determinar la vía molecular involucrada en dicho proceso.
2. Examinar el papel de P2X7R en el deterioro del UPS asociado con la enfermedad de Alzheimer y epilepsia.
3. Estudiar el potencial del bloqueo de P2X7R como una nueva estrategia de tratamiento para rescatar el deterioro del UPS asociado a enfermedades neurológicas.
4. Evaluar P2X7R como un potencial biomarcador para la enfermedad de Alzheimer.

2 Materials

2.1 Equipment

The different equipment used for the performance of the Biochemical and Molecular Biology techniques that are described in this thesis are listed in **Table 5**.

Table 5. List of the different equipment used.

Technique	Instrumentation and Model	Commercial provider
Cell Culture	Optical Microscope TE-200	Nikon
	AH-100 horizontal laminar flow Hood	Tel-Star
	Incubator 5215-2	Shel-Lab
Gene Expression	SimpliAmp™ Thermal Cycler, StepOnePlus Real-Time PCR	Applied Biosystem
	Wide mini-sub cell GT Horizontal Electrophoresis System	Bio-Rad
	Biophotometer Plus Spectrophotometer	Eppendorf
	Gel Logic 200 Imagin System Tranluminator	Kodak
	Gel Doc XR^{+} System	Bio-Rad
Immunological Techniques	Cryostat CM1950	Leica
	DTS-2 Digital Thermo Shaker	Elmi
	Stereo Microscope Zoom WF 10x/20	VWR
	Microscope TE-200	Nikon
	DFC310 FX Camera, Magnifier MZ10F, Confocal Microscope TCS SPE, DM 1000 Microscope	Leica
	pE-300^{white} fluorescence LED	CoolLED
Western Blotting	Mini-Protean 3 Electrophoresis System	Bio-Rad
	Mini-Trans-Blot Electrophoretic Transfer Cell Transfer System	Bio-Rad
	CP1000 Autoradiography Developer	AGFA
	LAS500 chemiluminescence detector	GE Healthcare
	LI-COR Odyssey	Biosciences

Stereotaxic injection	Stereotaxic equipment	UNO Anesthesia
	EVERFLO Oxygen Concentrator	Respironics
	Fluovac (Adsorption anesthesia)	Harvard Apparatus
	Anesthesia equipment	MPB
	Infusion pump	KdsScientific
Proteasome activity assay	LS55 Fluorimeter	PerkinElmer
Elisa	Microplate Reader VARIAN Cary	Agilent Technologies

2.2 Reagents and chemicals

Table 6 lists the compounds and reagents used in the experiments performed, as well as the commercial providers that supplied them.

Table 6. List of reagents and chemicals used.

Experimental Technique	Reagents and chemicals	Commercial provider
Cell Culture	Antibiotic / Antifungal Solution, Glutamax, Trypsin, Fetal Bovine Serum	Thermo Fisher Scientific
	Trypan Blue, DMEM, DMSO	Sigma-Aldrich
Transfection Procedures	Lipofectamine 2000	Thermo Fisher Scientific
Elisa	Human P2X Purinoceptor 7 (P2RX7) ELISA Kit	CUSABIO
DNA and RNA Techniques	NaOH, Tris-HCl, Agarose, *SYBR-Safe DNA Stain, RNase-Zap*, Moloney Murine Leukemia Virus Reverse Transcriptase, dNTPs, Kapa2G Fast HotStart Ready mix with dye	Sigma-Aldrich
	DNA ladder mix (100-5000)	PanReac Applichem
	Ultratools polymerase, HotSplit polymerase, MasterMix polymerase, TaqMan Fast Universal PCR Master Mix, LuminoCt Ready Mix, SpeedTools Total RNA Extraction Kit	Biotools

	EconoTaq Plus Green 2x Master Mix	Lucigene
Western Blotting	Hepes, EDTA, NaCl, NaF, Sodium Ortovanadate, NP40, Nitrocellulose Membranes, SDS, Glycine, Tris, *Tween-20*, Ponceau Red, Skimmed Milk Powder, BSA, glycerol	Sigma-Aldrich
	Bradford Reagent, Bromophenol Blue	Merck-Millipore
	Complete TM Protease Inhibitor Cocktail Tablets	Roche
	Ammonium persulfate, Bis-acrylamide 30%, Temed, β-mercaptoethanol, Molecular weight standards	Bio-Rad
	Okadaic acid	Calbiochem
	Methanol, Protein Marker VI (10-245) prestained	PanReac Applichem
	ECL Pro	Perkin Elmer
	Autoradiography films, developer liquid and fixative	AGFA
Immunofluorescence/ Immunohistochemistry	H_2O_2, PBS, BSA, FBS, TritonX-100, Xylene, Ethanol, diaminobenzidine	Sigma-Aldrich
	Elite Vectastain kit	Vector Laboratories
	FluorSave^{TM} Reagent	EMD Millipore
	Microscope Slides	Thermo Fisher Scientific

All media and solutions were prepared with high quality, high purity Merck salts dissolved in deionized water. In general, any substances not specified in the tables were provided by Sigma-Aldrich or Merck-Millipore.

2.3 Pharmacological compounds

Table 7. List of the different pharmacological compounds used.

	Compound	[] final *in vitro*	[] final *in vivo*	Commercial provider
Purinergic agonists and antagonists	ATP	100 µM		Sigma-Aldrich
	BzATP	1-200 µM	30 mM	Sigma-Aldrich
	A-438079	40 µM		Merck-Millipore
	GSK-1482160A		100 mg/Kg	GlaxoSmithKline
	Suramine	100 µM		Tocris
Kinase inhibitors	LY294002	50 µM		Merck-Millipore
	GF-109203X	50 µM		Merck-Millipore
	SB216763	5 µM		Merck-Millipore
	U0126	10 µM		Merck-Millipore
	KN93	5 nM		Merck-Millipore
	H89	1 µM		Merck-Millipore
Proteasome substrates	Succ-LLVY-AMC	100 µM		Sigma-Aldrich
	Z-LLE-β-2-NAP	200 µM		Sigma-Aldrich
	BPC-LSTR-AMC	100 µM		Sigma-Aldrich
Proteasome inhibitor	MG132	10 µM		Sigma-Aldrich
Neuroexcitotoxic agent	Kainic Acid		25mg/Kg	Abcam

2.4 Antibodies

Primary and secondary antibodies used for immunofluorescence (IF) or western blot techniques are listed in **Table 8-9**. For nucleic labelling, the 4'-6-diamidino-2-phenylindole nucleic acid marker (DAPI, Invitrogen) was used.

Table 8. List of the different antibodies used.

Primary Antibody	Species	Commercial provider	Dilution WB	Dilution IF
Anti-P2X7R	Rabbit	Alomone Labs	1/1000	1/100
Anti-αTubulin	Mouse	Sigma-Aldrich	1/10000	
Anti-GFP	Rabbit	Life Technologies	1/1000	1/500
Anti-GFP	Chicken	AVESLab		1/400
Anti-GAPDH	Rabbit	Sigma-Aldrich	1/10000	
Anti-β-Actin	Mouse	Sigma-Aldrich	1/10000	
Anti-β-Catenine	Mouse	BD Bioscience	1/500	
Anti-phospo-Akt	Rabbit	Cell Signaling	1/1000	
Anti-Akt (total)	Mouse	Cell Signaling	1/1000	
Anti-β1	Mouse	Enzo Life Science	1/1000	
Anti-β2	Mouse	Enzo Life Science	1/1000	
Anti-β5	Rabbit	Enzo Life Science	1/1000	1/500

Anti-LMP2	Mouse	Enzo Life Science	1/1000	
Anti-MECL-1	Rabbit	Enzo Life Science	1/1000	
Anti-LMP7	Mouse	Enzo Life Science	1/1000	
Anti-Rpn12	Mouse	Enzo Life Science	1/1000	
Anti-Rpt3	Rabbit	Enzo Life Science	1/1000	
Anti-CORE 20S	Rabbit	Enzo Life Science	1/1000	
Anti-phospho-GSK-3	Rabbit	Cell Signaling	1/1000	
Anti-GSK3 α/β	Mouse	Invitrogen	1/1000	
MCP-8017.3	Rabbit	Homemade (Martìn-Clemente, B., et al. 2004)	1/1000	
Anti-c-Myc	Mouse	Sigma-Aldrich	1/1000	
Anti-FK2	Mouse	Enzo Life Science	1/5000	1/1000
Anti-phospo-STAT3	Rabbit	Cell Signaling	1/500	
Anti STAT3 total	Mouse	Cell Signaling	1/1000	
Anti-Nrf2	Rabbit	Invitrogen	1/1000	
Anti-Iba1	Rabbit	Wako		1/300
Anti-NeuN	Mouse	Merck-Millipore		1/500
Anti-GFAP	Mouse	Sta Cruz Biotechnology		1/200

Table 9. List of the different antibodies used.

Secondary Antibody	Species	Commercial provider	Dilution Western Blot	Dilution IF
Anti-rabbit Alexa 488	Goat	Invitrogen		1:400
Anti-chicken Alexa 488	Goat	Invitrogen		1:400
Anti-rabbit Alexa 555	Goat	Invitrogen		1:400
Anti-rabbit Alexa 594	Goat	Invitrogen		1:400
Anti-mouse Alexa 594	Goat	Invitrogen		1:400
Anti-mouse IRDye 800 CW	Goat	LI-COR	1:15.000	
Anti-rabbit IRDye 680 CW	Goat	LI-COR	1:15.000	
Anti-rabbit IgG conjugated with horseradish peroxidase	Goat	DAKO	From 1:1000 to 1:10.000	
Anti-mouse IgG conjugated with horseradish peroxidase	Goat	DAKO	From 1:1000 to 1:10.000	
Streptavidin Alexa Fluor 488		Abcam		1:400

2.5 Oligonucleotides

For the genotyping of Ub^{G76V}-GFP mice, oligonucleotides **A** and **B** showed in **Table 10** were used to amplify sequences of 300 base pairs (bp) in size. Details of the construction inserted in the Ub^{G76V}-GFP mouse are described in section 1.6.2.1. For the genotyping of

transgenic mice lacking the P2X7 protein, oligonucleotides **C** and **D** were used obtaining sequences of 415 bp for the KO, and of 1142 bp for the WT; a full detailed description is provided in section **1.6.2.2.**

The 4 oligonucleotides used to identify the hemizygous P301S mice (**E, F, G, H**) amplify a WT sequence of 200 bp as positive control and a transgenic sequence of 450 bp. The transgene (PS19) inserted into chromosome (Chr) 3 (Build GRCm38/mm10) causes a 249 kilobase (Kb) deletion; details of the mutation are described in section **1.6.2.3.**

Oligonucleotides	Sequence	Amplifying protocol		
GFP	**A**:Fw 5'-CCTACGGCGTGCAGTGCTTCAGC-3'	(1) 94°C 5 min	(40) 94°C 30 sec 60°C 45 sec 72°C 45 sec	(1)72°C 10min
	B:Rv 5'-CGGCGAGCTGCACGCTGCGTCCTC-3'			
P2X7$^{-/-}$	**C**:Fw 5'-CTGGCAACTATCCATTTTCC-3'	(1) 94°C 5 min	(35) 94°C 30 sec 60°C 60 sec 72°C 125 sec	(1)72°C 10min
	D: Rv 5'-GTGTGAGTGAATGAGATCGTG-3'			
P301S	**E**: Fw 5'-GGCATCTCAGCAATGTCTCC-3'	(1) 94°C 5 min	(28) 94°C 15 sec 65°C 15 sec 72°C 10 sec	(1)72°C 10min
	F: Rv 5'-GGTATTAGCCTATGGGGGACAC-3			
	G: 5'-CAAATGTTGCTTGTCTGGTG-3'			
	H: 5'-GTCAGTCGAGTGCACAGTTT-3'			

Table 10: List of the different oligonucleotides and amplification protocols used for the genotype of Ub^{G76V}-GFP, P301S mice, and P2X7$^{-/-}$ mice. Numbers between brackets indicate the number of amplification cycles. The time of each step is indicated in minutes (min) or seconds (sec).

2.6 Biological Material

2.6.1 Human samples

Post-mortem samples from the cortex and hippocampus of patients diagnosed with Alzheimer's disease were provided by Banco de Tejidos Fundación CIEN (BT-CIEN, Madrid, Spain). Written informed consent for brain removal after death for diagnostic and research purposes was obtained from the brain donors and/or next of kin. Procedures, information, and consent forms were approved by the Bioethics Subcommittee of Fundación Cien Madrid, Spain (S19001). Cortical and hippocampal samples from healthy patients at the same age range as the Alzheimer's samples were used as a control group. **Table 11** summarizes the gender and age of the patients with AD and the controls from whom the hippocampal or cortex post-mortem samples were collected. Dr. Miguel Medina of Fundación CIEN, Madrid generously provided plasma samples from patients with Alzheimer's disease and mild cognitive impairment (MCI) as part of a collaboration through Project PurinesDX. Plasma samples from healthy patients in the same age range as the Alzheimer's samples or MCI were

used as a control group. **Table 12** summarizes the gender and age of the patients with AD or MCI and the controls from whom the plasma samples were collected.

Table 11. Lists case information for Control and Alzheimer's disease cases. M, male. F, female; qPCR, quantitative PCR, WB, Western Blot

Diagnosis	Sex	Age (years)	WB	qPCR	Proteasome Activity	Immunofluorescence/ Immunohistochemistry
Alzheimer	M	81	√	√	√	
Alzheimer	M	76	√	√	√	
Alzheimer	M	69	√	√	√	
Alzheimer	M	68	√	√	√	√
Alzheimer	M	63	√	√	√	√
Alzheimer	M	57	√	√	√	
Alzheimer	F	94	√	√	√	
Alzheimer	F	86	√	√	√	
Alzheimer	F	65	√	√	√	√
Control	F	83	√	√	√	√
Control	F	74	√	√	√	
Control	F	61	√	√		
Control	F	58	√	√		√
Control	M	73	√		√	√
Control	F	70	√		√	

Table 12. List case information for Control, Mild Cognitive Impairment, and Alzheimer´s disease plasma samples used for P2X7R ELISA. M, male. F, female; MCI (Mild Cognitive Impairment)

Diagnosis	Sex	Age (years)	Diagnosis	Sex	Age (years)	Diagnosis	Sex	Age (years)
Control	M	72	**Alzheimer**	F	70	**MCI**	F	80
Control	F	85	**Alzheimer**	F	75	**MCI**	F	77
Control	F	70	**Alzheimer**	F	78	**MCI**	F	72
Control	F	76	**Alzheimer**	F	71	**MCI**	M	82
Control	M	75	**Alzheimer**	F	76	**MCI**	M	77
Control	F	77	**Alzheimer**	M	67	**MCI**	M	80
Control	M	76	**Alzheimer**	M	74	**MCI**	F	74
Control	F	76	**Alzheimer**	M	79	**MCI**	F	79
Control	F	76	**Alzheimer**	M	82	**MCI**	M	76
Control	M	76	**Alzheimer**	M	81	**MCI**	M	76
Control	F	75	**Alzheimer**	F	81	**MCI**	F	75
Control	F	74	**Alzheimer**	M	71	**MCI**	F	75
Control	M	75	**Alzheimer**	F	87	**MCI**	F	73
Control	M	72	**Alzheimer**	M	81	**MCI**	M	76
Control	F	77	**Alzheimer**	F	86	**MCI**	M	75
Control	F	79	**Alzheimer**	F	82	**MCI**	M	72
Control	F	73	**Alzheimer**	F	81			
Control	M	75	**Alzheimer**	M	89			
Control	M	80	**Alzheimer**	F	84			
Control	M	73	**Alzheimer**	M	76			
Control	F	73						
Control	M	74						
Control	M	80						
Control	F	75						
Control	M	75						
Control	M	75						
Control	F	74						
Control	F	74						
Control	M	73						
Control	F	74						
Control	F	76						
Control	M	72						

2.6.2 Animal Models

The protocol for animal experiments was approved by the Committee of Animal Experiments at the UCM and the Environmental Counselling of the Comunidad de Madrid, Spain (PROEX 374/15 and PROEX 185/17). Four to five mice per cage were kept, with food and water available ad libitum, in a temperature-controlled environment with 12-hour light-dark cycles. The experimental procedures were carried out at the Universidad Complutense de Madrid under European and Spanish regulations (86/609 / EEC; RD1201 / 2005), following the guidelines indicated by the International Council of Laboratory Animals (ICLAS) and under the approval of the CAM (ES280790000086). All surgeries were performed under isoflurane anesthesia, and all efforts were made to minimize suffering. The mouse models used for this thesis were the following: UPS activity reporter mouse (Ub^{G76V}-GFP mouse), P301S Tg mice, expressing a mutant form of the human microtubule-associated protein tau (*MAPT*), and P2X7 null mice. A detailed description of each mouse model line follows below.

2.6.2.1 UPS activity reporter mouse (Ub^{G76V}-GFP mouse)

These transgenic mice ubiquitously express the ubiquitin fusion degradation substrate ubiquitinG76V-green fluorescent protein (Ub^{G76V}-GFP). The Ub-GFP reporter carries a ubiquitin fusion degradation (UFD) signal consisting of an N-terminally linked ubiquitin that serves as an acceptor for the polyubiquitin trees linked through the canonical Lys48 and the less common Lys29 residues. The G76V substitution prevents removal of the ubiquitin moiety by cellular ubiquitin C-terminal hydrolases, leading to efficient ubiquitination and proteasomal degradation of the Ub^{G76V}-GFP fusion (*Dantuma et a., 2000; Lindsten and Dantuma, 2003*). Therefore, by having the first Ub irreversibly bound to GFP, the tagged protein can only follow the path of degradation. This construct is under the control of the ubiquitous β-actin promoter and activator of the "immediate-early" Cytomegalovirus (CMVIE) (**Figure 13**). There are two lines of this reporter mouse, lines 1 and 2, coming from two different founders obtained from the microinjection of the construct into fertilized oocytes. Line 1 mice (Ub^{G76V}-GFP mice, also called U1 mice) were used in the experiments carried out in this Doctoral Thesis.

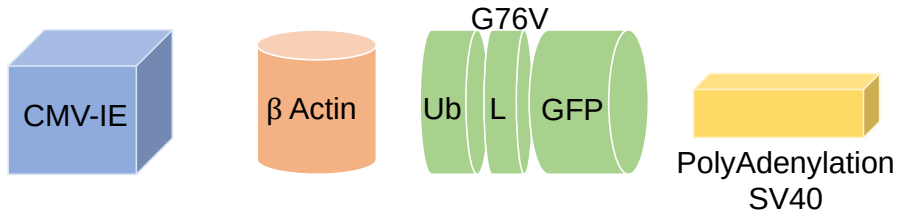

Figure 13: Schematic representation of the construction inserted in the Ub^{G76V}-GFP mouse. Construction is under the control of the ubiquitous chicken β-actin promoter. The transgene contains the cytomegalovirus (CMV) enhancer and the GFP protein with a Ub molecule attached to its N-terminal end whose last aa has been mutated (G76V) to prevent it from being eliminated by the deubiquitinating enzymes of the Ubiquitin-Proteasome System.

2.6.2.2 P2X7 null mice

P2X7 null mice (*P2rx7*$^{-/-}$, B6N-P2rx7$^{tm1d(EUCOMM)Wtsi/leg}$) mice were provided by Prof. Annette Nicke. *P2rx7*$^{-/-}$ mice were purchased from the European Mutant Mouse Archive. The knockout mouse is obtained by insertion of the L1L2_Bact_P vector at position 122652263 of Chromosome 5 upstream of the critical exon(s) (Build GRCm38). The cassette is composed of an FRT site followed by lacZ sequence and a loxP site. This first loxP site is followed by a neomycin resistance gene under the control of the human beta-actin promoter, SV40 polyA, a second FRT site and a second loxP site. A third loxP site is inserted downstream of the targeted exon(s) at position 122653166. The critical exon(s) is/are thus flanked by loxP sites. A "conditional ready" (floxed) allele can be created by flp recombinase expression in mice carrying this allele. Subsequent cre expression results in a knockout mouse (http://www.informatics.jax.org/mgihome/nomen/IKMC_schematics.shtml)(**Figure 14**).

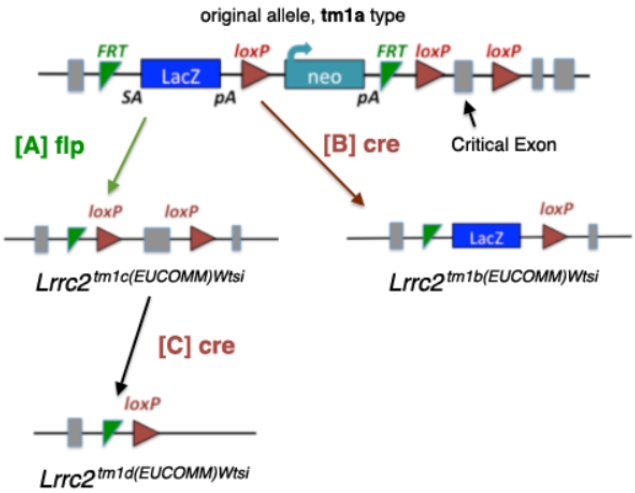

Figure 14: P2rx7$^{-/-}$ mouse construction scheme. Excising the tm1c allele with [C] cre removes the critical exon and creates a tm1d allele with only one FRT and one loxP site remaining.

2.6.2.3 Mice expressing a mutant form of the human microtubule-associated protein tau (MAPT)

P301S mice were kindly provided by Professor Jesús Ávila de Grado (CBM, Madrid), line B6; C3-Tg (Prnp-MAPT*P301S) PS19Vle/J, stock number 008169. The original B6C3H/F1 genetic background was changed to C57BL/6J by back-crossing them with C57 animals in our laboratory. The P301S model is a transgenic line carrying a hemizygous mutant form of the human microtubule-associated protein tau, MAPT, under the control of the mouse prion protein promoter (Prnp). The transgene encodes the disease-associated P301S mutation (mutation type: point, missense; codon change: CCG to TCG; genomic region: coding, exon 10; genomic position: rs63751438, **Figure 15**) and includes four microtubule-binding domains and one N-terminal insert (4R/1N) (*The Jackson Laboratory, 2019*). The expression of the hyperphosphorylated, insoluble mutant human MAPT is five-fold higher than the expression of the endogenous mouse MAPT; the protein accumulates with age, causing decreased microtubule binding/density (*Yoshiyama et al., 2007*). P301S mice have a median lifespan of approximately nine months, with approximately 80% dying by 12 months. P301S mice develop widespread neurofibrillary tangle-like inclusions in the neocortex, amygdala, hippocampus, brain stem, and spinal cord from five months of age with progressive accumulation, but not amyloid plaques. The ultrastructure of the neurofibrillary tangle-like lesions detected is like that found in brain lesions of human Alzheimer's disease and tauopathy patients (*Holmes et al, 2014*).

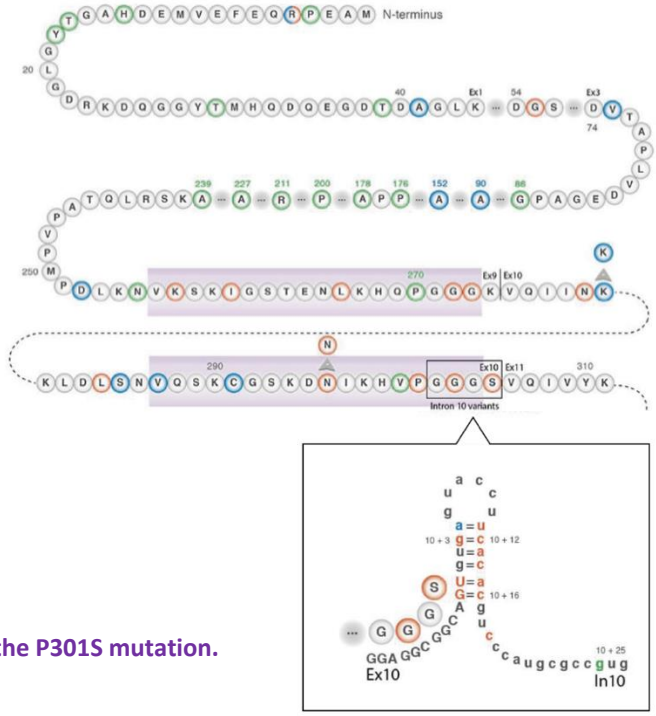

Figure 15. Scheme of the P301S mutation.

2.6.2.4 P2X7 null mice expressing the Ub^{G76V}-GFP reporter (Ub^{G76V}-GFP/ P2X7-/-).

To evaluate how the total absence of P2X7 receptor could affect UPS function, we generated P2X7 null mice expressing the Ub^{G76V}-GFP reporter (Ub^{G76V}-GFP/ P2X7-/-). Ub^{G76V}-GFP mice were crossed with P2X7 null mice; Ub^{G76V}-GFP/ P2X7+/- mice obtained from this crossbreeding were then backcrossed with P2X7-/- mice to finally generate Ub^{G76V}-GFP/ P2X7-/- mice (frequency of 25%), as shown in **Figure 16**.

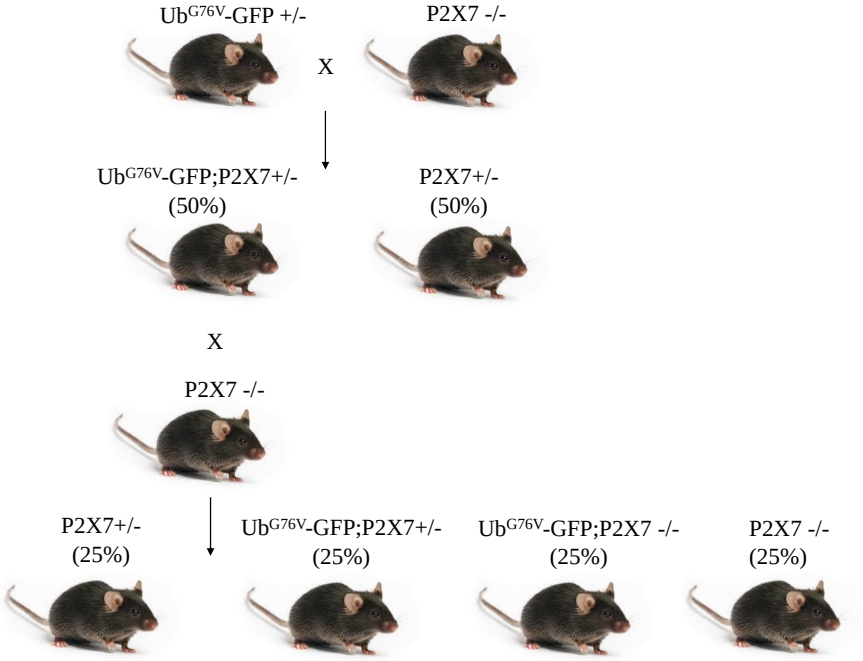

Figure 16: Schematic representation of the crossbreeding designed to obtain the double transgenic Ub^{G76V}-GFP/ P2X7-/-. The line was obtained by crossing heterozygous Ub^{G76V}-GFP mice with P2X7 null mice; Ub^{G76V}-GFP;P2X7+/- mice were then backcrossed with P2X7-/- to finally obtain Ub^{G76V}-GFP;P2X7.

2.6.2.5 UPS activity reporter mouse expressing a mutant form of MAPT (Ub^{G76V}-GFP/ P301S)

To analyse the UPS activity associated with tau toxicity, we generated a double transgenic mice line expressing the Ub^{G76V}-GFP reporter and a mutant form of MAPT (Ub^{G76V}-GFP; P301S). Heterozygous Ub^{G76V}-GFP mice were crossed with heterozygous P301S mice, as shown in **Figure 17**, with a production frequency of these double transgenic mice of 25%.

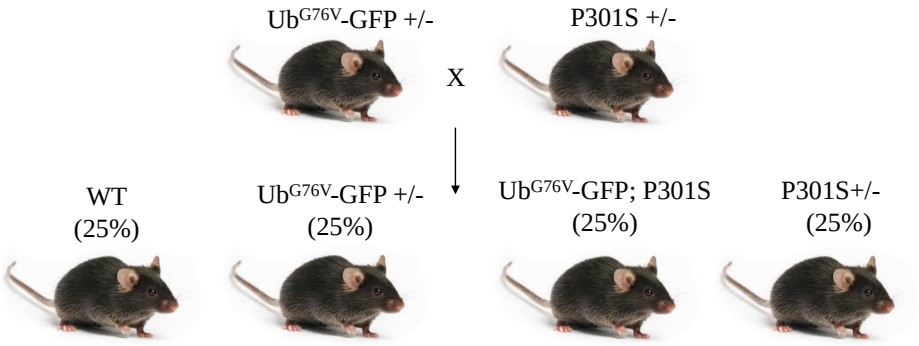

Figure 17: Schematic representation of the crossbreeding designed to obtain the double transgenic Ub^{G76V}-GFP/ P301S. The line was obtained by crossing Ub^{G76V}-GFP mice and P301S mice, both heterozygous.

2.6.3 Murine Neuroblastoma N2a Cells

All tissue culture procedures were performed in a lamina flow cabinet to maintain sterile conditions. The cell line used was Neuro-2a murine Neuroblastoma (N2a) which originates from a clone established by R.J. Klebe and F.H Ruddle from a spontaneous tumour of albino mouse strain A (*Ruddle et al.,1969*). These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with a high glucose content (4500 mg / L glucose), supplemented with 10% inactivated fetal bovine serum (FBSi), GlutaMAXTM (L-alanyl-L-glutamine), 100 U / ml of penicillin and 100 µg / ml of streptomycin in a controlled atmosphere of 5% CO_2, saturated with humidity and at a constant temperature of 37 ° C. For line maintenance, passages were given every two or three days: trypsin was added on top of the cells; cells were collected, centrifugated and finally reseeded 1:3 or 1:6 in new flasks as needed.

3 Methods

3.1 Transfection Procedures

For the different transfection approaches that were tested, a common setup was designed to be followed in all the experiments to have more solid and reproducible results. First, N2a cells were seeded into 6-well plates with DMEM a day before transfection. Cell transfections were performed using the Lipofectamine 2000 cation reagent. The transfection mix was prepared according to the manufacturer's protocol using a ratio of 3µl of Lipofectamine 2000 for every 1µg of total DNA transfected. A mixture with 4µg of DNA diluted in DMEM and Lipofectamine was incubated for 20 minutes at room temperature before adding it to the cells. The transfection mixture was added dropwise to N2a cells plated in 6-well plates in 2ml DMEM media. 4 to 6 hours post-transfection, the medium present in the cells was exchanged for a DMEM medium supplemented with FBSi. For co-transfection experiments, according to the manufacturer's protocol, DNA from different plasmids was mixed at specific ratios and only then added to the transfection reagent.

Firstly, to study the functionality of UPS, two different UPS reporters, Ub^{G76V}-YFP and GFP-CL1 were used, respectively (**Figure 18**). Cells expressing reporter substrates are sensitive to functional impairment of the UPS by the accumulation of the readily detectable fluorescent reporter substrate. Notably, while Ub^{G76V}-YFP is a properly folded protein, the GFP-CL1 is targeted for degradation through the presence of the C-terminal bulky hydrophobic motif CL1, which resembles a misfolded domain (*Gilon et al 1998*). Both reporter plasmids respond to treatments with various proteasome inhibitors, increasing their fluorescence intensity up to 1000 times (*Dantuma & Lindsten 2010, Kessler et al 2003*). As a control, the Ub-M-GFP construct (*Dantuma et al.,2000*) was used, a fusion protein that does not have the G76V substitution so that it is rapidly processed to release Ubiquitin and GFP.

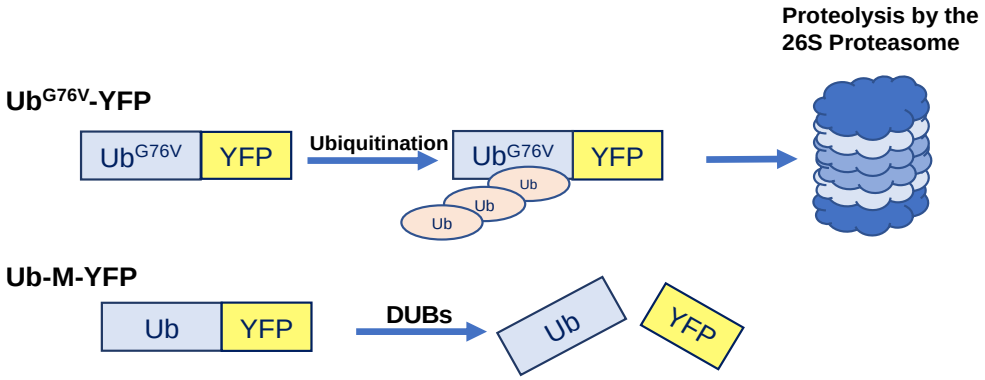

Figure 18: Schematic construction of Ub-M-GFP and Ub^{G76V}-GFP plasmids. Mutated Ubiquitin (G76V) prevents the action of deubiquitinating enzymes (DUBs). Binding to GFP is irreversible, so that the marked protein will be degraded by the proteasome.

For P2X7 receptor overexpression, the cDNA encoding the human P2X7 receptor was purchased from Geneservice (Genomics Service) (cDNA clone number MGC: 20089, IMAGE: 4298811; Cambridge, UK). The P2X7 receptor cDNA was isolated from the original plasmid (pOTB7) and the P2X7-GFP construct was subcloned into the corresponding sites of the pd2EGFP-N1 vector (Clontech) for expression in cells. Furthermore, the P2X7 was inserted into the pIRES.eGFP vector (BD Bioscience) to obtain the expression of both unfused proteins. The GFP expression allowed the identification and monitoring of the transfected cells (*Diaz-Hernandez et al.,2009*).

Silencing of the P2X7 receptor was performed by expressing the corresponding shRNA ("small hairpin RNA"). For this, the expression vector pSuper-neo / gfp (Oligoengine, Seattle, WA, USA) was used, which, by expressing shRNA and green fluorescent protein (GFP) in parallel, allowed the identification and monitoring of the transfected cells (*Diaz-Hernandez et al.,2009*).

For overexpressing *Psmb5* or *Psmb6*, N2a cells were transfected with proteasome subunit beta type 5 (*Psmb5*; RefSeq NM_002797) Myc-DDK-tagged- open-reading-frame (ORF) plasmid and proteasome subunit beta type 1 (*Psmb6*; RefSeq NM_002798) Myc-DDK-tagged ORF purchased from OriGene Technologies,Inc. MD,USA. The c-Myc expression allowed the identification and monitoring of the transfected cells.

3.2 Drugs administration

24 hours after transfection, cells were stimulated with different compounds indicated in **Table 7**, following the protocol represented in **Figure 19**. In all cases, before treatments, the serum was withdrawn to arrest the cell cycle. When combined treatments were applied (agonist/antagonist or agonist/inhibitor), the inhibitor or antagonist was added 20 minutes before the agonist. After the corresponding treatments, proteins were extracted. Firstly, the culture medium was completely removed, and cells were washed with ice-cold PBS. Secondly, cells were lysed in lysis buffer containing 50 mM Tris/HCl, 150 mM NaCl,1% Nonidet P40 and Complete™ Protease Inhibitor Cocktail Tablets pH 7.4. Subsequently, cells were collected in microfuge tubes and homogenized for 1h at 4° C. Finally, the tubes were centrifuged for 10 minutes at 15000 rpm to collect the supernatant containing the cytosolic proteins. The protein content was determined by Bradford assay.

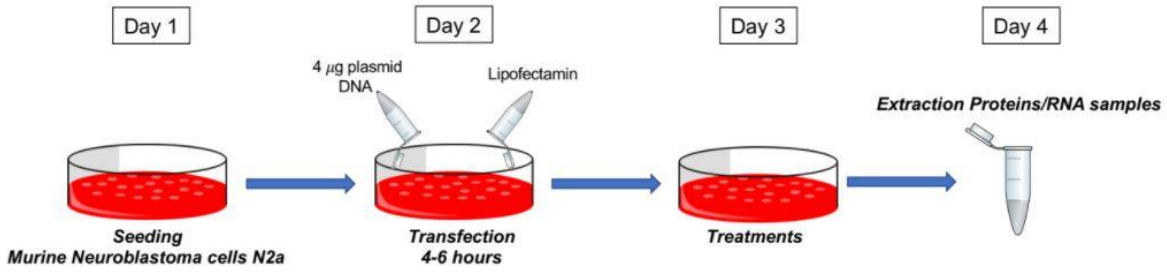

Figure 19: Schematic representation of the protocol followed for *in vitro* experiments with N2a cells

3.3 Stereotaxic injection

Intracerebroventricular (i.c.v) administration of the different compounds was performed by stereotaxic surgery in 6-months-old adult mice. Animals were anesthetized with isoflurane (1-chloro-2, 2, 2-trifuoroethyl-difluoromethylether, Isovet®, BRAUN) nebulized with 50% O_2. Mice were kept under anesthesia during the surgery. The scalp was incised along the midline, and one hole was made at the appropriate stereotaxic coordinates from Bregma (mediolateral, 1 mm; anteroposterior, 0.5 mm; dorsoventral, 2.5 mm) (**Figure 20**). 2 µl of BzATP or 2 µl of PBS were intracerebroventricularly infused with a Hamilton syringe at a rate of ≈ 1 µl/min in the right hemisphere. After the injection, the needle was kept for an additional 3 min and then slowly withdrawn (at a rate of 1mm/ minute). The skin

was sutured with clips (Reflex). Mice were euthanized 24 h after surgery and the injected area was micro-dissected for biochemical analysis.

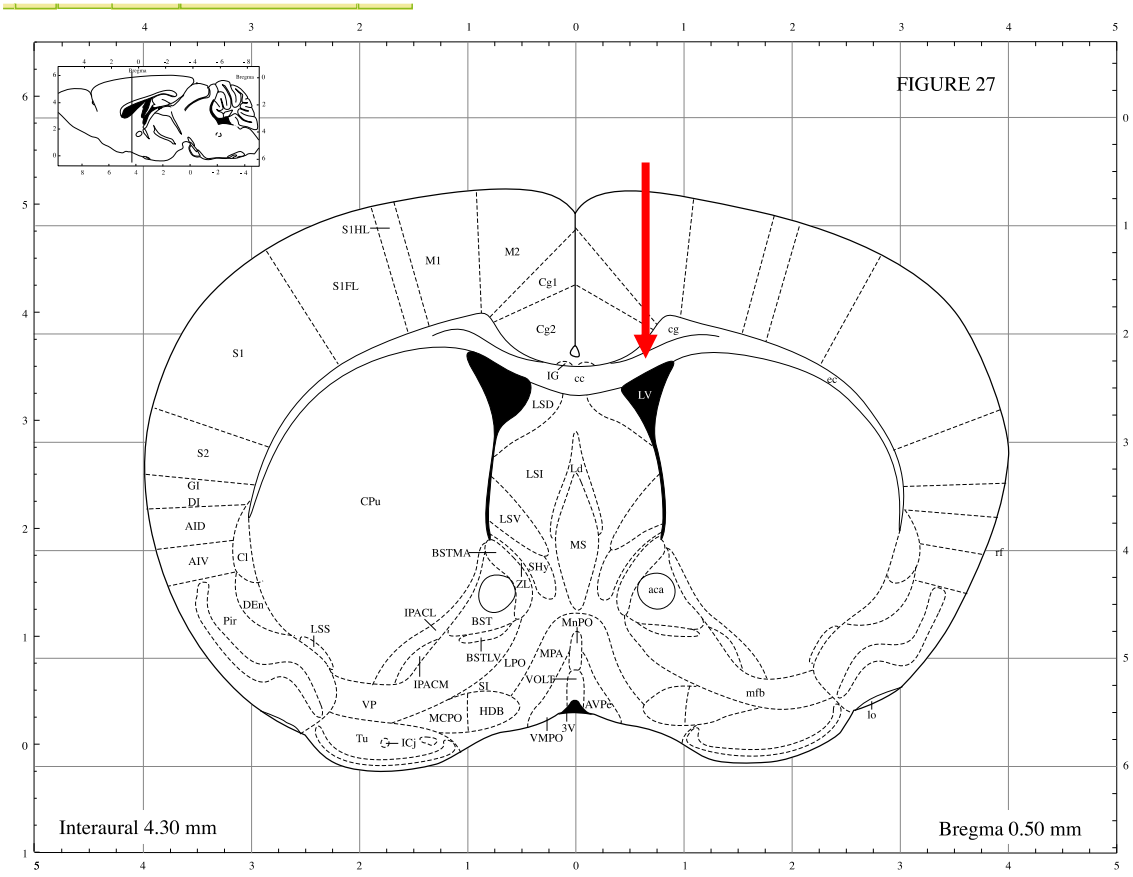

Figure 20: **Intracerebroventricular injection**. Scheme of a coronal section of the brain of a 6-month-old mouse. The red arrow indicates the path of the Hamilton Needle. The head of the arrow indicates the point where the treatment has been introduced. *Image obtained from Paxinos and Franklin 2012*

3.4 Drug preparation and administration

P2X7 specific negative allosteric antagonist GSK 1482160A was diluted at 10 mg/ml in vehicle solution. Vehicle solution was calcium- and magnesium-free sterile phosphate-buffered saline (PBS) plus 20% hydroxypropyl-b-cyclodextrin and 0.2% DMSO. 10 µL of GSK solution corresponding to a dosage of 100 mg per Kg of body weight or the same volume of vehicle solution were daily intraperitoneally injected to 9-months-old P301S and wild-type (WT) mice for three weeks. The treatment protocol followed a single-blind design, by which the experimenter was unaware of the genotype and treatment applied to each mouse (n = 5-7 mice per group and condition). After treatment, mice were sacrificed, and the brain tissue was processed to perform immunoblot and immunohistochemical analyses.

3.5 Genotyping of the different transgenic lines

Genomic DNA was extracted from a tail fragment of the animal. For P301S and Ub^{G76V}-GFP tail fragments, by incubation with 600 µL of NaOH 50 mM at 99°C for 30 minutes. The reaction was then neutralized by adding 50 µL of Tris-HCl 1M pH 8,0 and the samples were centrifuged for 6 minutes at 13.000 rpm to separate the organic and aqueous phases.
For *P2rx7*$^{-/-}$ mice, tail fragments were incubated at 55°C overnight with 200 µL of lysis buffer at pH 7,5 (100 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl, 0,2% SDS) and proteinase K. The day after, samples were centrifuged at 13.000 for 10 minutes to separate the organic and aqueous phases. The supernatant was then transferred to a new tube with 2-Propanol and the samples were mixed until the DNA became visible. Samples were later centrifuged at 13.000 rpm for 5 min and washed with EtOH 70%. Finally, the pellet was diluted in H_2O milliQ.

Simple PCR reactions were carried out using: for P301S mice DNA Kapa2G Fast HS Master Mix, specific primers (0.5 µM each) and 2 µL of genomic DNA in a final volume of 12 µL; for P2X7$^{-/-}$ mice Econo PLUS Green 2x Master Mix, specific primers (10 µM each) and 1 µL of genomic DNA in a final volume of 25 µL; for Ub^{G76V}-GFP mice DNA Amplitools Master Mix ,specific primers (10 µM each) and 2 µL of genomic DNA in a final volume of 24 µL. DNA amplification was carried out on a SimpliAmp™ Thermal Cycler (Applied Biosystem). Specific oligonucleotides and amplification protocols are shown in the **Table 10**. PCR amplification products were electrophoresed on a 1.5% (w/v) agarose gel and stained with SYBR® Safe DNA Gel Stain (Life Technologies CA, USA). PCR bands were visualized by the gel imaging system Gel Logic 200 Imaging System (Kodak, Rochester, NY, USA) or Gel Doc XR^+ System (Bio-Rad).

3.6 RNA extraction and quantification, Reverse Transcriptase (RT-PCR), Quantitative Real-Time PCR (qRT-PCR)

After the corresponding treatments, total RNA was extracted and purified from cells or mice's hippocampus following the protocol described in the commercial kit "SpeedTools Total RNA Extraction Kit". Once the extraction of the total RNA was completed, the DNA was digested in situ by incubating the samples in the presence of RNase-free DNase for 15 minutes at room temperature to eliminate the contaminating DNA. At the end of this time, the DNase was inactivated, and the highly purified total RNA fraction was recovered. The RNA concentration was determined by quantifying the absorbance at 260 nm of the samples

obtained using the Biophotometer Plus spectrophotometer. To estimate RNA purity, the ratio of samples' absorbance at 260 nm and 280 nm (A_{260}/A_{280}) was calculated; typical requirements for A_{260}/A_{280} ratios are 1,8-2,2.

The process of reverse transcriptase (retrotranscription or RT-PCR) for the synthesis of complementary DNA (cDNA) was performed using 1 µg of total RNA, 6 µg of random primers, 350 µM dNTPs, and M-MLV reverse transcriptase. The reaction was carried out at 37°C for 90 minutes, followed by incubation at 70°C for 15 minutes to inactivate the enzyme.

Quantitative Real-Time PCR (qRT-PCR) was performed on cDNA using reaction mixtures containing DNA Master SYBR Green I mix. Specific oligonucleotides and amplification protocols are shown in **Table 13**; reactions were carried out on a StepOnePlus Real-Time PCR System. Expression levels of mRNA were represented as $2^{-\Delta\Delta Ct}$, where the average cycle threshold (Ct) was obtained from triplicates of each sample. First, ΔCt means were normalized to parallel amplification of GAPDH as an endogenous control. Next, ΔΔCt means were normalized to the average of the corresponding control.

Oligonucleotides	Sequence	Amplifying protocol	
Psmb5	Fw: 5'-ATCGAAATGCTTCACGGAAC-3'	(1) 94°C 20 sec	(40) 95°C 1 sec 60°C 20 sec
	Rv: 5'- CGTTCCTATTGCGAAGCTC-3'		
Psmb6	Fw: 5'-CTGGGAAAACCGGGAAGTCT-3'	(1) 94°C 20 sec	(40) 95°C 1 sec 60°C 20 sec
	Rv: 5'- GGTTGTCCTGGAGTCCGCT-3'		
Psmb7	Fw: 5'- TGCCTTATGTCACCATGGGTT-3'	(1) 94°C 20 sec	(40) 95°C 1 sec 60°C 20 sec
	Rv: 5'- CTTGGCTTCTTCCTCCTCCA -3'		
GAPDH	Fw: 5'-CACCACCAACTGCTTAGCCC-3'	(1) 94°C 20 sec	(40) 95°C 1 sec 60°C 20 sec
	Rv: 5'-TGTGGTCATGAGCCCTTCC-3'		
Ub^{G76V}-GFP	Fw: 5'-TGCACCTGGTACTCCGTCT-3'	(1) 94°C 5 min	(40) 94°C 30 sec 60°C 30 sec
	Rv: 5'-TCCAGCTCGACCAGGATG-3'		

Table 13: List of the different oligonucleotides and amplification protocols used for RT-qPCR. The numbers in parentheses indicate the number of cycles. The duration time of each step is expressed in minutes (min) or seconds (sec).

3.7 Quantification of P2X7R levels in human plasma

P2X7R was measured quantitatively in human plasma using the Human P2X purinoceptor 7 (P2RX7) ELISA kit (Cusabio, Houston, TX, USA) following manufacturer's instructions. Standards and samples diluted in Reagent Diluent were added to the 96-well

plates and incubated for 2 hours at 37 °C. After the indicated time, the liquid was removed, without washing, and 100 μL of Biotin antibody was added to each well and the plates were incubated for 1 hour at 37 °C. The wells were then aspirated and washed three times with Wash Buffer (200 μL). After washing, HRP-avidin (100 μL) was added to each well and again plates were incubated for 1 hour at 37 °C. Then HRP-avidin was removed and the washing step was repeated five times. After this step, 90 μL of TMB substrate was added to each well and incubated for 15-30 minutes at 37 °C in dark. A Stop solution (50 μL) was added to each well and the optical density was read within 5 minutes, using a microplate reader set to 450 nm. A standard curve was constructed from serial dilutions of P2X7R Standard that was provided with the commercial kit.

3.8 Wester Blotting

Specific proteins were detected and quantified by western blotting. Protein samples were prepared with 5X loading buffer (4% SDS [sodium dodecyl sulfate] (w/v), 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v), 0.004% bromophenol blue (w/v), 0.125M Tris-HCl). Samples were then heated at 95°C for 5 mins to denature the proteins. Proteins were separated by SDS polyacrylamide gel electrophoresis in running buffer (25 mM Tris, 200 mM glycin, 0,1 %(w/v) SDS, pH 8,3) at 120V in a Mini- PROTEAN Tetra cell electrophoresis chamber. Gels and nitrocellulose (Whatman) membranes were equilibrated in Transfer Buffer (25 mM Tris, 190 mM glycine, 20% (v/v) methanol, 0,1% (w/v) SDS, pH 8,3). The transfer was carried out on a BioRad mini-Trans Blot system, at 150 mA for 40 minutes for proteins with lower molecular weights or at 230 mA for 70 minutes for proteins with higher molecular weights. After the transfer, the membranes were stained for 5 minutes with Ponceau red dye to verify the correct separation and transfer of the proteins. Finally, they were washed with a PBS buffer containing 0.1% (v / v) Tween-20 (PBS-Tween or washing solution). Membranes were later incubated with blocking buffer (5% milk powder or 3% BSA in PBS-Tween) for 1 hour at RT on an orbital shaker and finally incubated with primary antibodies at 4°C ON. Primary antibodies used for the experiments are listed in **Table 8**.

On the second day, membranes were washed for 10 min with PBS-Tween three times and incubated with secondary antibodies (listed in **Table 9**) for 1 h at room temperature, followed by enhanced chemo luminescence detection. This system contains luminol, which is the specific substrate for peroxidase, and when oxidized, generated a luminescent signal that

can be detected by autoradiographics. For this purpose, Agfa X-ray films were used together with the CP1000 Agfa autoradiography developing machine or ImageQuant LAS 500 chemiluminescent detector or LI-COR Odyssey. Gel band images were analyzed using ImageJ software. Protein expression was normalized with respect to the expression of housekeeping proteins, like α-Tubulin, β-actin, GAPDH, Akt, or GSK3 from the same experiment. In the figures, the representative Western blot images show only the quantified bands.

3.9 MTT Tetrazolium Cell Viability Assay

This experimental procedure allows the determination of cell viability based on the mitochondrial activity of living cells. It is based on the ability of mitochondrial dehydrogenases to break the tetrazolium ring of 3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium (which in solution is yellow), releasing insoluble purple crystals.

Murine neuroblastoma cell line N2a was planted at 5 X 10^5 cells/well in 12-well plates and cultured with DMEM supplemented with Glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 % heat-inactivated fetal bovine serum. Cells were grown at 37 °C in a humidified atmosphere containing 5 % of CO_2. 24 h after being plated, N2a cells were stimulated with BzATP (200 μM), A438079 (40 μM), or MG132 (10 μM) for 24 h. After eliminating the media from the cells, MTT(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide diluted in Buffer Locke (NaCl 140mM, KCl 4,7 mM, KH_2PO_4 1,2 mM, HEPES 10 mM, Glucose 5,5 mM) was added directly to the plate and incubated for 2-3 hours at 37 °C. After this step, Solubilizing Buffer (Triton 10%, HCL 1 N 10%, Isopropanol 80%) was added to the plate. The plate was placed in the orbital agitator for 1 hour. The last step, the cells were scraped, and the relative absorbance was read at 570 nm on a spectrophotometer. As a blank, an MTT solution was used, reconstituted in the absence of cells, and mixed with Solubilizing buffer. Values were normalized in respect to that obtained from untreated cells, considered as 100% survival.

3.10 Proteasome activity assay

To measure the catalytic activity of the proteasome, proteasome substrates bound to fluorophores Methylcoumarin (AMC) or 2-β-Naphthylamine (NAP) were used. Those substrates in the presence of proteolytic activity break their binding to the substrate and emit fluorescence. Chymotrypsin-like, Post-glutamyl-like, and Trypsin-like type enzymatic activities were analyzed in cell culture lysates, mouse cortex and hippocampus, and postmortem samples from patients diagnosed with Alzheimer's disease and control. The transgenic mice, previously intraventricularly injected, were sacrificed by inhalation of CO_2. Subsequently, a cubic region of the ipsilateral hippocampus that comprised the volume closest to the i.c.v administration area was dissected and extracted as shown in **Figure 21**.

For human samples, both control and AD, a little piece of the hippocampus was isolated and extracted. Both cell culture and tissue samples from humans and mice were lysed in lysis buffer (10mM Tris-HCl pH 7.8, 0.5mM dithiothreitol, 5mM ATP, 0.03% Triton X-100, and 5mM $MgCl_2$) and, in the case of tissue extracts, they were homogenized with a mini-pointer and suspended with 20G needles for their complete lysing. Subsequently, the lysates were centrifuged at 16,000 x g at 4 ° C for 20 min. The resulting supernatants were placed on ice and assayed for protein concentration by Bradford method. Extracts were adjusted to 0.5 µg /µL total protein by dilution with lysis buffer and incubated at 37 °C for 60 min. All assays were done in quadruplicate. Chymotrypsin-like activity was determinated using the substrate succinyl-LLVY-4-methylcoumaryl-7-amide (SUCC-LLVY-AMC; Sigma-Aldrich; 100 µM), trypsin-like activity was determined using the substrate Boc-LSTR-AMC (BOC; Sigma-Aldrich; 100 µM) and post-glutamyl-like activity was determined using the substrate Z-LLE-β-2-naphtylamine (NAP; Sigma-Aldrich; 200 µM). Assay mixtures containing 12 µg of protein, 32 µL of the substrate, and the assay buffer (100 mM HEPES-KOH pH 7.5, 5 mM $MgCl_2$, 1mM ATP, 10 mM EGTA) were made up in a final volume of 150 µL. The cleavage products AMC were analyzed after stopping the reaction with 1 mL of SDS 1 %, in a fluorimeter (excitation/emission 380/435 nm) (*Diaz-Hernandez et al.,2006).* Background activity (caused by non-proteasomal degradation) was determinated by the addition of the proteasome inhibitor MG132 at a final concentration of 500 µM.

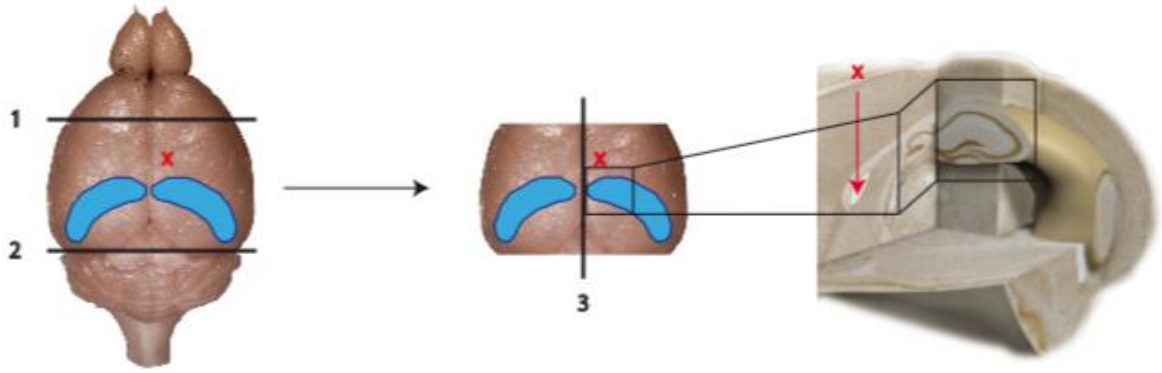

Figure 21: Schematic representation of the removal of the cubic region after intracerebroventricular administration. After brain extraction, the olfactory bulbs (1) and the cerebellum (2) are removed. Subsequently, a longitudinal cut is made (3) and the ipsilateral hemisphere is dissected to extract a cubic region of the hippocampus (blue) close to the treatment administration area. (X) indicates the coordinates where the administration of treatments has been carried out by stereotaxic surgery and the red arrow indicates the direction followed by the Hamilton needle until it reaches the ventricle.

3.11 Immunoaffinity purification of 20S proteasome under native conditions

Assembled 20S proteasome complexes were purified from N2a cells, treated or not with 200 µM of BzATP, with the rabbit IgG polyclonal antibody MCP-8017.3 antibody directed against the 20 S proteasome core (*Martìn-Clemente et al. 2004; Rodriguez-Vilariño, et al. 2000*). In each experiment, 6 million cells were seeded in two 75 cm^2 flasks, 2 treated with vehicle and 2 treated with BzATP. For the IP; firstly, the antibody was centrifugated at maximum speed for 5 minutes to remove possible protein aggregates. Secondly, protin A Sepharose beads (GE-Healthcare) were washed five times with PBS and measured the “dry” volume to resuspend them 1:1 with the antibody. The MCP antibody coupled with the Sepharose beads was incubated overnight at 4 °C under rotary agitation. Before used the MCP Sepharose antibody was washed five times with the BIP buffer (50 mM Tri-HCl (pH 7,5), 150mM NaCl, 0,5% NP40, 0.5mM PMSF, 10 µg/ml Leupeptin, 10 µg/ml Pepstatin).

24h after treatment, cells were harvested on ice, resuspended with ice-cold PBS, transferred to one or multiple Eppendorf and centrifugated for 5 minutes at 3000 rpms at 4 °C. The supernatant was discarded after centrifugation, and the cells were washed four times with ice-cold PBS. The pellet was then lysed in 500 µl of BIP buffer and consequently centrifugated for 30 minutes at 14000 rpms at 4 °C. After removal of cellular debris by centrifugation, protein amount was measured, and equal amounts of the supernatant were split into two aliquots. Each aliquot was incubated for 3 hours at room temperature with rotary shaking with the MCP-Sepharose antibody. The Sepharose beads were then washed

with 500 µl of ice-cold BIP buffer four times and proteins interacting with the beads were finally eluted in loading buffer (SDS-LB N-etilmaleimide to a final concentration of 25mM). The immunoaffinity-purified protein complexes were characterized by Western blotting.

3.12 Kainic Acid-induced *Status Epilepticus*

Status Epilepticus was induced in Ub^{G76V}-GFP and Ub^{G76V}-GFP; P2X7-/- mice aged 4 months old. Mice received an intraperitoneal injection of Kainic Acid (25mg/kg) emulsified in 0.9% normal saline (*Marathe et al.,2015*). This dosage was shown to induce seizures and neurodegeneration in the hippocampus (*Koeller et al.,2008*). The status was defined as a continuous convulsive condition lasting longer than 30 minutes. Forty minutes following the injection of KA, mice received intraperitoneal lorazepam (6mg/kg) to curtail *status epilepticus*. Mice were monitored for seizure behavior for 2 h after KA injection. Mice were sacrificed 24h later, and brain tissue was processed to perform immunoblot and immunohistochemical analyses.

During my secondment in Tobias Engel's Laboratory, we used an intra-amygdala injection of KA to elicit *status epilepticus* in WT and P2X7-/- mice (*Mouri et al.,2008*). Mice were sedated with isoflurane (5% induction, 1–2% maintenance) and kept normothermic with a feedback-controlled heat blanket during stereotaxic procedures. Mice were put on a stereotaxic frame after being thoroughly sedated, and a midline scalp incision was made to expose the skull. Dental cement was used to secure a guide cannula (coordinates from Bregma; AP = 0.94 mm, L = 2.85 mm). *Status epilepticus* was induced by microinjection of 0.3 µg KA in 0.2 µl phosphate-buffered saline (PBS); into the right basolateral amygdala 3.75 mm below the dura. Vehicle-injected control animals received 0.2 µl of PBS. To curtail seizures and minimize morbidity and mortality, the anticonvulsant lorazepam (6 mg/kg) was delivered i.p. 40 min following intra-amygdala KA or vehicle. The electroencephalogram (EEG) was recorded from cortical implanted electrodes, one on top of each hippocampus with the reference electrode on top of the frontal cortex. EEG was obtained using an Xltek recording device beginning 10 minutes before intra-amygdala KA injection. Hippocampal tissue was collected from either control mice injected with vehicle or animals treated with intra-amygdala KA 8 hours later.

3.13 Histological Techniques

3.13.1 Tissue processing

Mouse brain: mice were sacrificed by cervical dislocation and their forebrains were removed. The brain was divided into two hemispheres; one of them was immediately frozen into dry ice for later protein and RNA extraction. The other hemisphere was fixed with 4% paraformaldehyde and cryoprotected in a 30% sucrose solution. The samples were embedded in an OCT compound (Sakura) and frozen using dry ice. Finally, 20 µm floating sections were cut in sagittal planes with a cryostat and stored in a solution of 30% ethylene glycol, 30% glycerol, and 0.1 M PBS at -20°C until processed.

Human brain: human brain control and AD subjects were processed at the Fundación Centro de Investigación Enfermedades Neurológicas (CIEN) within the Vallecas Project. The whole brain was separated into two hemispheres, through a sagittal interhemispheric incision. One of the hemispheres was dissected and samples of the hippocampus were immediately frozen in dry ice and stored at -80°C for biochemical studies. The other hemisphere was fixed at 10% buffered formalin for at least 3 weeks. Samples (2-mm-thick) of the hippocampus were then obtained through dissection, embedded in paraffin following standard protocols (Leica, HistoCore Pearl), and later sectioned (5 µm) by using a microtome (Microm HM 355 S) and stored at -80°C until use.

3.13.2 Immunohistochemistry and immunofluorescence

For immunohistochemical analysis, sagittal sections (25 µm in thickness) of the mouse brain were pre-treated for 45 min at RT with 1% H_2O_2 in PBS to inactivate endogenous peroxidase. Afterward, sections were washed three times in PBS 1x for 10 minutes, blocked for 1 h with block solution (1% bovine serum albumin (BSA), 5% fetal bovine serum (FBS), and 0.2% TritonX-100 in PBS) and finally incubated overnight at 4°C with primary antibodies diluted in blocking solution (listed in **Table 8**). Later, sections were incubated with an avidin-biotin complex using the Elite Vectastain kit and staining reactions were performed with diaminobenzidine and 0.03% H_2O_2 for 10 min. Finally, brain sections were washed with PBS and mounted in FluorSave.

For immunofluorescence studies, mouse slices were washed three times in PBS 1x for 10 minutes, blocked for 1 h at room temperature (RT) with blocking solution, and then incubated at 37°C for 1 h or overnight at 4°C with primary antibodies (listed in **Table 8**). Subsequently, brain sections were washed with PBS buffer and incubated with secondary antibodies (listed in **Table 9**) and 4',6-diamidino-2-phenylindole (DAPI) staining (1:1000). After that, brain sections were washed on PBS and mounted.

For immunohistochemical analysis, human sections (5 µm in thickness) were pre-incubated at 55°C ON and then sequentially washed for 10 min in Xylene (catalog 131769.1611, Panreac), ethanol 100%, ethanol 96%, ethanol 70% and finally distilled H_2O, to remove paraffin and rehydrate them. Later, the sections were treated for 45 min at RT with 1% H_2O_2 in PBS to inactivate endogenous peroxidase and then boiled in citrate buffer (citric acid 1M in distillate H_2O) pH 5.9 for 5 min. Next, the sections were washed in PBS 1x for 10 minutes, blocked for 1 h with block solution, and finally incubated overnight with rabbit anti-P2X7R antibody (listed in **Table 8**). On the second day, sections were incubated with avidin-biotin complex using the Elite Vectastain kit, and staining reactions were performed with diaminobenzidine and 0.03% H_2O_2 for 10 min. Finally, brain sections were washed with PBS and mounted in FluorSave.

For immunofluorescence studies, human sections were pre-treated to remove paraffin, as previously described. Next, the sections were boiled in citrate buffer, washed in PBS 1x for 10 minutes, blocked for 1 h at room temperature (RT) with blocking solution and then incubated overnight at 4°C with rabbit anti-P2X7R antibody (listed in **Table 8**). Subsequently, brain sections were washed with PBS buffer, incubated for 1 h at RT with an avidin-biotin complex using the Elite Vectastain kit, then washed again with PBS buffer, and incubated with Streptavidin (**Table 9**). Sections were later incubated with mouse anti-FK2 primary antibody (**Table 8**) and, washed in PBS buffer and finally incubated with secondary antibody (listed in **Table 9**) and 4',6-diamidino-2-phenylindole (DAPI) staining (1:1000). After that, brain sections were washed on PBS and mounted.

3.13.3 Image Acquisition

Confocal images were acquired at RT with a TCS SPE microscope from Leica Microsystems equipped with a Plan Fluor 10× dry objective lens NA=0.30, 40× Apochromat NA=1.15 oil objective lens and 63× Apochromat NA=1.3 oil objective lens (Leica Microsystems GmbH) and 4 different laser lines (405, 488, 565 and 647nm). Pictures were acquired using the Leica software LAS AF v2.2.1 software (Leica Microsystems GmbH) and representative slices were converted to TIFF files using ImageJ software.

Transmitted light images were acquired using a microscope (DM 1000, Leica) with a DFC450 CCD camera (Leica Microsystems GmbH) using Leica Application Suite (v4.1). Sections were photographed with a Plan 4× dry objective lens (NA = 0.1) and insets with a Plan S-Fluor 20× or 40× dry objective lens (NA = 0.90, Nikon) at RT.

3.14 Statistics

Data are shown as mean values ± SEM. The numbers of mice per group used in each experiment are annotated as “n” in the corresponding figure legends. Figures and statistical analyses were generated using GraphPad Prism (v6.00, www.graphpad.com). To assess whether the data met the normal distribution, the Shapiro-Wilk or Kolmogorov-Smirnov tests were used. For the two-group comparison, data were analyzed with a two-tailed unpaired Student’s t-test. For multiple comparisons, data were analyzed using a one-way ANOVA followed by Dunnett's post hoc tests when all groups were compared against a control group or one-way ANOVA followed by Tukey´s post hoc tests when all groups were compared among them. When required, a two-way ANOVA followed by Tukey´s post hoc tests were used. The statistical test used, and the p-values are indicated in each figure’s legend. Significance was considered at *$P \leqq 0.05$, **$P \leqq 0.01$, ***$P \leqq 0.001$, or ****$P \leqq 0.0001$ throughout the study.

4 Results

4.1 BzATP-induced P2X7R activation causes an accumulation of UPS activity reporters

To study the functionality of UPS, a pharmacological approach was initially taken by using the N2a murine neuroblastoma cell line. These cells were transfected with the UPS-reporter plasmid Ub^{G76V}-YFP which enables monitoring the UPS activity once cells are stimulated with different P2X7R agonists and antagonists. As a first step, a thorough investigation of the effect that BzATP activation of P2X7R has on UPS activity was conducted. For this, N2a-Ub^{G76V}-YFP cells were stimulated with BzATP for 1 hour, 4 hours, 8 hours, and 24 hours. The results shown in **Figure 22A** demonstrate that BzATP activation caused considerable changes in the expression levels of the Ub^{G76V}-YFP reporter protein. Long-term stimulation (8hours and 24hours) resulted in a substantial rise in the levels of this protein. Based on these findings, the stimulation period for the next trials was set at 24 hours. Then, we chose to do a dose-response study using the BzATP agonist. In **Figure 22B** we observe that said compound caused an increase in reporter levels in a dose-dependent manner, therefore we set a concentration of 200 µM for the following studies.

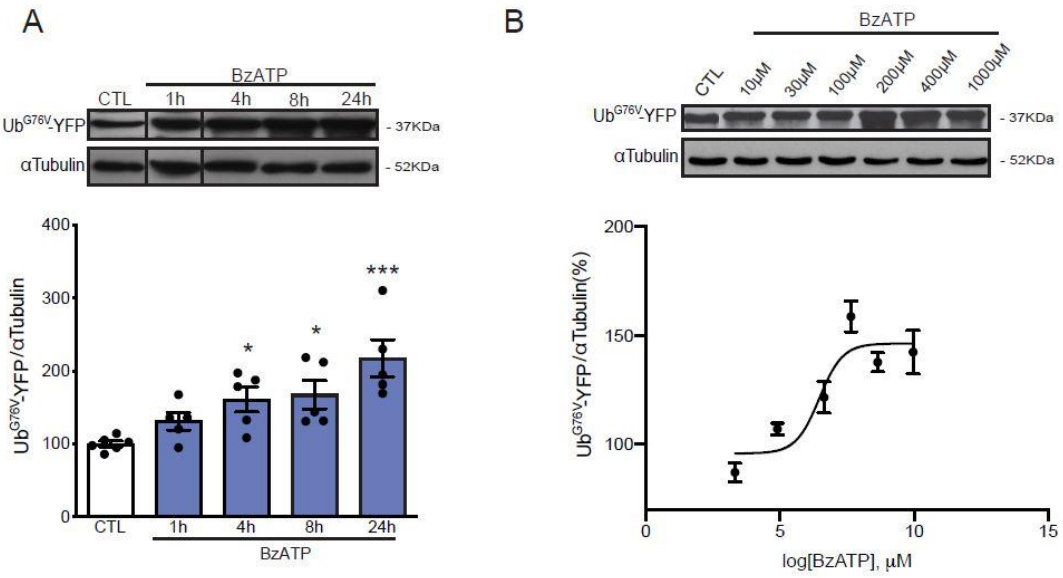

Figure 22: Time- and dose-response curve of BzATP on N2a-Ub^{G76V}-YFP (A) Representative Western Blot Image and graph showing the analysis of Ub^{G76V}-YFP levels in extracts from N2a cells expressing Ub^{G76V}-YFP after being stimulated with BzATP at different time points (1h,4h,8h, and 24h) (n=5) Amounts of protein were normalized to α-Tubulin. Values are represented as the mean ± s.e.m ***p < 0.001, *p < 0.05 using ANOVA (B) Representative Western Blot Image and graph showing the effect produced by the BzATP agonist on the levels of reporter at different concentrations range from 10 µM to 1mM. The data are represented as the percentage

of variation of the reporter levels with respect to the baseline values, the value being 100% the reporter levels before stimulation with the BzATP agonist.

Secondly, N2a-Ub^{G76V}-YFP cells were treated or not with BzATP in the presence or absence of the antagonist A438073 (40 µM) for 24 hours. We observed that the accumulation of the UPS reporter caused by P2X7R activation by BzATP was reverted when cells were stimulated with A43 (**Figure 23**).

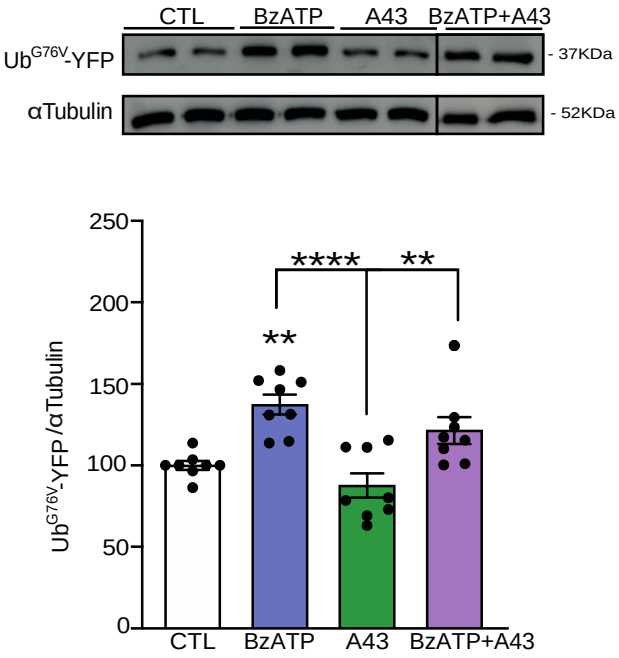

Figure 23: BzATP-P2X7R activation determines an accumulation of the specific reporter Ub^{G76V}-YFP Representative Western blot image and graph showing the analysis of Ub^{G76V}-YFP levels in extracts from N2a cells expressing Ub^{G76V}-YFP after being stimulated or not with BzATP in the presence or absence of the antagonist A438073 (n=4 experiments performed in duplicate). Amounts of protein were normalized to α-Tubulin. Values are represented as the mean ± s.e.m. **** $p < 0.0001$, **$p < 0.01$ using ANOVA

To confirm the data obtained with the Ub^{G76V}-YFP, we chose to utilize another reporter molecule, the GFP-CL1, which is likewise capable of monitoring the activity of the UPS (*Gilon et al., 1998*). This reporter, which is described in section 2.1 of Materials and Methods, also functions as a proteasome degradation signal. **Figure 24A** demonstrates that cells transfected with GFP-CL1 and stimulated with 200 µM BzATP for 24 hours showed a substantial increase in the reporter protein when compared to control cells. As a positive control, cells were tested with 10 µM of MG132, a proteasome inhibitor. To ensure that the increase in reporter accumulation was not due to a change in the translation of the reporters' proteins, we transfected the cells using the plasmid Ub-M-GFP (*Dantuma et al., 2000*). This reporter molecule is swiftly digested by the UPS and does not report the G76V mutation, thus only the

GFP protein is recognized. **Figure 24B** demonstrates that N2a cells transfected with the plasmid Ub-M-GFP and stimulated with BzATP for 24 hours showed no significant changes in GFP levels with respect to N2a cells treated with the vehicle solution.

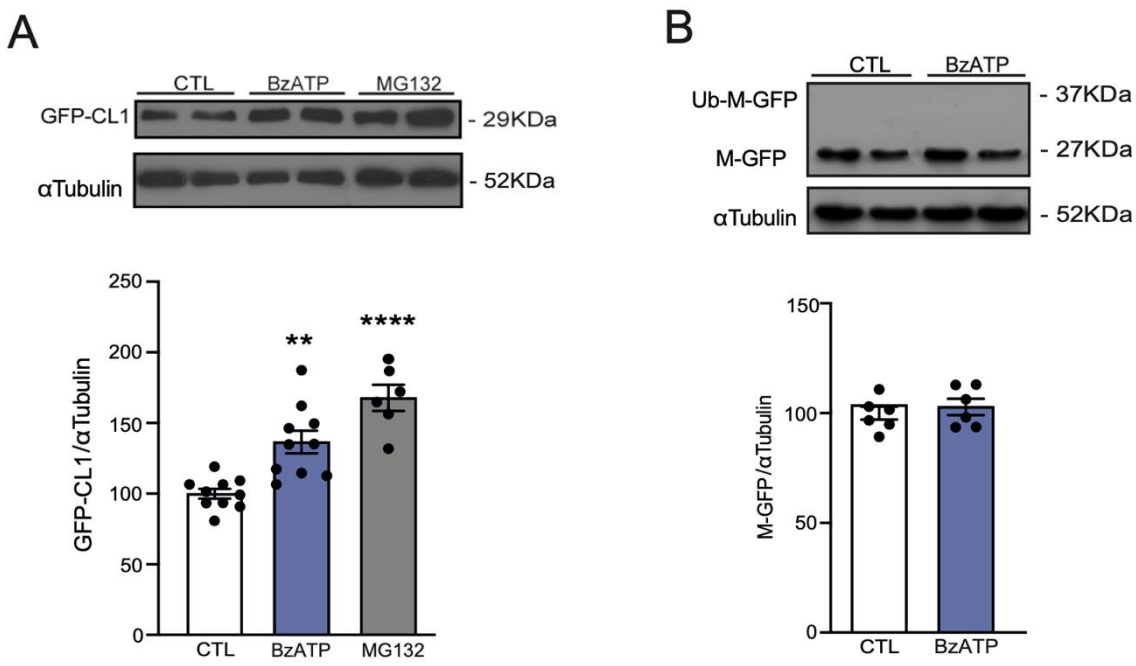

Figure 24: Study of UPS activity through the employment of different reporters. (A) Representative Western blot image of the detection of the GFP-CL1 protein obtained from cells treated with BzATP or MG132 together with their corresponding quantification (B) (n=5 experiments performed in duplicate). (C) Representative Western Blot image of the detection of the Ub-M-GFP protein on N2a cells treated with BzATP together with their corresponding quantification (D) (n=3 experiments performed in duplicate). Amounts of protein were normalized to α-Tubulin. Values are represented as the mean ± s.e.m. ***p < 0.001, **p < 0.01 using ANOVA

Next, we sought to see if the increase in reporter levels caused by BzATP was attributable to changes at the transcriptional level. For this purpose, we measured, by RT-qPCR, Ub^{G76V}-YFP mRNA levels from N2a-Ub^{G76V}-YFP cells treated or not treated with 200 µM BzATP for 24 hours. **Figure 25A** shows that there were no significant differences in Ub^{G76V}-YFP protein mRNA levels between treatments. Furthermore, to rule out any harmful effects of stimulating N2a cells for 24 hours with the agonist BzATP, we used the MTT method to assess cell viability. We found that BzATP-induced P2X7R activation had no cytotoxicity effect on N2a cells (**Figure 25B**). Cells treated with hydrogen peroxide (500 µM H_2O_2) were used as positive control. Following that, we established if the increase in reporter levels produced by BzATP was connected to a change in the number of proteasomes 20S core particles. As shown in **Figure 25C**, there were no significant differences in the quantities of 20S core particles

between the two treatments. We also looked at whether P2X7R activation alters the expression of certain subunits of the 19S regulatory complex, which, as mentioned in the introduction, catalyzes the last step in the ubiquitin-mediated protein degradation process. We searched for the expression of the Rpt3 subunit, which is involved in the ATP-dependent degradation of ubiquitinated proteins, as well as the Rpn12 subunit, which is a regulatory component of the 26S proteasome and is also involved in the ATP-dependent degradation of ubiquitinated proteins. BzATP stimulation caused no substantial changes in the expression of the Rpt3 (**Figure 25D**) or the Rpn12 subunits (**Figure 25E**).

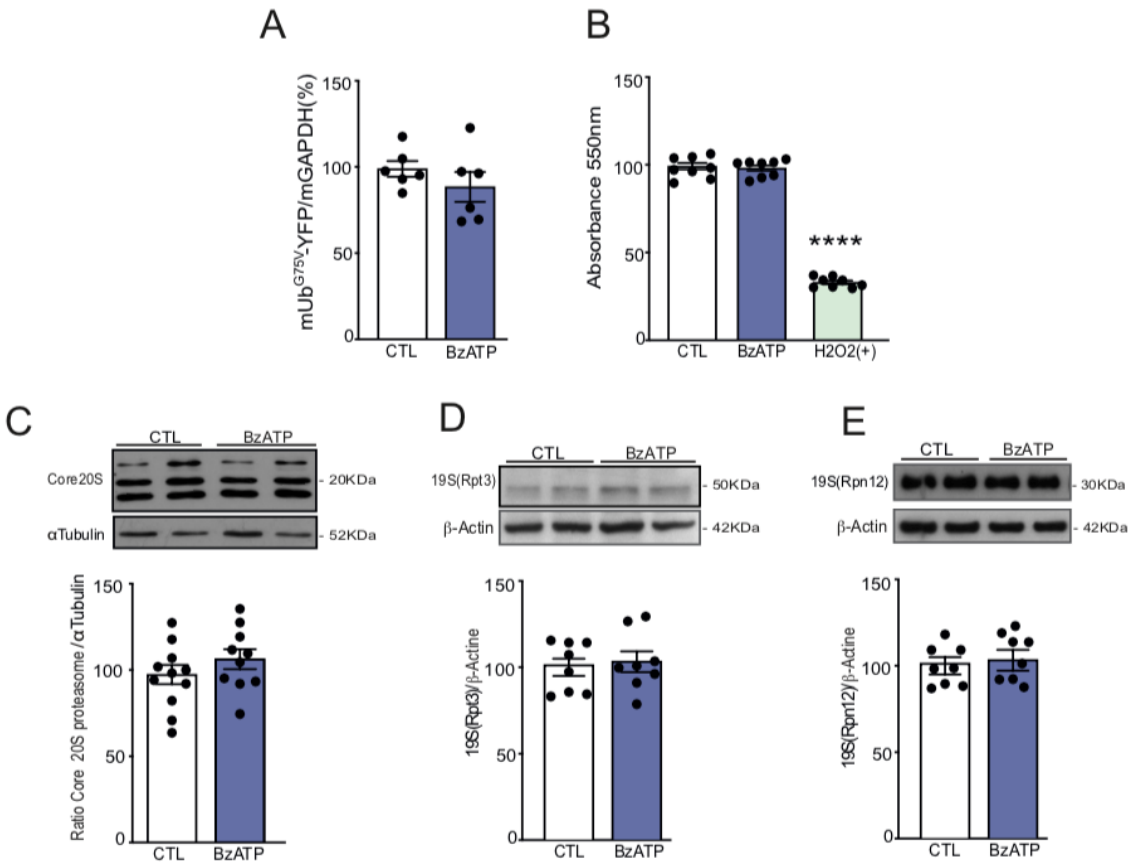

Figure 25: Different pharmacological and molecular biology techniques used for UPS monitoring (A) Representative graph of the relative levels of Ub^{G76V}-YFP mRNA measured by quantitative PCR and normalized with respect to the internal GAPDH control. (B) Cell viability assays measured by MTT. Representation of the percentages with respect to the untreated control cells. (C) Representative Western blot image and graph of the 20S catalytic core of the proteasome. Amounts of protein were normalized to α-tubulin (n=5 experiments performed in duplicate. (D) Representative Western blot image and graph of the analysis of the expression of the Rpt3 subunit with ATPase activity. (E) Representative Western blot image and graph of the analysis of the expression of the Rpn12 subunit with non-ATPase activity. Amounts of protein were normalized to β-Actin (n=4 experiments performed in duplicate). Values are represented as the mean ± s.e.m.**** $p < 0.0001$ using ANOVA.

Furthermore, given that A438073 reverts the effect caused by BzATP in N2a-Ub^{G76V}-YFP (**Figure 23**), we sought to test if this drug might recover the cytotoxic impact induced by MG132 (*Das & Bhattacharyya 2014*). For that aim, N2a cells were pretreated for 20 minutes with 40 µM A438073 and then for 24 hours with proteasome inhibitor, 10 µM MG132. The MTT test was then used to determine cell viability. When N2a cells were cotreated with A438072, we discovered that MG132-induced cellular death was avoided (**Figure 26**).

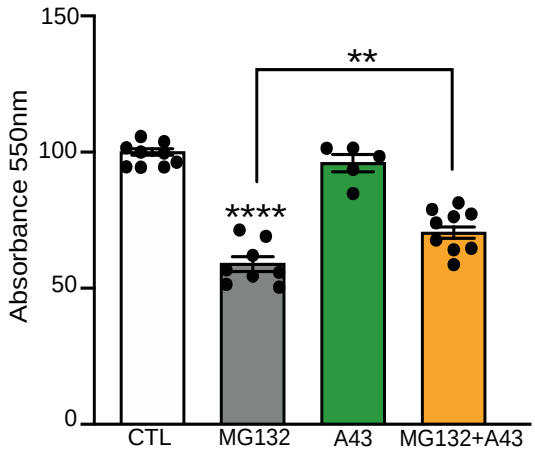

Figure 26: The A438073 antagonist can revert the cytotoxic effect induced by MG132. Effect of the A438073 antagonist on the cytotoxicity induced by MG132 proteasome inhibitor. Representation of the percentages with respect to the control cells treated with vehicle solution, measured by MTT. Values are represented as the mean ± s.e.m. of 4 independent trials done in duplicate **** $p < 0.0001$; **$p < 0.01$ using ANOVA

To confirm that the modifying impact on UPS activity is due to BzATP-induced P2X7R activation, N2a cells were co-transfected with Ub^{G76V}-YFP and P2X7R-GFP to generate P2X7R overexpression or a P2X7R silencing plasmid (ShP2X7R). After transfection, cells were treated for 24 hours with BzATP or PBS. In accordance with our earlier findings, BzATP administration caused a substantial intracellular accumulation of Ub^{G76V}-YFP when P2X7R was overexpressed (OxP2X7R)(**Figure 27A**). However, when the P2X7R was silenced, no changes in reporter levels were detected (**Figure 27B**).

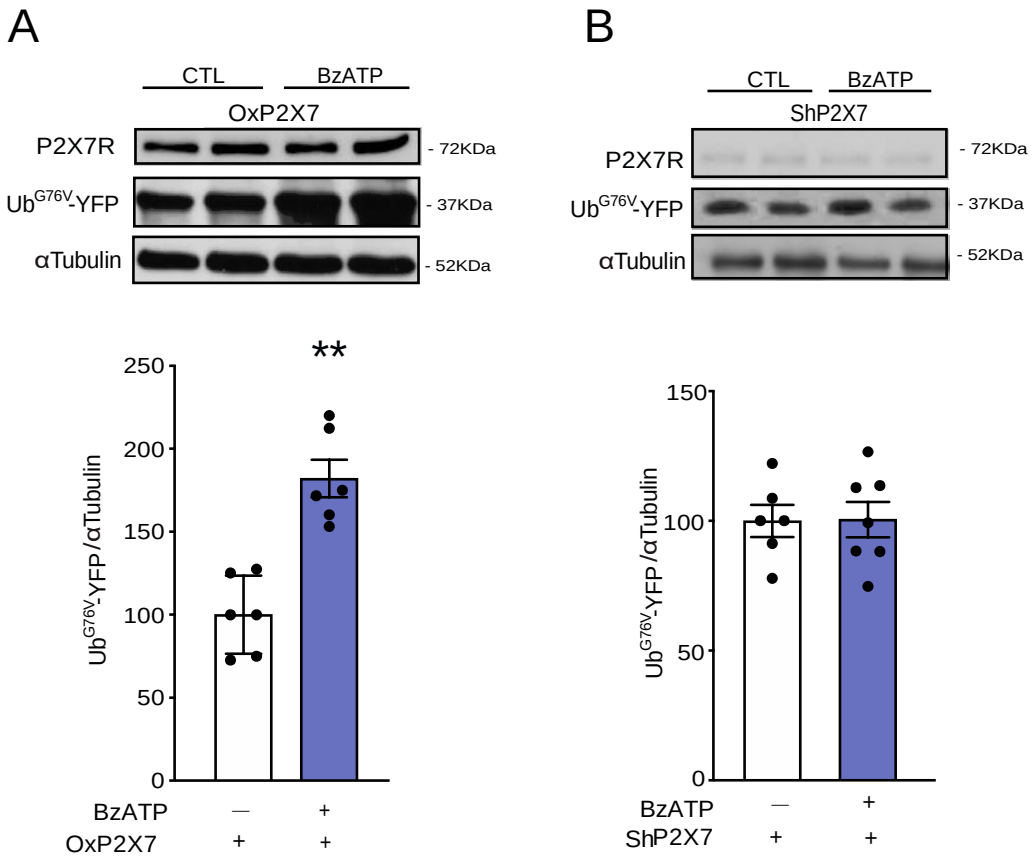

Figure 27: **Stimulation with BzATP causes an increase in the accumulation of UPS activity reporter in cells co-transfected with Ub^{G76V}-YFP and P2X7R-GFP.** Representative Western blot images and graphs showing the analysis of Ub^{G76V}-YFP levels in extracts from N2a cells expressing Ub^{G76V}-YFP co-transfected with plasmid overexpressing P2X7 (A) or shP2X7R (B) (n=3 experiments performed in duplicate). Amounts of protein were normalized with to α-Tubulin. Values are represented as the mean ± s.e.m $^{**}p < 0.01$ using *Unpaired t test*

4.2 *In vitro* activation of P2X7 receptor modulates the catalytic activities of the proteasome and the expression of the constitutive β subunits

To investigate the molecular mechanisms by which P2X7R can modulate UPS activity, we chose to examine the proteasome's 26S catalytic activities in N2a cells stimulated or not with BzATP (200 µM) in the presence or absence of the antagonist (A438073 40 µM) for 24 hours. BzATP stimulation substantially decreased both chymotrypsin-like (CT-L) and postglutamyl-like (PG-L) peptidase activities but did not affect trypsin-like activity (T-L) (**Figure 28**). Furthermore, CT-L and PG-L activity were vastly improved in N2a cells treated with A438073, a selective P2X7R antagonist.

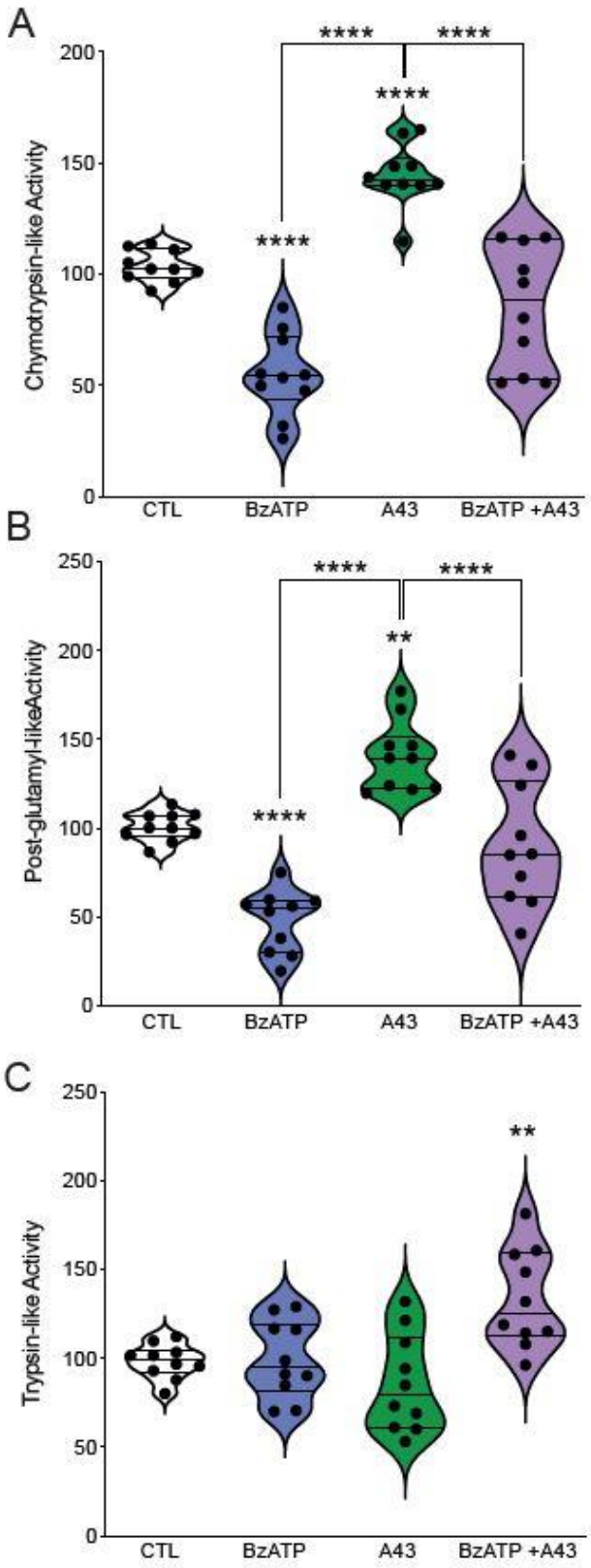

Figure 28: Activation of P2X7R by BzATP decreases proteasome chymotrypsin-like (CT-L) and postglutamyl-like (PG-L) activities. (A,B,C) Violin plots represent the measurement of 26S proteasome peptidase activities in N2a cells treated with BzATP for 24h in the presence or absence of the antagonist A438073 (40 µM). The specificity of the fluorogenic reaction was verified by adding the MG132 proteasome inhibitor. Violin plots depict the frequency distribution of data, with each point representing the results of three independent experiments carried out in triplicate. **** $p < 0.0001$, **$p < 0.01$ using ANOVA test

As a second step, we investigated whether the drop in CT-L and PG-L activities was attributable to a decrease in the expression of the constitutive β subunits or the inducible subunits of the immunoproteasome (βi). For this purpose, the expression of the different β and βi subunits was measured using Western Blot in N2a cell extracts following stimulation or not with BzATP (200 µM). **Figure 29A** shows that stimulating cells with BzATP 200 µM for 24 hours resulted in a significant decrease in the expression of β5 and β1 subunits, but their equivalent inducible subunits did not show any significant changes in expression levels (**Figure 29B**). There were no significant changes in the expression levels of the β2 and β2i subunits following BzATP stimulation.

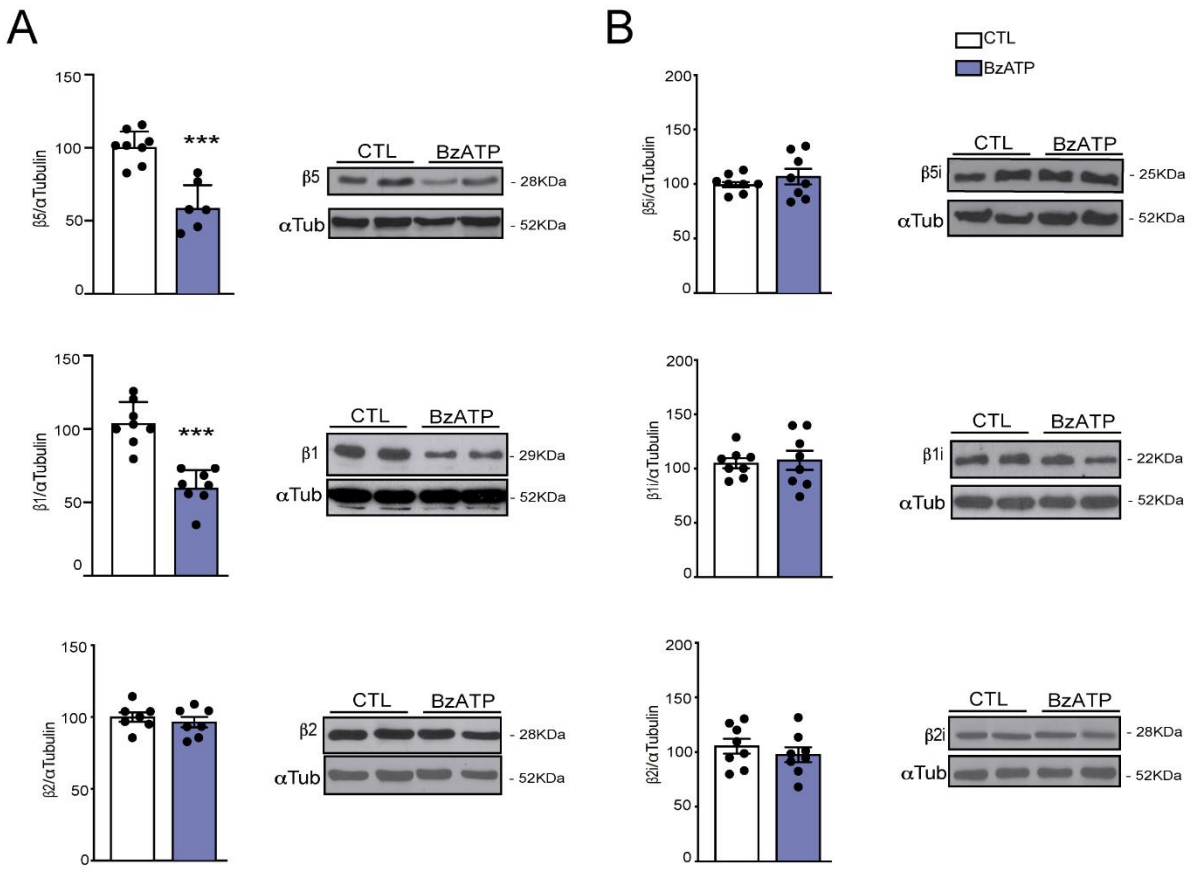

Figure 29: Analysis of the expression of the constitutive and inducible subunits of the 26S proteasome. (A) Representative Western Blot images and graphs of the expression of the constitutive β subunits B) Representative Western Blot images and graphs of the expression of the inducible β subunits. Amounts of protein were normalized to α-Tubulin. Values are represented as the mean ± s.e.m. of at least five independent experiments run in duplicate. ***p < 0.001, *Unpaired t test*

Afterward, we sought to elucidate if the reduction in β5 and β1 protein levels caused by BzATP was related to transcriptional changes. As a result, we measured, by RT-qPCR, β5(*Psmb5*), β1(*Psmb6*), and β2(*Psmb7*) mRNA levels from N2a-Ub^{G76V}-YFP cells treated or not

with 200 µM BzATP for 24 hours. As demonstrated in **Figure 30**, activation of P2X7R by BzATP resulted in a substantial reduction in mRNA levels of β5(*Psmb5*) subunit and β1(*Psmb6*), compared to untreated cells. We found no significant variation in mRNA levels of the β2(*Psmb7*) subunit in BzATP-treated cells, as expected.

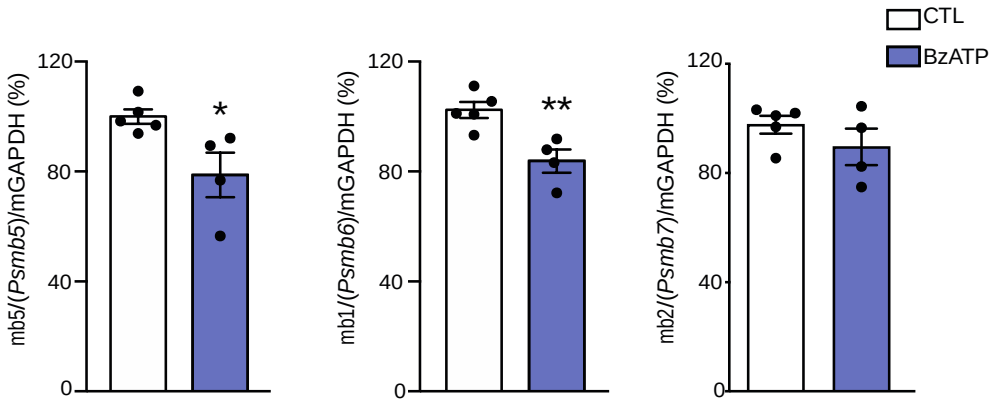

Figure 30: Reduction in CT-L and PG-L activity is caused by a decrease in the expression of mRNA levels of proteasome subunit (Psmb5) β5 and (Psmb6) β1. Representative graphs of the relative levels of β5**,** β1, and β2 mRNA measured by quantitative PCR and normalized with respect to the internal GAPDH control. Values are represented as the mean ± s.e.m. of 3 independent experiments run in duplicate. **p < 0.01, *Unpaired t test*

4.3 P2X7R modulates UPS activity by regulating the assembling of 20S proteasome catalytic subunits

To investigate the function of P2X7R activation in proteasomal assembly, we purified 20S proteasome complexes from N2a cells that had been treated or not with 200 µM of BzATP, using the rabbit IgG polyclonal antibody MCP-8017.3, which is directed against the 20S proteasome core. P2X7R activation caused by BzATP decreased the total number of β5 catalytic subunits composing the 20S proteasome core particle, according to immunoprecipitation assays (**Figure 31**). We used β2 expression to normalize our results since its expression didn't change either at protein or messenger levels when we stimulated with BzATP.

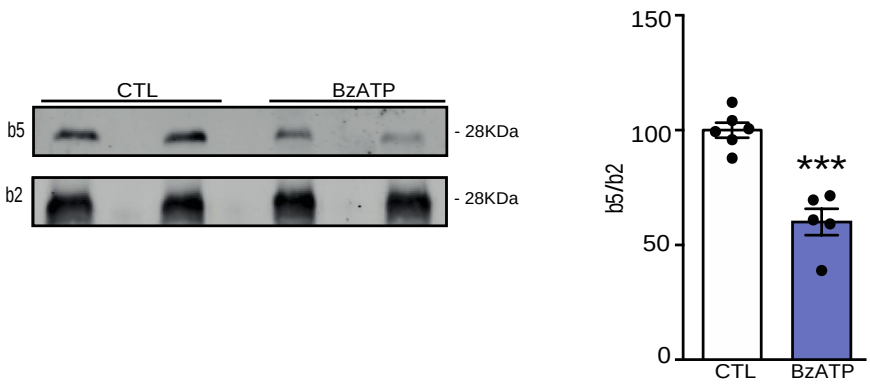

Figure 31: P2X7R activation modifies the expression of the β5 subunit assembling the 20S core. Representative Western Blot image and graph of the expression of the β5 subunit in N2a cells after immunoprecipitation. Amounts of protein were normalized to the β2 subunit. Values are represented as the mean ± s.e.m. of three independent experiments run in duplicate. ***p < 0.001, *Unpaired t test*

In addition, to show that P2X7R activation impairs UPS and prevents it from appropriately assembling into the 20S proteasome core, N2a cells were co-transfected with Ub^{G76V}-YFP and proteasome subunit beta type 5 (*Psmb5*) or with proteasome subunit beta type 1 (*Psmb6)* to promote the overexpression of β5 and β1, respectively. After transfection, cells were treated with BzATP or PBS for 24 hours**.** **Figure 32** depicts how the overexpression of β5 and β1 subunits prevents UPS impairment caused by P2X7R activation triggered by BzATP.

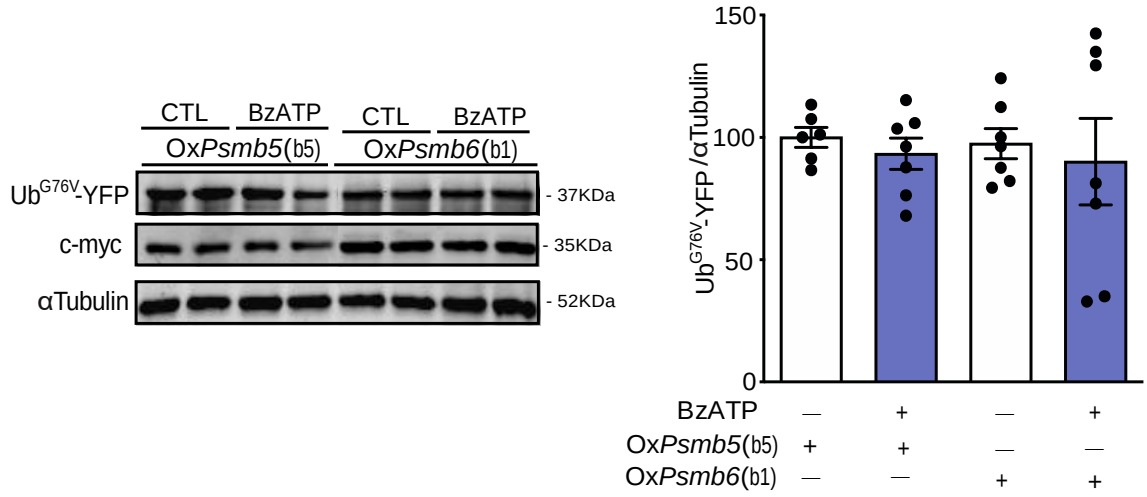

Figure 32: **Overexpression of β5 and β1 subunits rescued the UPS impairment caused by P2X7R activation.** Representative Western blot images and graph showing the analysis of Ub^{G76V}-YFP levels in extracts from N2a cells expressing Ub^{G76V}-YFP co-transfected with a plasmid overexpressing Psmb5(β5) or a plasmid overexpressing Psmb6(β1) (n=3 experiments performed in duplicate). Amounts of protein were normalized with to α-Tubulin. The c-Myc expression allowed the identification and monitoring of the transfected cells. Values are represented as the mean ± s.e.m

4.4 The Akt/GSK/Nrf2 pathway seems to be involved in the modulatory effect that P2X7R exerts on UPS

After confirming that P2X7R is capable of altering the proteolytic activity of the UPS, we attempted to identify the intracellular signaling route implicated in this phenomenon. To achieve this aim, we used a broad battery of selective inhibitors of the main intracellular kinases associated with this receptor. Since previous studies reported that P2X7R might regulate the PI3K/Akt/GSK3 intracellular pathway (*Gomez-Villafuertes et al.,2015; Diaz-Hernandez et al.,2012*), we wondered whether these kinases were engaged in this signaling pathway. Results showed that when N2a-Ub^{G76V}-YFP cells were treated with the selective inhibitor of PI3K (LY294002 50 µM), the baseline levels of the reporter increased. However, when cells were pretreated with BzATP and then treated with LY294002, no additive impact was seen. No substantial increase in UPS reporter accumulation was seen once N2a cells were treated with the selective inhibitor of Glycogen synthase kinase 3 (GSK3) (SB216763 5 µM) alone. Nonetheless, in N2a cells cotreated with GSK3 antagonist and BzATP, the UPS impairment caused by BzATP was reversed (**Figure 33**).

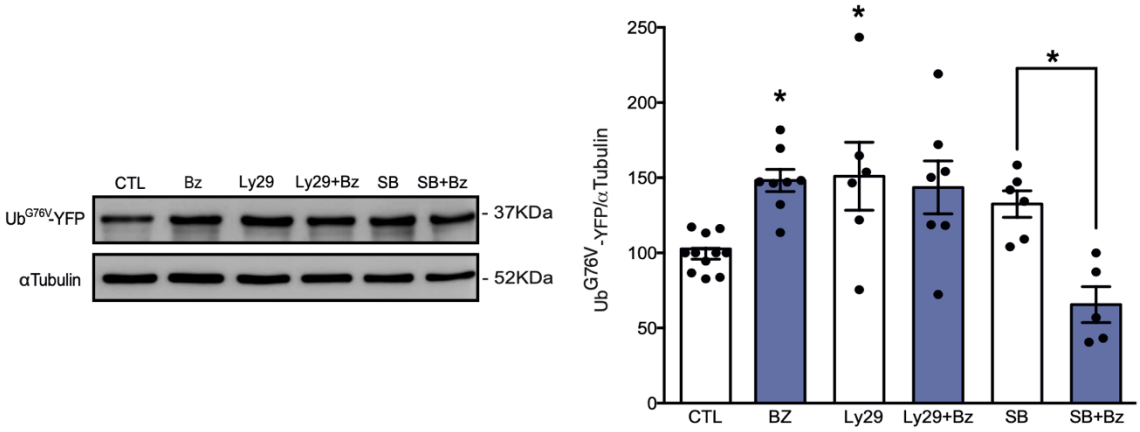

Figure 33: Involvement of PI3K and GSK3 kinases in the response induced by BzATP on reporter levels. Representative Western blot image and graph of the effect produced by the inhibitors LY294002 (50 µM) and SB (5 µM) in the presence or absence of BzATP on the levels of Ub^{G76V}-YFP reporter. Amounts of protein were normalized to α-Tubulin. Values are represented as the mean ± s.e.m. **p < 0.01 using *ANOVA*

To validate these prior findings, we decided to take a different approach, measuring CT-L proteasome activity in N2a cells stimulated or not with BzATP (200 µM) in the presence or absence of specific PI3K (LY294002 50 µM) and GSK3 (SB216763 5 µM) inhibitors for 24 hours. The combination of the GSK inhibitor and BzATP dramatically enhanced CT-L in N2a cells compared to cells treated solely with the inhibitor (**Figure 34**).

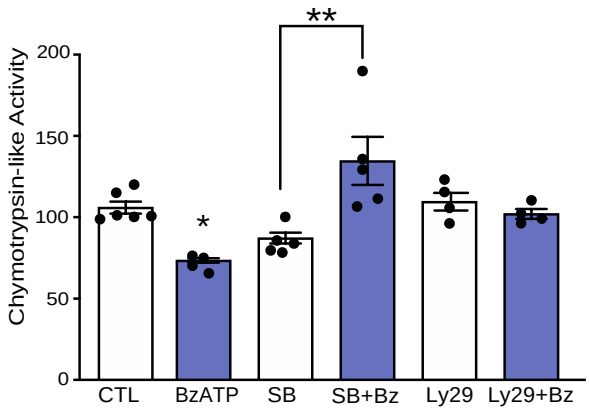

Figure 34: Involvement of PI3K and GSK3 kinases in the response induced by BzATP on chymotrypsin levels. Measurement of chymotrypsin activity in N2a cells treated with BzATP for 24h in the presence or absence of the inhibitors LY294002 (50 µM) and SB (5 µM). The specificity of the fluorogenic reaction was verified by adding the MG132 proteasome inhibitor. Values are represented as the mean ± s.e.m **p < 0.01, *p < 0.05 using ANOVA test

To determine P2X7R function in this pathway, we looked at how P2X7R activation affected the activity of Protein Kinase B (PKB or Akt) and GSK3. When compared to PBS-treated cells, we observed a substantial reduction in the phosphorylation status of both kinases at early stages (4h and 8h) (**Figure 35**). This finding suggests that P2X7R activation causes Akt inhibition and subsequent GSK3 activation and that it may be involved in the regulation of this signaling pathway.

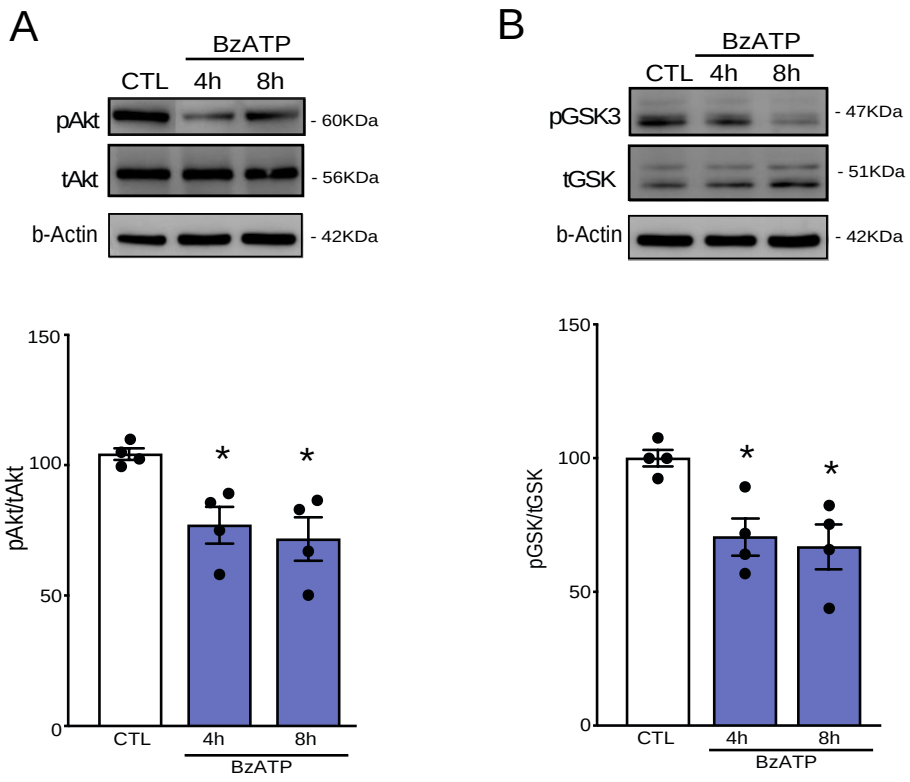

Figure 35: The Akt/GSK pathway seems to be involved in the modulatory effect that P2X7R exerts on UPS Representative Western Blot images and graphs of the expression of the phospho and total levels of Akt (A) and GSK3 (B) in extracts from N2a cells treated or not with BzATP at different time points (4h and 8h) (n=4 performed in duplicate). Graphs represent the mean ± s.e.m of the ratio of pGSK3/tGSK3 or pAkt/tAKt. β-actin expression was used as an internal control. **p < 0.01 *p < 0.05; using *ANOVA*

Previous works have reported that GSK3 mediates the phosphorylation of the Nuclear factor erythroid 2-related factor 2 (Nrf2) (*Chowdhry et al.,2013*), one of the main transcriptional factor involved in the oxidative stress response (*Jaramillo and Zhang., 2013*). Furthermore, Nrf2 regulates the basal and inducible expression of genes of multiple subunits of the 20S proteasome, including *Pmsb5* (β5) (*Kwak et al.,2003; He et al.,2020*). Because previous research has shown that GSK-3 inhibits Nrf2 by nuclear exclusion, promoting its proteasomal degradation (*Salazar et al.,2006; Rojo et al.,2008; Rada et al.,2015*), we decided to look into Nrf2 expression levels in our experimental conditions to see if this transcriptional factor is involved in the intracellular pathway by which P2X7R modulates the UPS. When compared to PBS-treated cells, P2X7R activation resulted in a substantial reduction in NRF2 expression levels in N2a cells (**Figure 36**). All these findings suggest that P2X7R can control UPS *in vitro* by influencing the transcription of catalytic subunits β5 and β1 via an intracellular route involving Akt/GSK3/Nrf2.

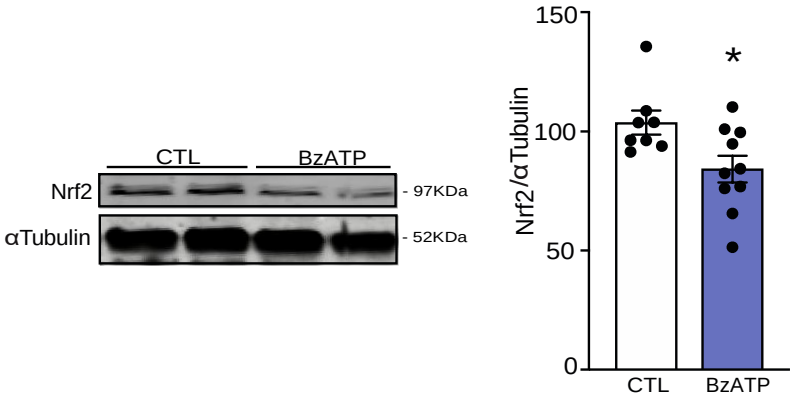

Figure 36: The Nrf2 transcriptional factor seems to be involved in the modulatory effect induced by P2X7R activation(A-B) Representative Western Blot image and graph of the expression of the levels of Nrf2 in extracts from N2a cells treated or not with BzATP for 24h (n= 5 experiments performed in duplicate) Amounts of protein were normalized to α-Tubulin. Values are represented as the mean ± s.e.m. *p < 0.05 using *t test*

4.5 *In vivo* activation of P2X7 receptor produces a decrease in the catalytic activities of the proteasome and the expression of the constitutive β subunits

Since *in vitro* studies indicate that P2X7R controls UPS activity, we investigated whether P2X7R activation *in vivo* may also regulate UPS. We administrated BzATP (2µL of BzATP 30mM) or vehicle solution (PBS) by i.c.v to both WT mice and P2X7 -/- mice. After 24 hours of administration, animals were sacrificed, and the most proximal hippocampus region adjacent to the injection site was dissected and removed to assess the proteasome's CT-L and PG-L activities. In this part of the study, Trypsin-like activity was not analyzed as no modifications were observed *in vitro* in the proteolytic activity or in the expression levels of its corresponding subunits. **Figure 37A** and **37C** show that *in vivo* administration of BzATP in WT produced a significant decrease in CT-L and PG-L activities, whereas in mice lacking P2X7R this administration did not induce significant differences in proteasomal activities. Subsequently, in these same extracts, the expression levels of the β and βi subunits were measured. Figures **37E** and **37G** show that *in vivo* administration of BzATP in WT produced a decrease in the expression of β1 subunit and β5, which was not seen in P2X7 -/- mice (**Figure 37F-H**).

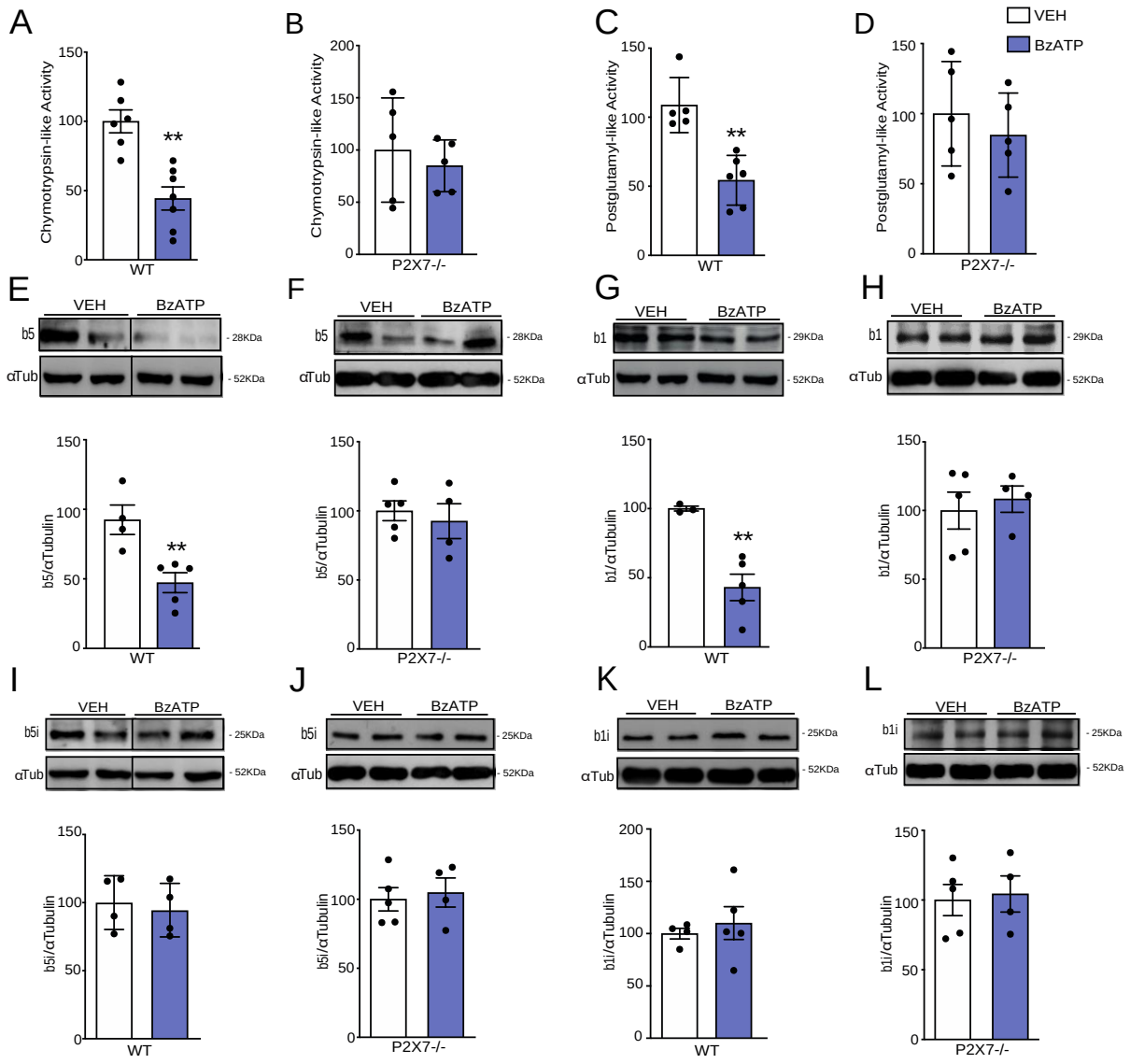

Figure 37: Effect of P2X7R activation on the expression of β subunits in extracts of hippocampus from WT and P2X7 -/- mice. (A) Measurement of CT-L in WT mice and (B) P2X7-/- mice after i.c.v. injection of BzATP (blue) or vehicle solution (white) (C) Measurement of PG-L in WT mice and (D)P2X7-/- mice. In all reactions, the specificity of the fluorogenic reaction was verified by addition of the MG132 proteasome inhibitor. (E-H) Representative Western Blot images and graphs of the expression of the β5 and β1 in WT mice and P2X7-/- mice (I-L) Representative Western Blot images and graphs of the expression of the β5i and β1i in WT mice and P2X7-/- mice. Amounts of protein were normalized to α-Tubulin. **p < 0.01 using *Unpaired t test.* Data in bar graphs represent mean ± s.e.m.

Next, using RT-qPCR, we determined β5(*Psmb5*) and β1(*Psmb6*) mRNA levels in WT mice injected with BzATP or vehicle solution. *In vivo* administration of BzATP resulted in a substantial reduction in mRNA levels of β5 (*Psmb5*) and β1(*Psmb6*) subunits, as seen in **Figure 38**, compared to vehicle treated mice.

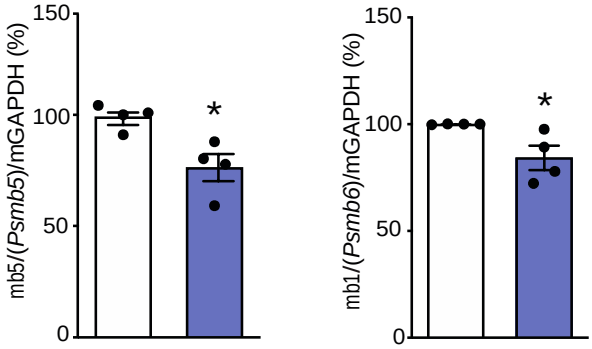

Figure 38: Reduction in CT-L and PG-L activities is caused by a decrease expression of mRNA levels of proteasome subunit β5 and β1 in WT mice. Representative graph of the relative levels of *Psmb5*(β5) and *Psmb6*(β1) mRNA measured by quantitative PCR and normalized with respect to the internal GAPDH control in hippocampus extract of WT mice after i.c.v. injection of BzATP (blue) or vehicle solution (white) (n=4) Values are represented as the mean ± s.e.m. *p < 0.05, *Unpaired t test*

4.6 Akt/GSK/Nrf2 pathway is involved in the modulatory effect that P2X7R exerts on the activity of UPS *in vivo*

Since *in vitro* studies conducted in N2a cell suggest that the intracellular signalling pathway Akt/GSK/Nrf2 might be involved in the modulatory effect that P2X7R exerts on UPS function, we proceeded to check whether *in vivo* activation of P2X7R is also capable of modulating those kinases. We measured possible variation in the phosphorylation of Akt, Akt-downstream target (GSK3) and Nrf2 by Western Blot in hippocampi extracts from WT mice and P2X7 -/- mice i.c.v injected with Veh or BzATP for 24hours. WT-BzATP treated mice presented a significative decrease in the phosphorylated rate of Akt and GSK3 in Ser 9/21 sites. Decrease that was not visible in P2X7-/-BzATP treated mice. Furthermore, compared to WT-Veh mice, WT-BzATP treated mice had reduced levels of Nrf2 (**Figure 39A**). Previous studies demonstrated that P2X7R-activation promotes the inhibition of GSK3 since phosphorylation of Ser 9/21 sites in GSK3 has been related to a decreased GSK3 enzyme activity (*Diaz-Hernandez et al., 2008; Diaz-Hernandez et al., 2012*). Confirming those data, as we previously demonstrated *in vitro*, activation of P2X7R by BzATP causes a reduction in phospo-Akt levels, which resulted in Akt inhibition and consequently GSK3 activation. The implication of P2X7R in the modulatory effect on UPS through the Akt/GSK-3/Nrf2 pathway was confirmed by the lack of any modification at protein levels of those kinases in P2X7-/-BzATP treated mice (**Figure 39B**).

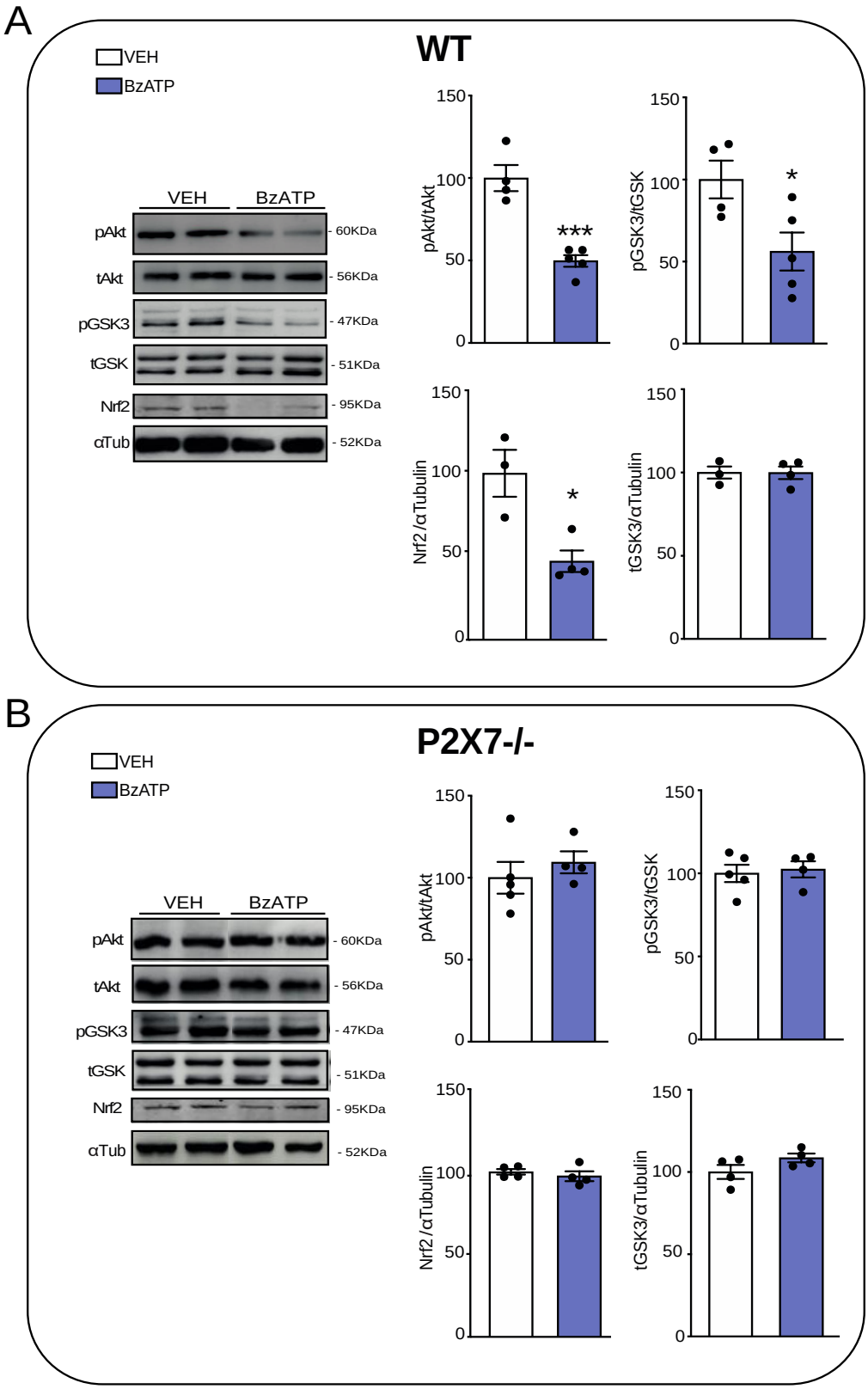

Figure 39: *In vivo* intracellular pathway Akt/GSK/Nrf2 pathway is involved in the UPS impairment induced by P2X7R activation. Representative Western Blot images and graphs of the expression of the phospo and total levels of Akt, GSK3 and Nrf2 inhippocampal extract of (A) WT (n=5) and (B) P2X7-/- mice (n=4) i.c.v injected with Veh (white) or BzATP (blue).Values are represented as the mean ± s.e.m of the ratio of pGSK3/tGSK3, pAkt/tAKt or Nfr2/ α-Tubulin. α-Tubulin expression was used as an internal control related to tGSK ***p < 0.001, *p < 0.05 using *Unpaired t test*

4.7 *In vivo* administration of BzATP causes an accumulation of Ub^{G76V}-YFP in UPS reporter mice in both neurons and microglia cells

To evaluate the effect of P2X7R activation induced by BzATP on UPS activity we used mice expressing the Ub^{G76V}GFP reporter, UbGFP mice. We administrated BzATP or vehicle solution i.c.v to UbGFP mice and animals were sacrificed after 24 hours, and brains sections were analysed by immunofluorescence. As shown below in **Figure 40**, i.c.v administration of BzATP increased the total number of GFP positive cells accumulating UPS reporter in the ipsilateral hippocampus as compared to the contralateral hippocampus. In addition, i.c.v of vehicle solution in UbGFP mice did not result in any significant difference in the total number of GFP hippocampal cells accumulating UPS reporter in both hippocampi (**Figure 40**).

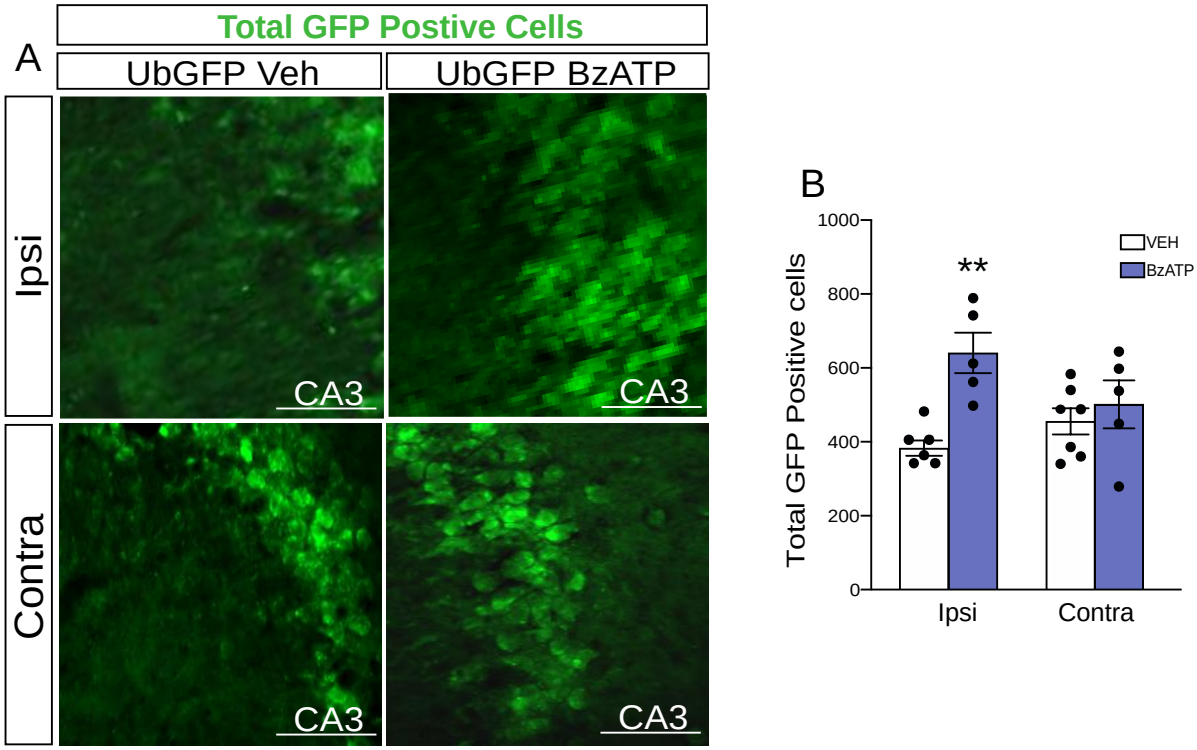

Figure 40: Ub-GFP-BzATP mice showed an accumulation of the Ub^{G76V}-GFP reporter in the ipsilateral hippocampus (A) Representative immunofluorescence image of GFP fluorescence (green) in coronal sections of the contralateral and ipsilateral hippocampus of UbGFP mouse treated with BzATP or vehicle solution. (B) Graphs represent the mean ± s.e.m of the total number of positive GFP cells of the contralateral and ipsilateral hippocampus of UbGFP mouse treated with BzATP (blue) or vehicle solution (white). (n=5 mice per treatment and n=3 sections per mouse).

Thanks to the use of different specific antibodies that allow us to identify the different cell lineages present in the hippocampus, we were able to determine that Ub^{G76V}-GFP reporter accumulation occurred mainly in neuronal cells. The injection of BzATP enhanced the number of neuronal and microglial cells with a considerable accumulation of the Ub^{G76V}-GFP reporter (**Figure 41**). On the other hand, it should be noted that administration of BzATP did not produce significant modifications in the number of astrocytes that presented a significant reporter accumulation.

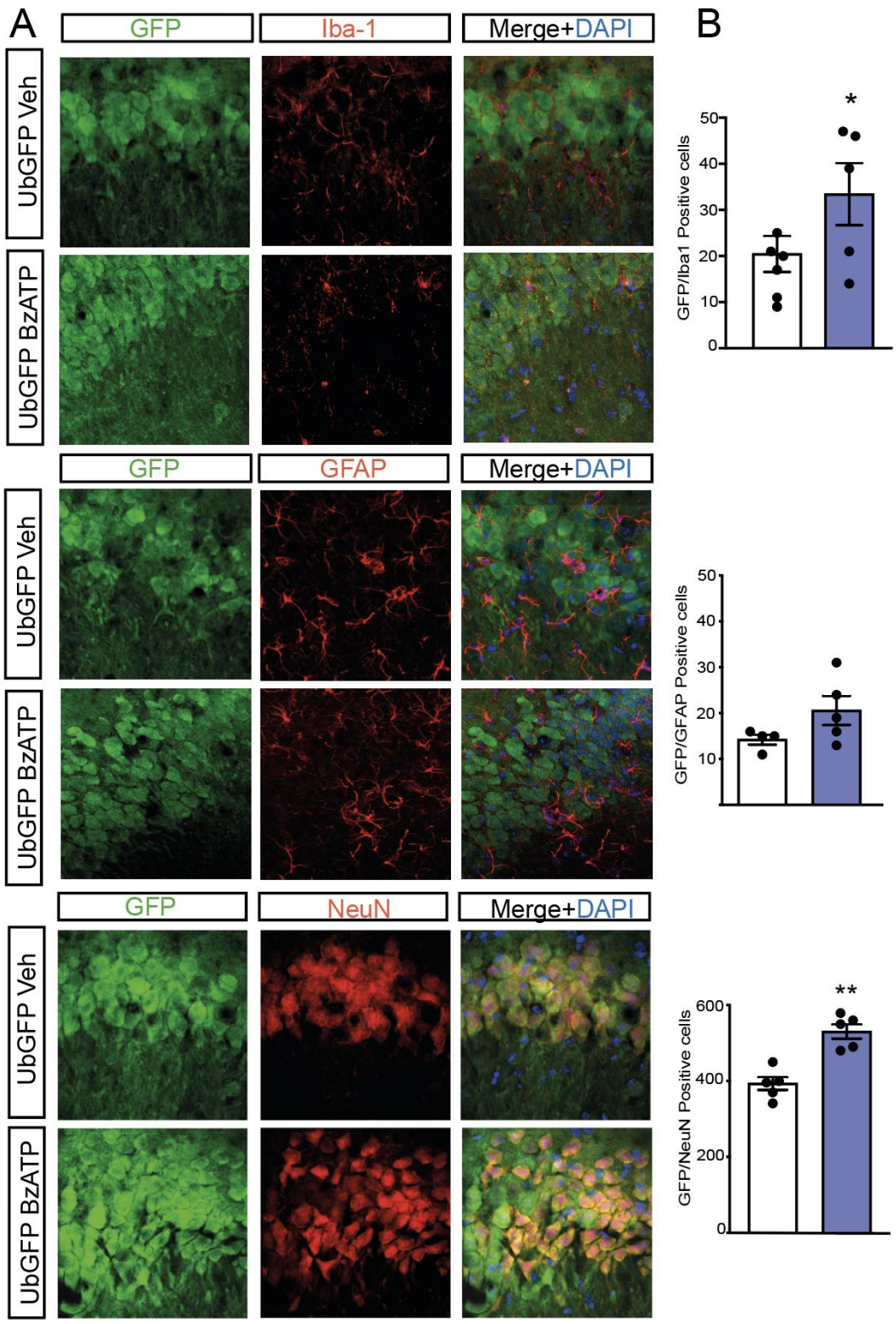

Figure 41: Accumulation of the Ub^{G76V}-GFP reporter by *in vivo* administration of BzATP and Vehicle solution in the UbGFP mice. (A) Representative images and (B) quantification graphs corresponding to colocalization studies of hippocampal cells accumulating Ub^{G76V}-GFP (green) stained by immunofluorescence with markers (red) of astrocytes (GFAP), microglia (Iba-1) and neurons (NeuN) in UbGFP mice i.c.v injected with Vehicle solution (white) or BzATP (blue). (n=5 mice per treatment and n=3 sections per mouse). Graphs represent the mean ± s.e.m **p < 0.01 using *Unpaired t test*

4.8 Impaired proteasome function in Alzheimer's disease

Since there is compelling evidence that UPS is impaired in many tauopathies such as AD, and may be the underlying factor leading to the accumulation of ubiquitinated proteins found on these diseases (*de Vrij et al., 2004; Ciechanover and Kwon, 2015*), we wonder if P2X7R activation might contribute to this impairment. To investigate this process and confirm that proteasome activity was altered in AD, studies were conducted on post-mortem hippocampi from AD and age and sex-matched controls individuals that were provided by the collaboration with Fundación CIEN, Madrid. Our analyses revealed that AD patients presented a higher amount of polyubiquitinated proteins than controls (**Figure 42A**). Furthermore, accordingly with previous observations (*Keller et al., 2000; Lopez Salon et al., 2000*) CT-L and PG-L activities were reduced in AD brains compared with control subjects (**Figure 42B-C**). To determine if the loss of proteasome activity in the brain of AD was due to decreased levels of proteasome subunits, we analysed the levels of proteasome constitutive and inducible subunits by Western blot analysis (**Figure 42 D-G**). The reduction in CT-L activity was consequent to a significant decrease in β5 levels, whereas the reduction in PG-L activity was due to a significant decrease in the expression levels of β1. Furthermore, like we previously did *in vitro* and *in vivo* in WT mice, we analysed, by RT-qPCR, β5(*Psmb5*) levels in hippocampi from AD and age and sex-matched controls. As shown in **Figure 42H**, AD patients presented reduced β5(Psmb5) mRNA levels than controls.

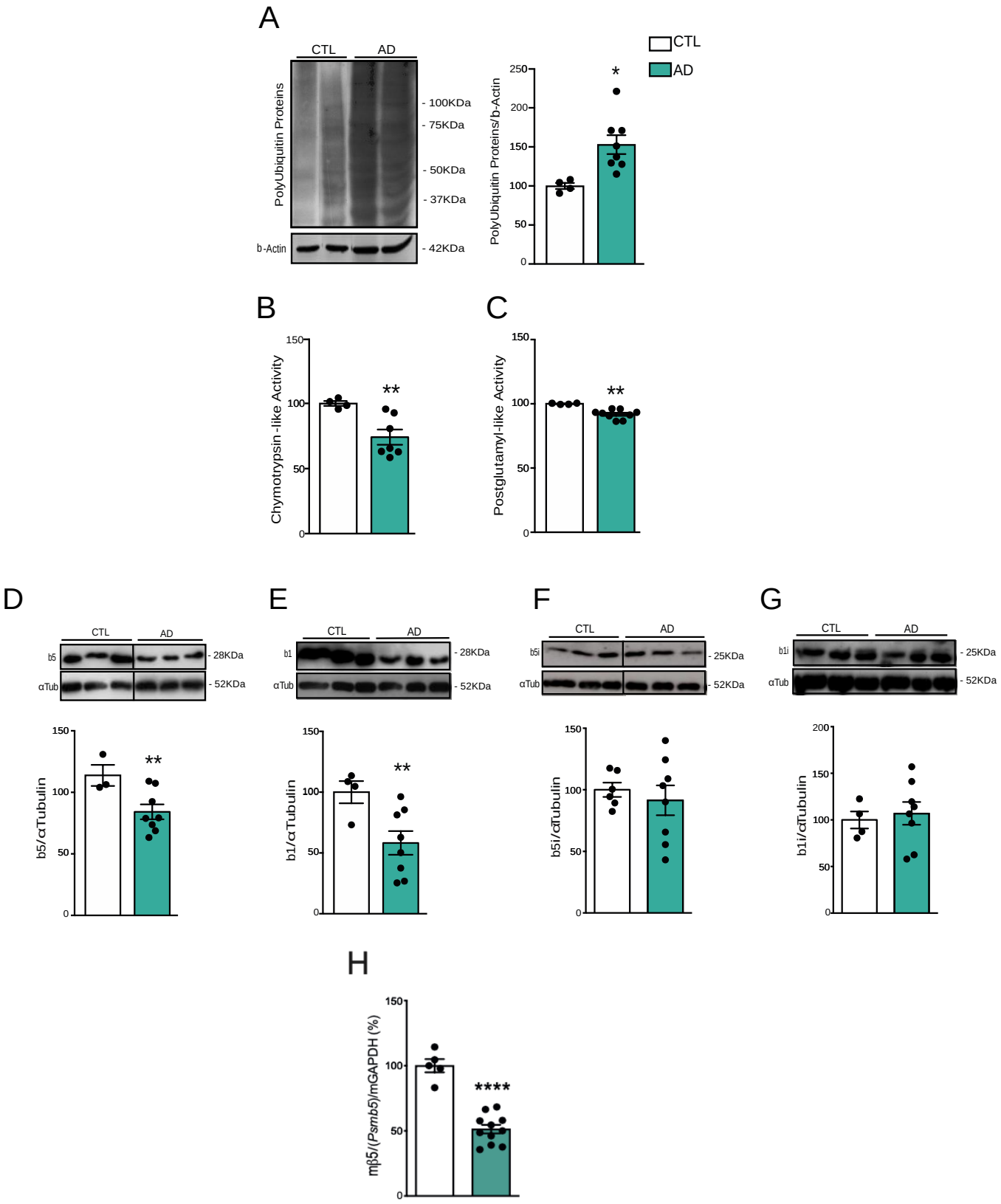

Figure 42: Reduced CT-L and PG-L activities in postmortem AD brains. (A) Representative Western Blot images and graph of the expression of Polyubiquitin Proteins in AD and control hippocampi. Amounts of protein were normalized to β-Actin. (B-C) Measurement of CT-L and PG-L activities in postmortem hippocampus of AD (n=7) and controls (n=4) brains. In all reactions, the specificity of the fluorogenic reaction was verified by addition of

the MG132 proteasome inhibitor. (D-G) Representative Western Blot images and graphs of the expression of the β5 and β1 constitutive and inducible subunits in AD and control hippocampi. Amounts of protein were normalized to α-Tubulin. (H) Representative graph of the relative levels of β5 mRNA measured by quantitative PCR and normalized with respect to the internal GAPDH control in hippocampus of AD patients(green) or controls (white) ****p < 0.0001 ;**p < 0.01; *p < 0.05 using *Unpaired t test*. Data in bar graphs represent mean ± s.e.m.

4.9 P2X7R inhibition reverts the UPS impairment associated with Alzheimer's disease

The microtubule-associated protein tau, as indicated in the introduction, is a crucial factor in the development of Alzheimer's disease (*Wang and Mandelkow,2012*). In many neurodegenerative disorders, Tau aggregates are prevalent in many neurodegenerative diseases and correspond with the degree of memory loss in Alzheimer's disease. The UPS participates in tau clearance, with a specific enzyme cascade involved in tau ubiquitination and destruction. Any disruption of the UPS may block tau protein degradation and, in conjunction with hyperphosphorylation, increase tau tangle development (*Brunello et al.,2019*). We employed P301S mice, a well-characterized AD mouse model, to assess the role of P2X7R in UPS dysfunction associated with AD (*Yoshiyama et al., 2007*). These animals produce 5-fold more tau protein and develop phospho-tau disease at 6 months of age (*Yoshiyama et al., 2007*). Our major objective was to investigate if inhibiting P2X7R *in vivo* impacted UPS activity in P301S mice. To this purpose, a set of 9 months old P301S and WT mice were daily treated with the selective P2X7R antagonist GSK 1482160A (GSK, 100 mg/kg, i.p) or the same volume of vehicle solution (Veh) for 3 weeks. After 3 weeks of administration, animals were sacrificed, and the hippocampus was extracted to perform biochemical and immunofluorescence studies. At this age, Veh-P301S mice phenocopy the UPS dysfunction seen in human AD patients, exhibiting a substantial accumulation of polyubiquitinated proteins compared to Veh-WT mice. Importantly, the pharmacological blockage of P2X7R reverted the accumulation of polyubiquitin conjugates in GSK-P301S mice compared to Veh-P301S (**Figure 43A**). A later immunohistochemistry study confirmed that Veh-P301S presented a higher number of hippocampal cells accumulating polyubiquitin aggregates than GSK-P301S in the dental gyrus (DG); polyubiquitin conjugates were not detected in Veh-WT mice (**Figure 43B**).

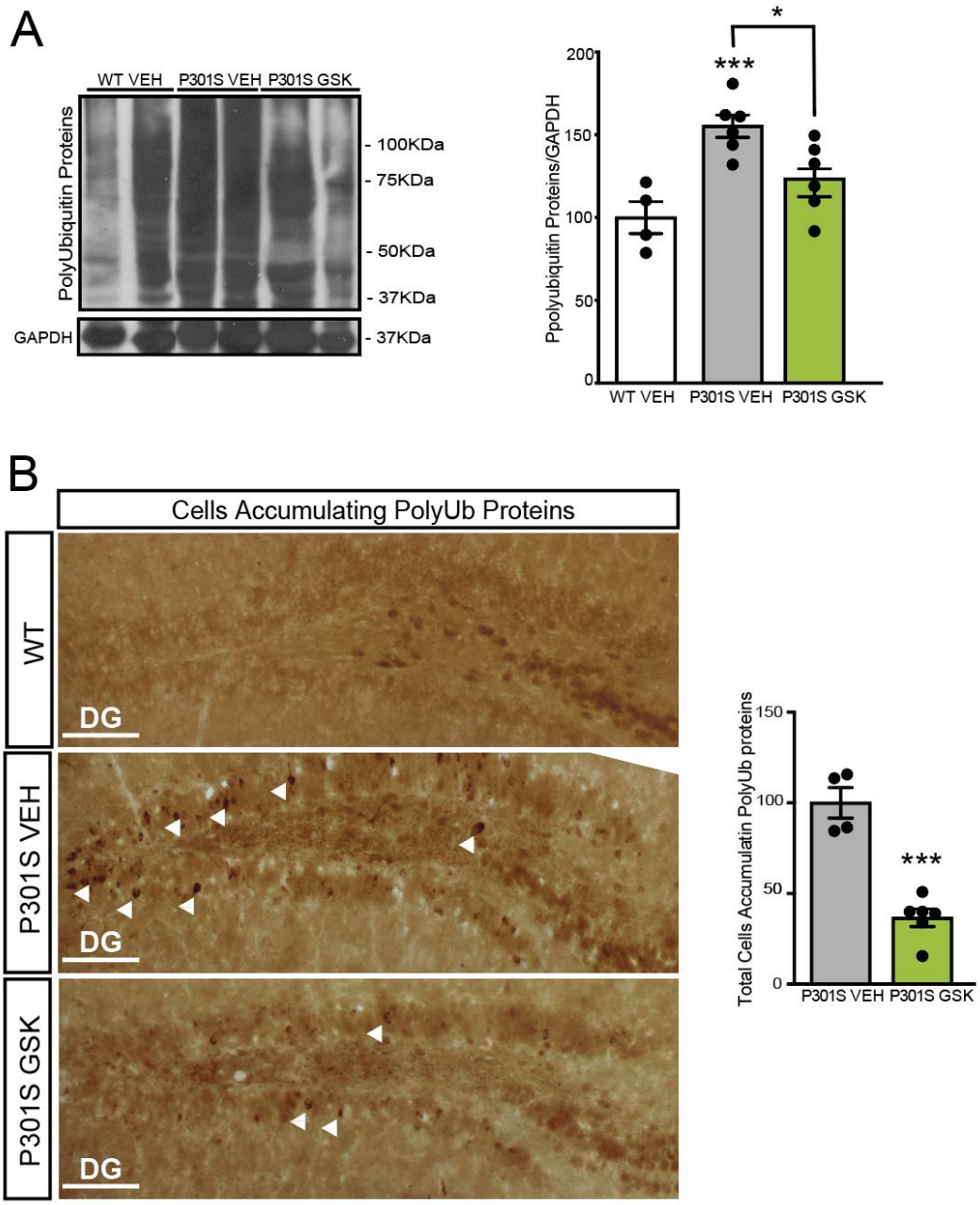

Figure 43: Veh-P301S mice phenocopy the UPS dysfunction present in AD patients (A) Representative Western Blot images and graph of the expression of Polyubiquitin Proteins in WT and P301S mice treated with P2X7R antagonist GSK 1482160A (GSK, 100 mg/kg, i.p) or vehicle solution (Veh). Amounts of protein were normalized to GAPDH. (n≧4 per genotype and treatment) (B) Representative images and graph of hippocampal sections from WT-Veh mice and P301S mice treated with GSK or Veh that were stained with an antibody to detect polyubiquitin conjugates (FK2). (n≧4 per genotype and treatment and n≧4 sections per mouse). Dash lines indicate the granular layer of DG area. Scale bar: 100μm.

Later, we measured the different catalytic activities of the proteasome in WT and P301S mice treated with P2X7R antagonist GSK 1482160A. As shown below in **Figure 44A-B** Veh-P301Smice presented a significant reduction in CT-L and PG-L activities compared to Veh-

WT mice. Accordingly, pharmacological inhibition of P2X7R improves CT-L and PG-L activity in P301S mice **(**Figure 44A-B). Then, in these same hippocampi extracts, we analysed the expression levels of the β constitutive subunits by Western Blot. Like we previously saw in AD patients, the reduction in CT-L activity was due to a significant decrease in β5 protein levels and the decrease in PG-L activity was concomitant to a significant reduce in the expression levels of β1 (Figure 44C-D). Finally, we investigated, by RT-qPCR, mRNA levels of β5(*Psmb5*) and β1(*Psmb6*). Veh-P301S presented a significant reduction in mRNA levels of β5 (*Psmb5*) and β1(*Psmb6*) subunit, compared to Veh-WT mice. More importantly, the blockage of the P2X7R, rescues the mRNA reduction of β5 and β1 in P301S mice (Figure 44E-F)

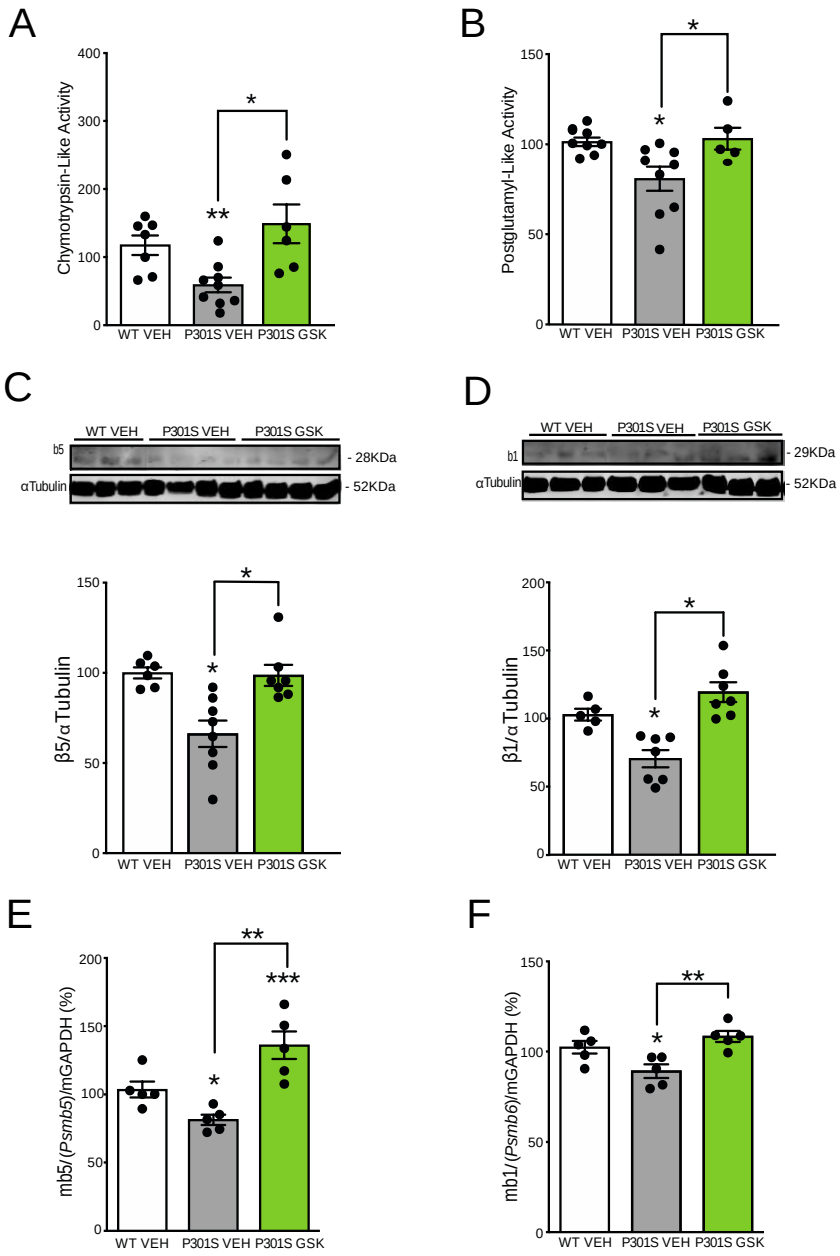

Figure 44: Pharmacological inhibition of P2X7R affects UPS activity in P301S mice. (A-B) Measurement of CT-L and PG-L activities in WT and P301S mice treated with P2X7R antagonist GSK 1482160A (GSK, 100 mg/kg, i.p) or vehicle solution (Veh). In all reactions, the specificity of the fluorogenic reaction was verified by addition of the MG132 proteasome inhibitor (C-D) Representative Western Blot images and graphs of the expression of the β5 and β1 constitutive subunits in the hippocampus from WT and P301S mice treated with Veh or GSK (n=5 mice per treatment at least). Amounts of protein were normalized to α-Tubulin. (E-F) Representative graph of the relative levels of β5 and β1 mRNA measured by quantitative PCR and normalized with respect to the internal GAPDH control in hippocampus of WT mice (white) or P301S mice treated with Veh (grey) or GSK (green)***p < 0.001; **p < 0.01; *p < 0.05 using *ANOVA*. (n≧4 per genotype and treatment). Data in bar graphs represent mean ± s.e.m.

To identify the cells most vulnerable to the UPS-impairment associated with Alzheimer's disease, we created a double transgenic mouse that expressed both the UPS reporter and a mutant version of the human microtubule-related protein tau (Ub^{G76V}-GFP;P301S). Comparative immunohistochemistry analysis revealed that UbGFP;P301S mice presented a higher number of cells accumulating UPS reporter in the CA3 region than their control-littermates UbGFP mice (**Figure 45**).

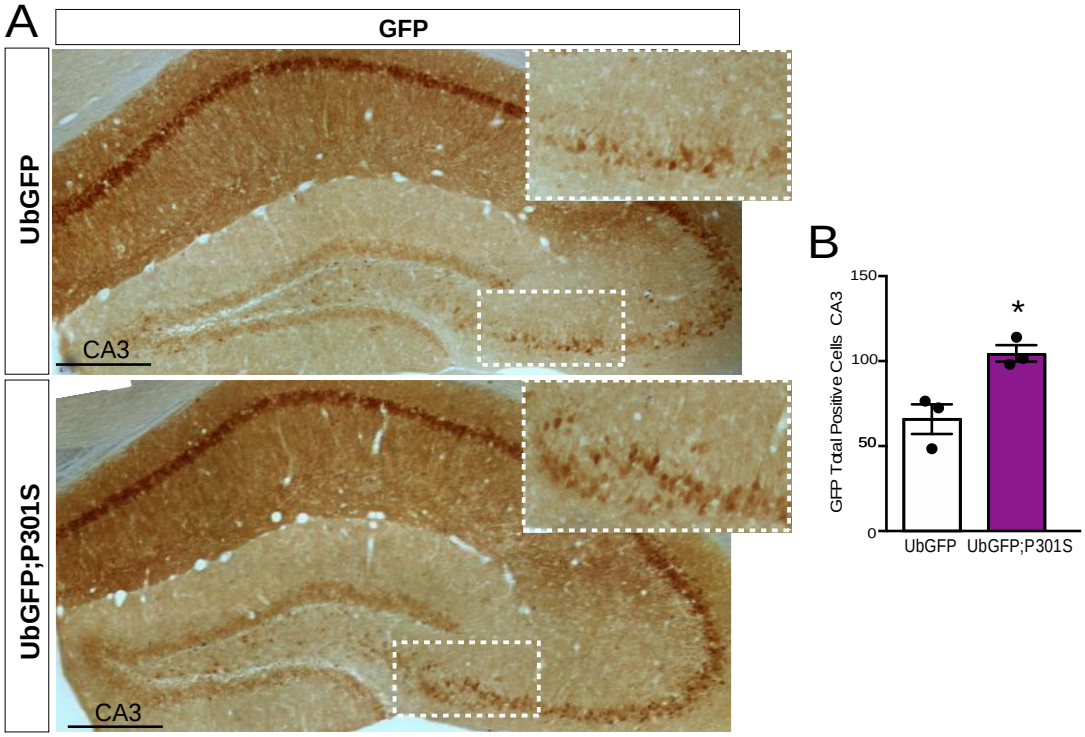

Figure 45: Accumulation of the Ub^{G76V}-GFP reporter in double transgenic mice expressing a mutant form of the protein tau (A) Representative images of hippocampal sections from UbGFP and UbGFP;P301S mice stained with GFP. Dash lines indicates the granular layer of CA3 area. Scale bar: 100 μm. (B) Graph represents the quantification of GFP positive cells in the CA3 area of the hippocampus (n=3 mice per genotype with n=3 sections/mouse) *p < 0.05 using *Unpaired t test*. Data in bar graphs represent mean ± s.e.m.

Later, we chose to investigate whether P2X7R blocking increases neuronal survival in tauopathies, since it has previously been shown that P301S animals had a decrease in the number of neurons across the pyramidal cell layer in the CA3 area of the hippocampus (*Yoshiyama et al., 2007*). To answer this question, we quantified the total number of positive neuronal cells, identified by the neuronal marker Neun, in the CA3 area of the hippocampus of WT and P301S mice treated with either vehicle or GSK solution. We observed that Veh-P301S mice have a significant reduction in the number of hippocampal neurons, compared to Veh-WT mice (**Figure 46**). Furthermore, in line with the previously shown beneficial effects of P2X7R antagonist GSK, in vivo pharmacological inhibition of P2X7R reduced neuronal loss in CA3 in P301S mice (**Figure 46**). Overall, our findings suggest that P2X7 is implicated in the UPS impairment associated with AD, and that blocking it may prevent UPS impairment-induced cellular death.

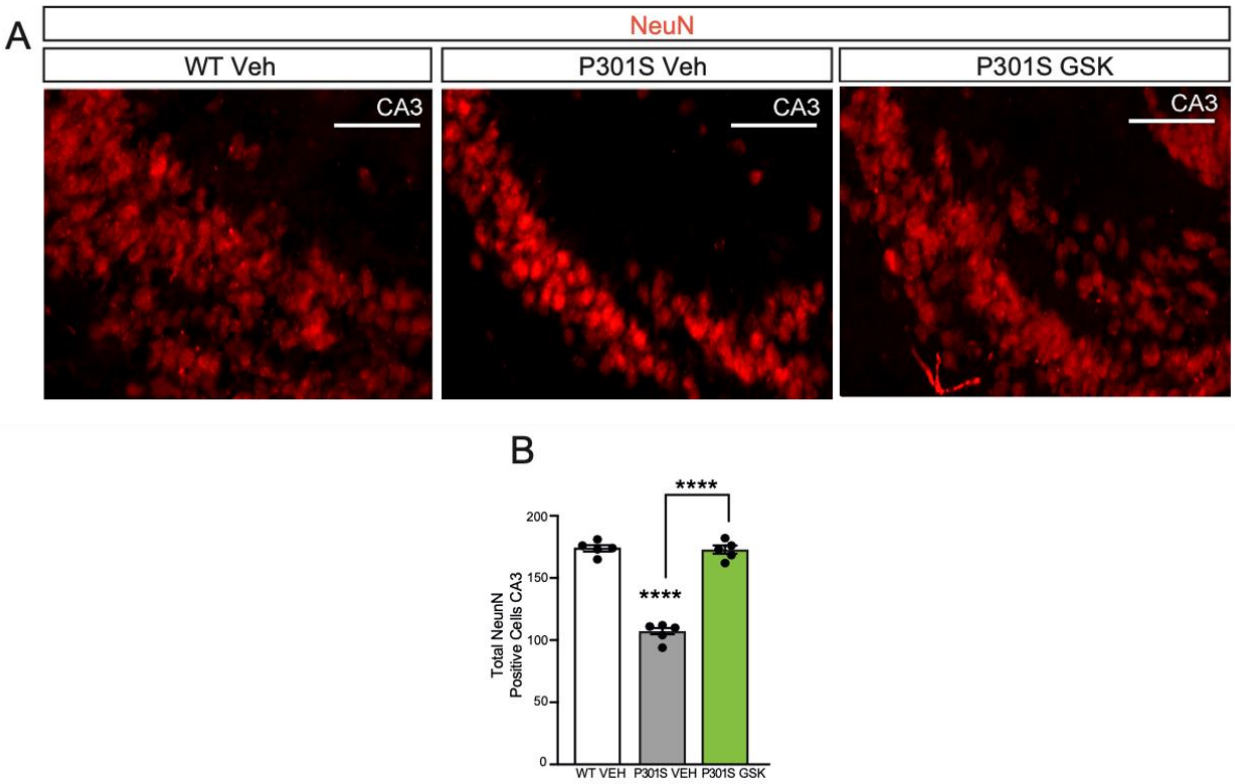

Figure 46:. *In vivo* P2X7 inhibition enhances neuronal survival in P301S mice (A) Representative immunofluorescence images of the hippocampal CA3 area stained with NeuN from WT and P301S mice treated with Veh solution or P2X7 inhibitor GSK 1482160A (GSK, 100 mg/kg, i.p). Scale bar = 100 µm. Graph represents the quantification of neuronal hippocampal cells identified as NeuN positive (n≧4 mice per genotype and treatment and n≧4 sections per mouse). Data in bar graphs represent mean ± s.e.m.

4.10 P2X7R might be involved in UPS dysregulation after seizures

Since it has been demonstrated that prolonged seizures (*status epilepticus*) can impair UPS function (*Engel., et al 2017*), during my secondment period in Tobias Engel Laboratory at RCSI (Dublin), we decided to investigate the role of P2X7R in the UPS dysregulation that occurs in hippocampal cells following seizures induced by Kainic Acid administration (KA). To answer this question, we employed a well-known mouse model of *status epilepticus* produced by an intra-amygdala microinjection of the excitotoxin KA (0,2 µg KA in 0,2 µg of PBS) (*Mouri et al., 2008*). To reduce mortality and morbidity, an anticonvulsant drug was administrated 40 min following KA injection (*Mouri et al., 2008*). We looked at polyubiquitinated conjugates in the hippocampus of WT mice 8 hours after *status epilepticus*, which is when hippocampal P2X7R expression rises in the intra-amygdala KA animal model of *status epilepticus* (*Engel et al.,2012b*). Epileptic mice showed an increase in polyubiquitinated substrates 8 hours after *status epilepticus* as compared to control WT mice (**Figure 47A**). Second, to investigate the role of P2X7R in the regulation of UPS following seizure, we produced *status epilepticus* in WT and P2X7 -/- mice and evaluated the amounts of polyubiquitinated proteins in the hippocampal extract. P2X7 -/-mice showed a reduction in polyubiquitinated conjugates 8 hours after *status epilepticus* compared to WT mice (**Figure 47B**). Furthermore, following seizures induced by intra-amygdala KA injection, we measured proteasome CT-L activity in the hippocampus of P2X7 -/- mice. CT-L activity was shown to be higher in the hippocampus of P2X7 -/- mice than in WT epileptic animals (**Figure 47C**).

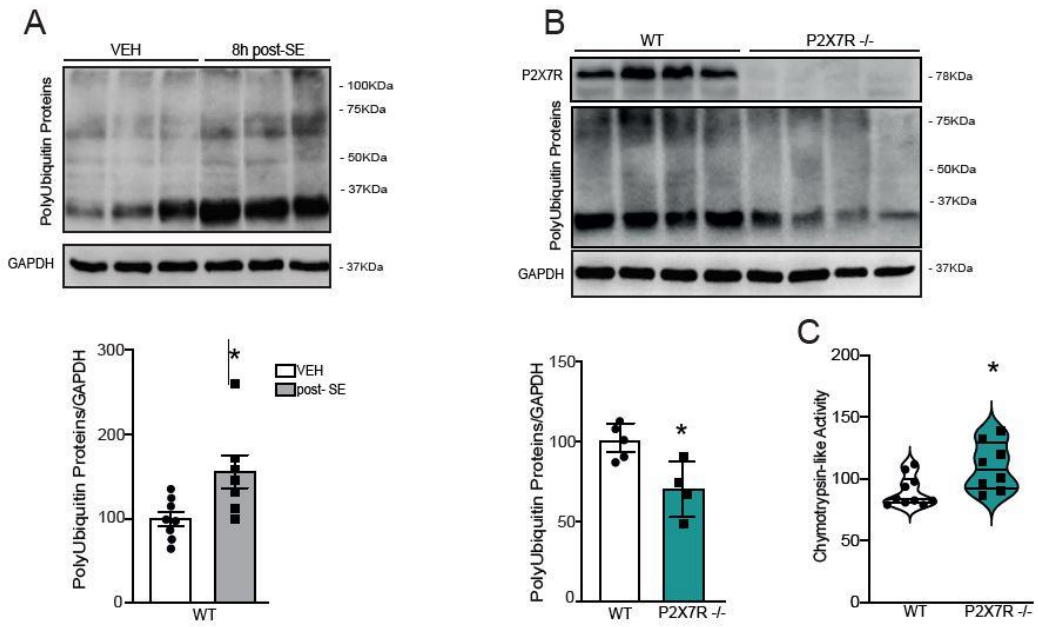

Figure 47: P2X7R seems to be involved in the UPS dysregulation that takes place after seizures. (A) Representative Western Blot images and graph of the expression of Polyubiquitin Proteins in the hippocampus of WT mice pre (n=8) and 8h post *status epilepticus* (n=7) induced by intra-amygdala KA injection. (B) Representative Western Blot images and graph of the expression of Polyubiquitin conjugates in the hippocampus of WT mice and P2X7 -/- 8h post *status epilepticus* (n=7). Amounts of protein were normalized to GAPDH. (C) Violin plots represent the measurement of CT-L in the hippocampus of WT mice and P2X7 -/- 8h post *status epilepticus.* In all reactions, the specificity of the fluorogenic reaction was verified by the addition of the MG132 proteasome inhibitor. *p < 0.05 using *Unpaired t test* Data in bar graphs represent mean ± s.e.m.

To investigate further the role of P2X7R in the regulation of UPS dysfunction following status epilepticus, we utilized a UPS reporter mouse expressing modified GFP (Ub^{G76V}-GFP). Based on previous studies (*Engel et al.,2017; Marathe et al.,2015; Koeller et al.,2008*), we injected UbGFP mice with a 25 mg/kg intraperitoneal (i.p.) dosage of KA to elicit seizures in our laboratory. An equivalent volume of vehicle was administered i.p to control mice (0.9% saline). To curtail seizures and minimize morbidity and mortality, the anticonvulsant lorazepam (6 mg/kg) was delivered i.p. 40 min following KA or vehicle injection. 24h after the injection, mice were sacrificed, and we performed an immunofluorescence study using antibodies against GFP and P2X7R. Double-staining revealed a co-localization of GFP and P2X7R in the CA1 area of the hippocampus of UbGFP mice 24h after seizures, compared to control mice (**Figure 48**).

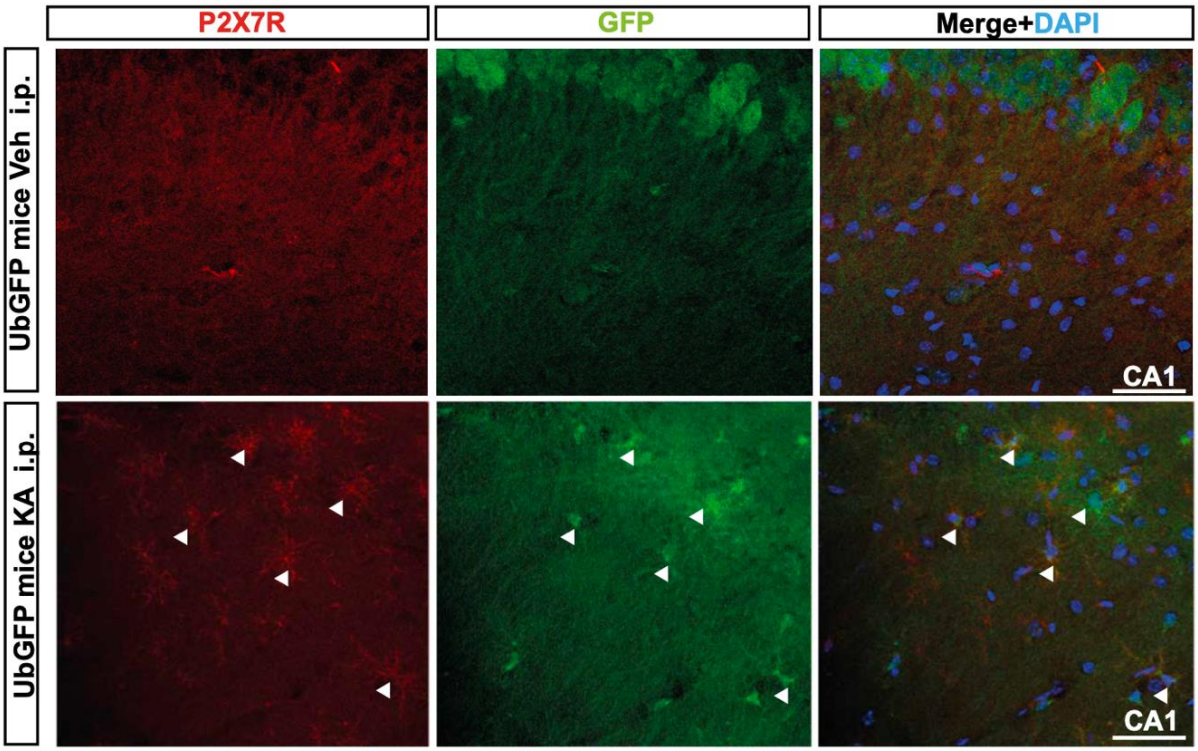

Figure 48: Cell-specific UPS impairment after status epilepticus Representative immunofluorescence images show co-localization between the neuronal GFP (green) and P2X7R (red) in the hippocampal subfield CA1, 24 h post-status epilepticus in Ub^{G76V}-GFP reporter mice. Scale bar: 50 µm.

Then, we looked at how much the absence of the P2X7 receptor affected UPS dysfunction following *status epilepticus*. To address this question, we generated a double transgenic mouse expressing the UPS reporter and lacking the P2X7 receptor (Ub^{G76V}-GFP / P2X7 -/-). The generation of said mouse is described in detail in section **1.6.2.4** of Materials and Methods. We induced *status epilepticus* in UbGFP and UbGFP; P2X7-/- by an intraperitoneal injection of KA (25mg/Kg) following the protocol already described. Hippocampal samples were collected 24h after seizures and we analyzed the number of cells showing a Ub^{G76V}-GFP reporter accumulation. Notably, in UbGFP; P2X7-/- the number of cells that presented an accumulation of reporter was significantly lower from that observed in UbGFP mice in the CA3 area of the hippocampus after KA injection (**Figure 49**).

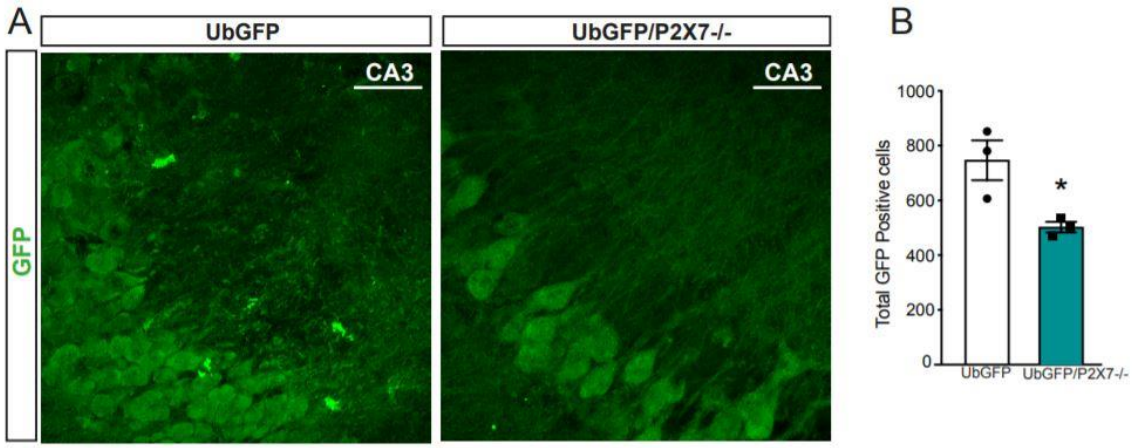

Figure 49: ***In vivo* administration of KA in Ub^{G76V}-GFP; P2X7 -/- mice produces a significant decrease in the number of cells accumulating the reporter** (A) Representative hippocampal CA3 images of the detection of the Ub^{G76V}-GFP reporter (green) by IF technique in Ub^{G76V}-GFP; P2X7-/- and Ub^{G76V}-GFP mice i.p injected with KA (25mg/Kg). Scale bar: 50 µm (B)The graph shows the quantification of the number of cells accumulating Ub^{G76V}-GFP in the hippocampus of Ub^{G76V}-GFP; P2X7-/- and Ub^{G76V}-GFP mice 24h after *status epilepticus* induced by KA(n=3 mice per genotype and n=3 sections per mouse). *p < 0.05 using *Unpaired t test* Data in bar graphs represent mean ± s.e.m.

Administration of KA to mice produces epileptic seizures resulting in neuronal damage in the hippocampus and other limbic structures. The CA (cornu ammonis) 1 subfield and the hilar region of the dentate gyrus are the most affected regions, but the CA3 subfield also frequently exhibits cell loss (*Engel & Henshall, 2009*). Since, as previously mentioned, P2X7R is involved in regulating cell death in neurons (*Sugiyama 2010; Ohishi 2016*), we decided to elucidate if the lack of the receptor could improve neuronal survival in the most affected area of the hippocampus after seizures induced by KA. We quantified the number of NeuN positive cells in CA1 area of hippocampus from UbGFP and UbGFP; P2X7-/- 24h after *status epilepticus* induced by i.p injection of KA. Our analysis showed that UbGFP, compared to UbGFP; P2X7-/- mice, present a significant reduction in the number of hippocampal neurons (**Figure 50**). These results seem to indicate that the P2X7R plays a crucial role in altering the balance of cellular proteostasis triggered by the administration of KA.

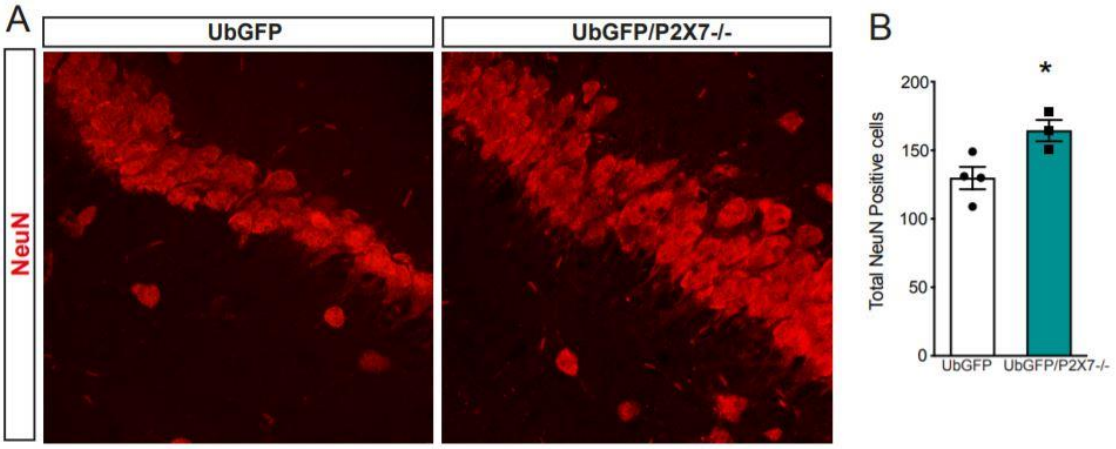

Figure 50: **The lack of the P2X7 receptor improves neuronal survival in UbGFP mice after *status epilepticus*.** (A) Representative immunofluorescence images of the hippocampal CA3 area stained with NeuN from Ub^{G76V}-GFP; P2X7-/- and Ub^{G76V}-GFP mice injected with KA(25mg/Kg, i.p) Scale bar = 50 µm (B) Graph represents the quantification of neuronal hippocampal cells identified as NeuN positive (n=3 mice per genotype and n=3 sections per mouse). *p < 0.05 using *Unpaired t test* Data in bar graphs represent mean ± s.e.m.

4.11 P2X7R may be considered a biomarker for Alzheimer's Disease

It has already been mentioned that P2X7R has been implicated in several disease conditions, ranging from infections (*Moreira-Souza et al.,2017*) to neurodegeneration (*Jimenez-Pacheco et al.,2016*), from autoimmune diseases (*Faliti et al.,2019*) to cancer (*De Marchi et al.,2019*). It is well known also that P2X7R is a potent stimulant of NLRP3 inflammasome activation and IL-1β release (*Orioli et al.,2017; Giuliani et al.,2017*). A recent study found that P2X7R is released into the circulation in both patients and healthy people, and that it is mostly associated with microvesicles/microparticles (*Giuliani et al.,2019*). Increased circulating levels of P2X7R might be associated with inflammation and could be a useful diagnostic or prognostic marker. In the second part of my project, we focused on the well-known relationship between P2X7R and Alzheimer's disease, intending to determine blood purines as a novel diagnostic for Alzheimer's disease. Plasma samples from control healthy subjects (CTL), Mild Cognitive Impairment (MCI), and Alzheimer's Disease (AD) patients were tested for the presence of P2X7R by ELISA. Results show that MCI and AD plasma samples present a higher P2X7R concentration ranging from 26,6 to 295,5 pg/mL compared to controls (**Figure 51**).

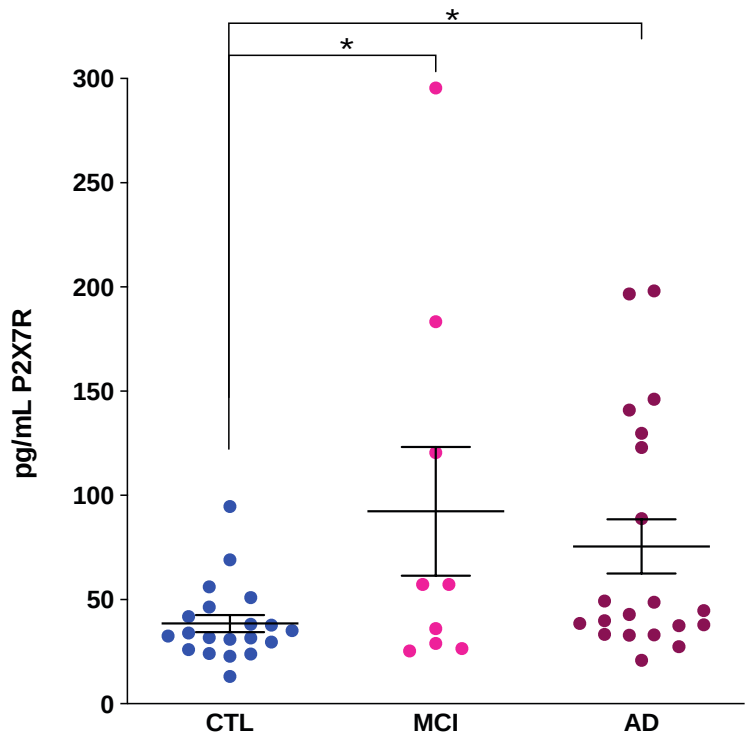

Figure 51: P2X7R plasma concentration is higher in AD and MCI patients compared to control subjects. ELISA analysis of plasma P2X7R levels from 19 healthy control subjects (CTL), 9 Mild Cognitive Impairment patients (MCI), and 18 Alzheimer's Disease patients (AD). *p < 0.05; Mann Whitney test between groups Data represent mean ± s.e.m.

The difference in plasma levels of P2X7R was more pronounced in female MCI compared to female control, rather than in male subjects (**Figure 52A**), whereas no difference was detected in AD patients comparing gender with control subjects (**Figure 52B**).

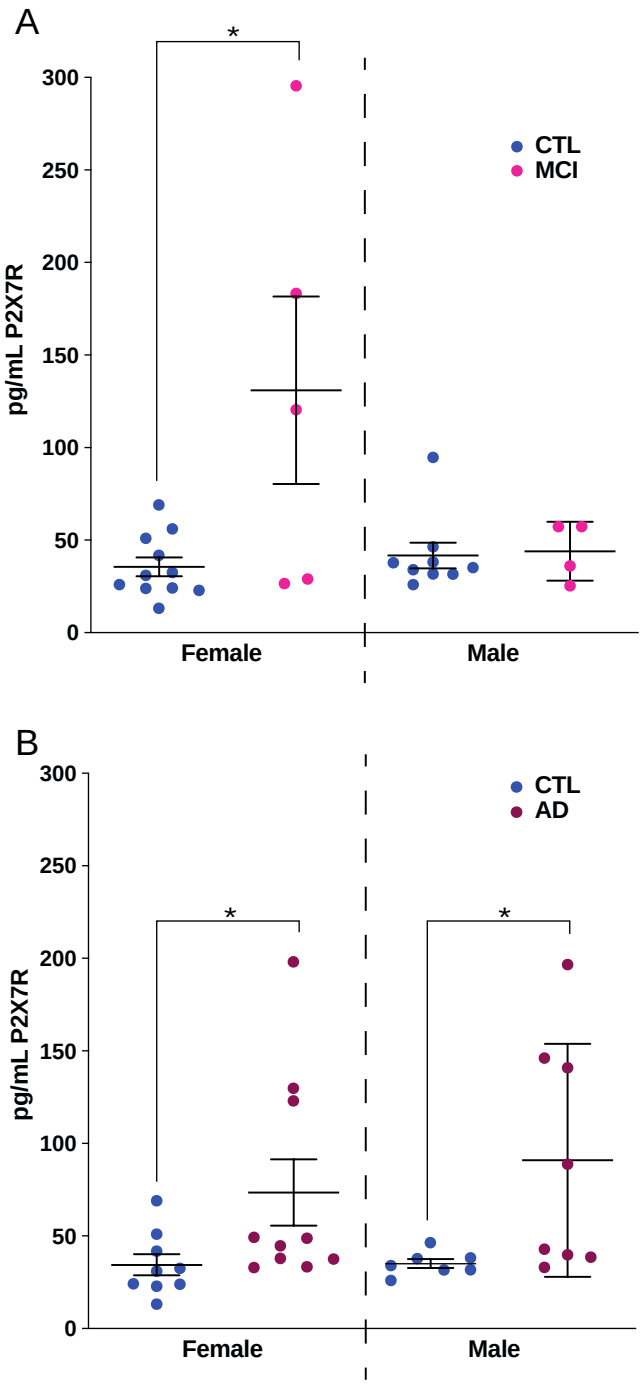

Figure 52: P2X7R plasma levels are higher in MCI females rather than in male subjects. ELISA measurements of the correlation between P2X7R concentrations and sex in CTL and MCI patients (A) and between CTL and AD(B). Data represent mean ± s.e.m. *p < 0.05 using the Mann Whitney test between groups

To further assess the strength of our finding, we performed a linear regression analysis between P2X7R plasma concentrations and age. Linear regression analysis showed no correlations between plasma P2X7R levels and age, neither in MCI nor in AD patients (**Figure 53**).

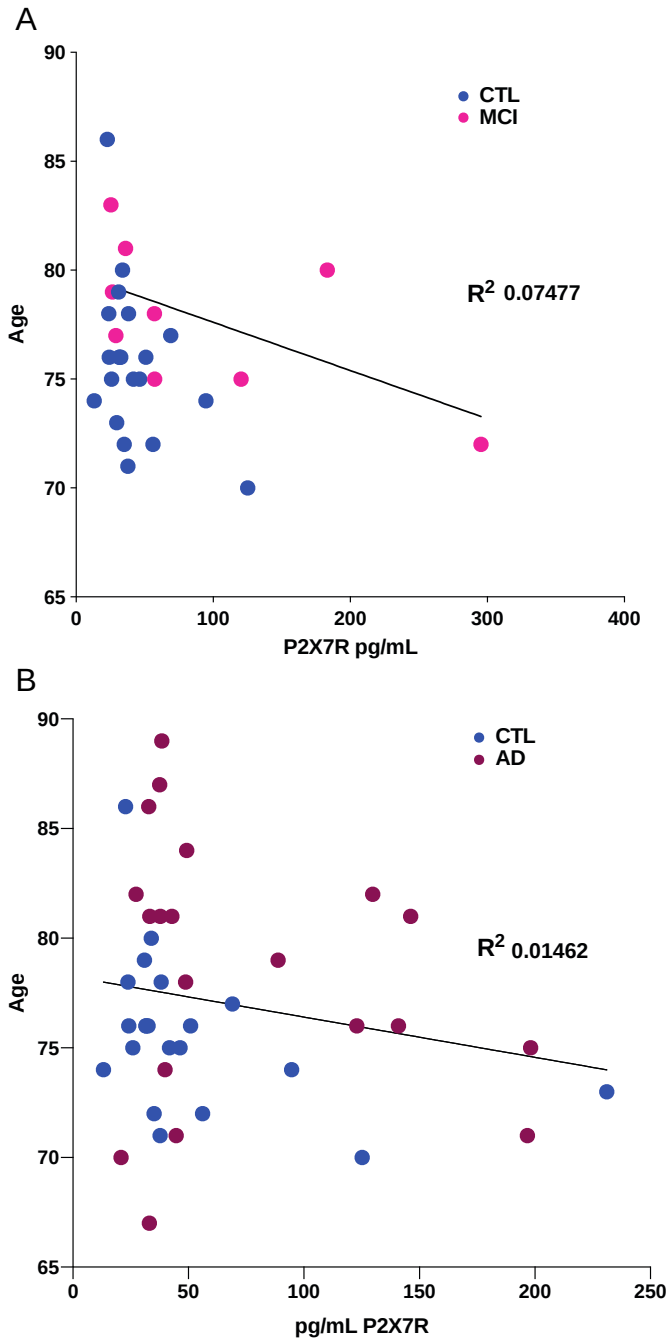

Figure 53: No correlation between age and P2X7R plasma levels (A)Linear regression analysis between age and P2X7R concentrations in plasma samples from CTL (blue) and MCI (pink) subjects. R^2= 0.07477 CTL=19; MCI=9 (B) Linear regression analysis between age and P2X7R concentrations in plasma samples from CTL (blue) and AD (violet) patients. R^2= 0.01462 CTL=20; AD=20

As a result of our partnership with Fundación CIEN in Madrid, we were able to obtain data that allowed us to do some regression analysis between P2X7R plasma levels and the Total Mini-Mental Status Examination (MMSETOT). In 2001, the American Academy of Neurology advised that individuals with MCI be tested and followed based on their risk of dementia development using general or short cognitive screening instruments (*Petersen et*

al.,2001). The Mini-Mental State Examination (*Folstein et al.,1975*), or MMSE, is a simple pen-and-paper test of cognitive function based on a total possible score of 30 points; it includes tests of orientation, concentration, attention, verbal memory, naming, and visuospatial skills. Linear regression analysis showed a strong negative correlation between MMSETOT and P2X7R levels, indicating that the more advanced is the impairment in MCI patients, the higher is the concentration of P2X7R in plasma (**Figure 54**).

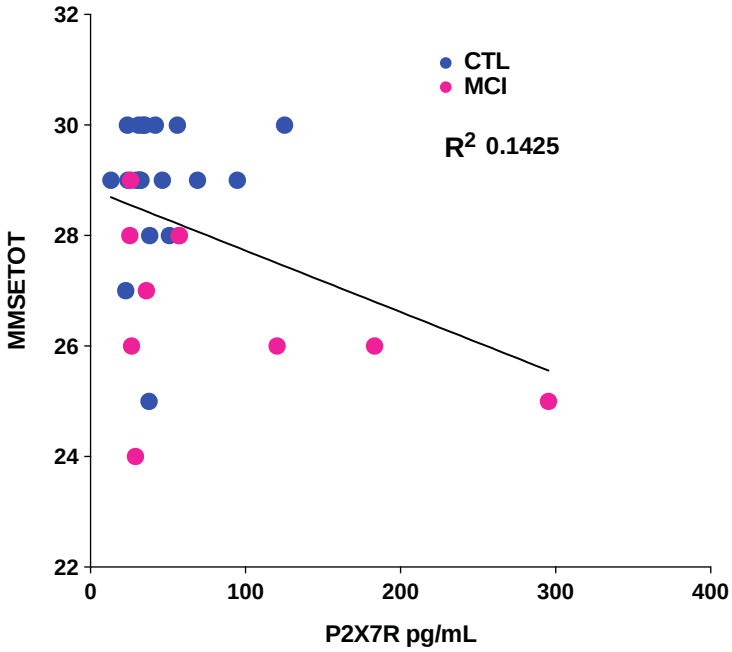

Figure 54: The high levels of P2X7R in blood from MCI patients correspond with a lower score on the MMSETOT(A)Linear regression analysis between P2X7R plasma levels and the Total Mini-Mental Status Examination (MMSETOT) in plasma samples from CTL (blue) and MCI (pink) subjects. R^2= 0.1425 CTL=16; MCI=9

Later, we conducted an additional longitudinal study to examine if P2X7R could be considered an early biomarker. The study's major goal was to compare the temporal trajectory of P2X7R in subjects with MCI and patients relative to healthy controls (HCs). In this longitudinal study, 12 participants (6 MCI patients and 6 control subjects) were enrolled. We examined plasma P2X7R levels in 6 HCs that remained stable after 5 years and 6 patients that began as controls but progressed to MCI after 5 years According to our findings, plasma P2X7R levels in MCI patients rose considerably with time (**Figure 55**). In agreement with our earlier findings, controls' P2X7R plasma levels were significantly lower than those of MCI patients after 5 years (**Figure 55**). Even if we cannot conclude that P2X7R is an early biomarker because there was no significant difference in P2X7R levels between controls and MCI patients within the first year, it would be interesting to continue this longitudinal study with more subjects.

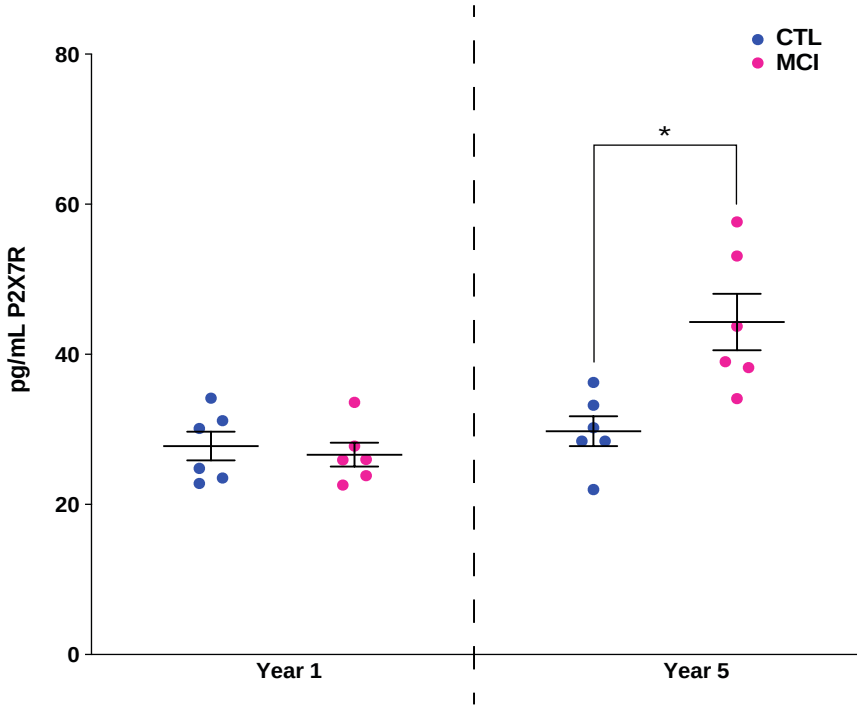

Figure 55: P2X7R plasma concentration is higher in MCI patients compared to control subjects in the longitudinal study. ELISA analysis of plasma P2X7R levels from 6 healthy control subjects (CTL) and 6 Mild Cognitive Impairment patients (MCI) in a longitudinal study. Data represent mean ± s.e.m. *$p < 0.05$; using Mann Whitney test between groups

5 Discussion

5.1 The P2X7 Receptor plays a key role in the alteration of cellular proteostasis by modulating UPS activity

In this Doctoral Thesis, using *in vitro* and *in vivo* approaches, we demonstrated that extracellular purines regulate via P2X7R the UPS activity in the central nervous system. The Neuro-2a murine neuroblastoma cell line (N2a) served as our *in vitro* model since it has been widely used as a neural model to investigate signaling pathways, secretory events, and neuronal differentiation, thus being a well-characterized system (*Gomez-Villafuerte et al., 2009; Gutierrez-Martin et al.,2011*). N2a cells have the advantage of constitutively expressing $P2Y_2$ and P2X7 receptors, which are the only members of the ionotropic family of purinergic receptors that are functionally expressed in N2a cells (*Gomez-Villafuerte et al., 2009; Wu et al., 2009*). The pharmacological studies carried out in N2a cells expressing UPS reporters, Ub^{G76V}-YFP or GFP-CL1, demonstrated that P2X7R activation by BzATP had a decremental effect on UPS activity. Importantly, we were able to verify that the increase in reporter levels was not due to translational or transcriptional alterations of the reporter proteins used. The involvement of P2X7R in the modulation of UPS activity was confirmed using pharmacological and molecular biology tools, including the overexpression or the selective knockdown of the receptor by specific shRNA.

Using specific fluorogenic substrates to assess proteasome activity, we demonstrated that P2X7R activation induced a significant decrease in the proteasome's catalytic activities CT-L and PG-L. This decrease in proteolytic activities was linked to a lower expression of the constitutive catalytic subunits β5 and β1 both at protein and mRNA levels. Remarkably, overexpression of *Psmb5* (β5) and *Psmb6* (β1) subunits prevented BzATP-induced UPS dysfunction in N2a cells, as evidenced by lower accumulation of the Ub^{G76V}-YFP reporter. In addition, the immunoprecipitation assay revealed that P2X7R activation decreased the amount of β5 catalytic subunits assembled into the 20S proteasome core particle. It is essential to note that, while there was a decrease in the expression of these subunits, there was no modification in the proteasomal biogenesis of the 20S core. These findings appear to suggest that *in vitro* activation of P2X7R by BzATP, decreases the expression and avoids the correct assembling of the catalytic subunits β5 and β1 in the 20S core particle, which causes

a decrease in the CT-L and PG-L activities of the proteasome, ultimately leading to no effective degradation of the reporter proteins.

Pharmacological investigations to determine the intracellular signaling route by which P2X7R changes the expression of the proteasome's catalytic subunits found that Akt and GSK3 kinases were implicated in this pathway, as previously described (*Diaz-Hernandez et al., 2012; Diaz-Hernandez et al, 2008*). Moreover, we discovered that P2X7R activation causes a reduction in the cytoplasmic levels of the transcription factor Nrf2, implicated in the regulation of the expression of several proteasomal subunits, thus protecting the cell from an accumulation of toxic proteins (*Pajares et al.,2017)*. Previous works have reported that GSK-3β, downstream effector of Akt kinase, governs the subcellular location of Nrf2. In particular, GSK-3β mediates the phosphorylation of Nrf2, excluding this transcription factor from the nucleus and so favouring its ubiquitination and consequent proteasomal degradation (*Salazar et al.,2006; Rojo et al.,2008; Rada et al.,2015*). Given this, it is reasonable to conclude that P2X7R activation, and therefore Ca^{2+} entrance into the cell surface, inhibits the PI3K/Akt pathway. When the PI3K/Akt signaling pathway fails, GSK-3β activity increases, which in turn phosphorylates Nrf2, promoting Nrf2 nuclear export and degradation by the proteasome. In our interest, Nrf2 has been involved in the modulation of *Psmb5* (β5) subunit expression (*Kwak & Kensler, 2006; Pajares et al.,2017*). As a result, we may hypothesize that missed Nrf2 nuclear translocation leads to reduced β5 subunit expression and decreased proteasomal activity (**Figure 56**).

Regarding Nrf2, there is a correlation between the reduction of Nrf2 and *Psmb5* (β5) levels in the midbrain of a Parkinson's disease mouse model (*Yang et al.,2007*). Additionally, the natural compound sulforaphane (SFN) gives a more robust image of Nrf2 as a crucial modulator of the UPS. *In vitro* experiments with murine neuroblastoma Neuro2A cells revealed increased production of the proteasome's catalytic subunits as well as its peptidase activity in response to SFN (*Kwack et al.,2007*). Moreover, it was shown that SFN stimulation of Nrf2 increases proteasomal activity in a UPS reporter mouse, increasing significantly the breakdown of the mutant huntingtin protein in Huntington disease-derived cells and decreasing its associated cytotoxicity (*Liu et al, 2014*).

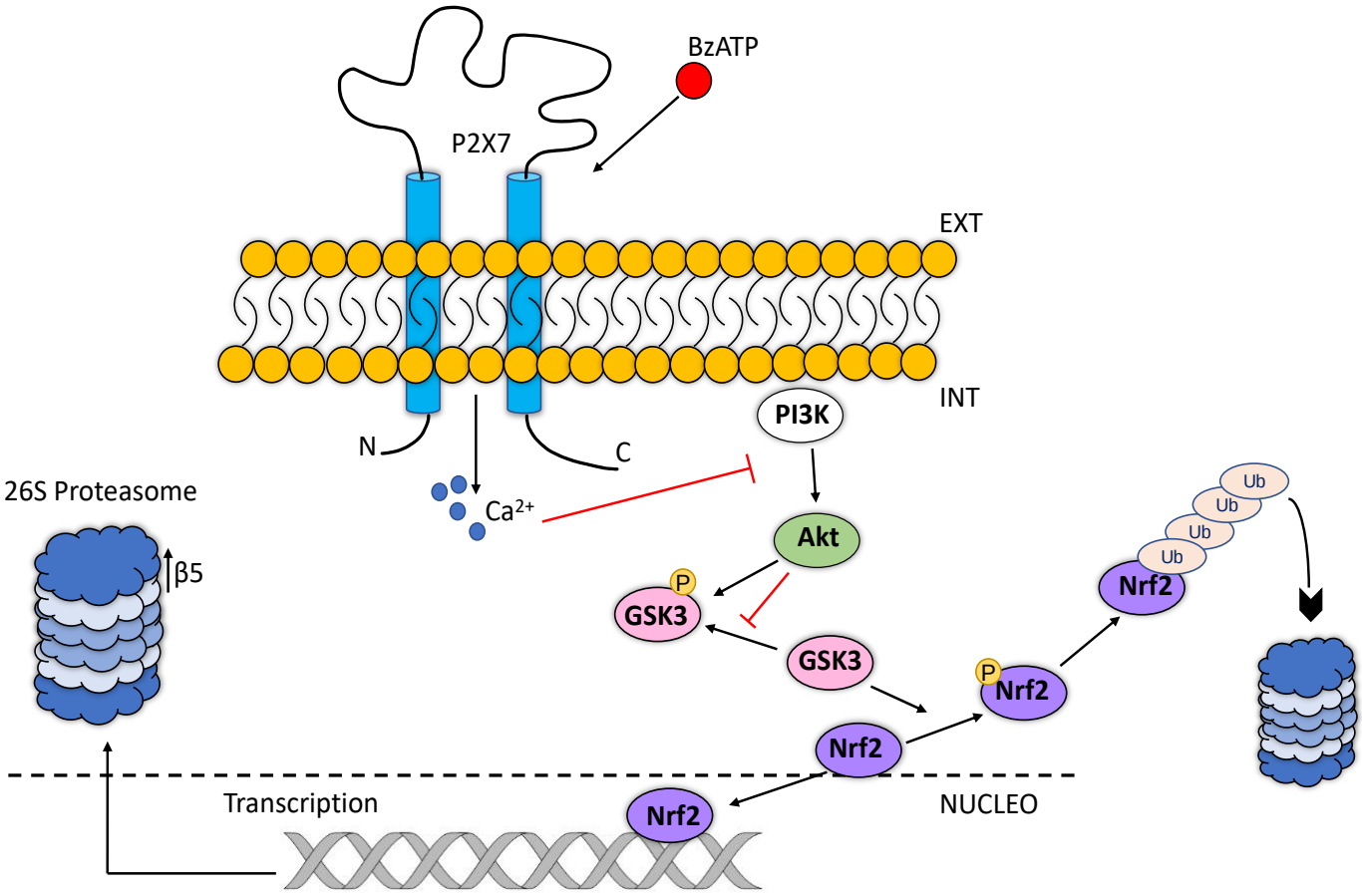

Figure 56: Schematic representation of the possible P2X7 receptor's signaling pathway through which it regulates UPS activity.

In line with the results obtained in our cellular model, *in vivo* intracerebroventricular (I.c.v) administration of BzATP induced a significant decrease in CT-L and PG-L activities in the hippocampus of wild-type (WT) mice. Moreover, this decrease was accompanied by a diminishing at protein and mRNA expression of the proteasomes' catalytic subunits β5 and β1. The absence of any change in the proteasome peptidase activities or the expression of the constitutive subunits in the hippocampus of P2X7 -/- mice i.c.v injected with BzATP, strongly supported the involvement of P2X7R in the control of UPS activity. Based on our previous results in N2a cells, we were able to identify that the Akt/GSK/Nrf2 pathway is implicated in the modulatory impact of P2X7R on UPS activity even *in vivo*.

Various studies have postulated that P2X7R may play a role in different pathological conditions such as neuroinflammation, oxidative stress, and neurodegenerative diseases (*Sebastian-Serrano et al.,2019'*). It is noteworthy that in these pathological conditions there is also an imbalance of cellular proteostasis by altering the proteasome activity (*Dantuma & Lindsten 2010; Glickman and Raveh,2005*). To evaluate the effect of P2X7R activation induced by BzATP on UPS activity, we used UPS reporter mice (Ub^{G76V}-GFP mice, also called mice U1), which allow detection of proteasome alterations at the cellular level (*Hernandez et al.,2004, Lindsten et al.,2003*). In this mouse line, the proteolytic activity of the proteasome must be decreased by up to 80% in order to detect the accumulation of the reporter Ub^{G76V}-GFP (*Dantuma et al.,2000, Kessler et al.,2003*). As a result, we saw that the accumulation of the reporter caused by BzATP was due to a substantial reduction in the catalytic activity of the proteasome in neuronal and microglia cells.

Previous research has indicated that neuroinflammation affects UPS via decreasing proteasomal activity, which is consistent with our findings (*de Diego Garcia et al., 2018; Jamart et al., 2014; Pintado et al., 2012*). Our laboratory previously reported that extracellular nucleotides, through the $P2Y_2$ receptor, can modulate the UPS dysfunction associated with neuroinflammation (*de Diego Garcia et al., 2018).* Particularly, $P2Y_2$ receptor activation can enhance CT-L and PG-L proteasome activities by increasing the expression of their catalytic subunits β5 and β1 (*de Diego Garcia et al., 2018; de Diego-Garcia et al., 2017*). The activation of Src, PI3K, and ERK is the intracellular signaling mechanism driving these alterations (*de Diego-Garcia et al., 2017*). Furthermore, $P2Y_2R$ activation reversed proteasomal dysfunction associated with LPS-induced neuroinflammation in astrocytes (*de Diego Garcia et al., 2018; de Diego-Garcia et al., 2017*). While $P2Y_2R$ activation promotes the expression of the proteasomal β5 subunit in astrocytes via a mechanism reliant on the Src/ERK pathway, P2X7R activation harms neurons and microglia cells by reducing proteasome activity. These findings suggest that UPS can be controlled by two distinct nucleotide receptors, each of which has opposing effects on UPS activity and in different cellular lineages. This is not the first time that these receptors have been shown to work antagonistically; previously, it was revealed that P2X7 and $P2Y_2$ receptors had opposite effects on α-secretase activity (*Leon-Otegui et al, 2011*). While $P2Y_2R$ has been associated with neuroprotective benefits, with lower expression levels in AD patients (Lai et al, 2008), P2X7R is increased in AD patients, particularly in microglial cells around senile plaques (*McLarnon et al., 2006; Parvathenani et al., 2003*).

Furthermore, whereas $P2Y_2$ haploinsufficiency increases plaque formation and enhances Aβ levels in the hippocampus of an amyloid mouse model (*Ajit et al, 2014*), P2X7R inhibition or genetic depletion reduces the number and size of amyloid plaques by reducing Aβ levels (*Diaz-Hernandez et al, 2012; Martin et al., 2019*).

5.2 The P2X7 Receptor is implicated in the UPS impairment associated with Alzheimer's disease, and blocking it may prevent the UPS impairment-induced cellular death

The integrity of the proteostasis network is critical for cell survival and is especially crucial in nonproliferative cells, notably neurons, whose failure leads to a variety of neurodegenerative disorders that are becoming more prevalent in the rising older population. Among these pathologies, Alzheimer's disease (AD) is one of the most prevalent disorders characterized by the accumulation of misfolded proteins (*Höhn et al.,2020*). AD is a chronic neurodegenerative disorder characterized by a progressive loss of cognitive functions, being the most prevalent and common type of dementia in our society. Neuropathologically, AD is characterized by a progressive accumulation in the extracellular space of senile plaques (constituted by the neurotoxic Aβ peptide) and intracellular neurofibrillary tangles (formed by the accumulation of hyperphosphorylated tau protein) (*DeTure and Dickson,2019*). The discovery of early Aβ deposition in numerous cortical regions 10-12 years before the first AD symptoms occurred (*Benzinger et al, 2013*) implies that Aβ accumulation is the initial event that triggers AD, followed by sequential tau accumulation (*Bateman et al, 2012; Hanseeuw et al, 2019*). It has been shown that Aβ can be degraded by the proteasome in cultured neurons and astrocytes, and treatment with the proteasome inhibitor lactacystin decreases the viability of cells exposed to Aβ (*Lopez Salon et al., 2003*). Moreover, postmortem tissue studies have shown an interesting region-specific reduction in proteasome activity in AD patients (*Keller et al. 2000; Keck et al.,2003*) and accumulation of ubiquitinated proteins (*de Vrij et al., 2004; Ciechanover and Kwon, 2015*). Other studies in cultured neuroblastoma cells show that unfolded tau is normally destroyed by the proteasome without prior ubiquitination, and induction of conformational changes in tau by treatment with SDS prevents its degradation (*David et al.,2002*). Taken together, several lines of evidence point to a reduced UPS function in AD and suggest that both Aβ and tau are important players in the game. However, it is unclear why the proteasome fails, whether a

decrease in UPS activity causes Aβ accumulation thus promoting the formation of amyloid plaques, or whether structural changes in Aβ or tau induce proteasome inhibition. As in other neurodegenerative diseases, AD has been associated with the prolonged maintenance of a state of neuroinflammation. Neuroinflammation, as previously stated, impacts UPS by reducing proteasomal activity (*de Diego Garcia et al., 2018; Jamart et al., 2014; Pintado et al., 2012*), increasing extracellular ATP levels (*Di Virgilio et al.,2020*), and increasing P2X7R expression (*Martinez-Frailes et al.,2019*). We have previously established that activating P2X7R *in vivo*, which requires high extracellular levels of ATP, induces UPS failure and affects cellular proteostasis in a neuroinflammatory environment as occurs in AD. Therefore, we sought to explore if P2X7R activation may have contributed to the AD-related UPS malfunction. Studies on post-mortem hippocampi from AD patients and age- and sex-matched controls revealed that AD patients had higher levels of polyubiquitinated proteins. As previously reported (*Keller et al., 2000; Lopez Salon et al., 2000*), CT-L activity was lower in AD brains than in the control individuals. In accordance with previously published data (*Keller et al., 2000*), we found a significant decrease both at the protein and mRNA level for the β5 subunit in AD patients. Interestingly, even though we discovered a small drop in PG-L activity, this was not followed by a decrease in β1 subunit protein levels. It is to note that we found no impairment at the protein level in any of the immunoproteasome (β5i and β1i) subunits in AD patients. Though this conclusion is consistent with our previous findings, it contradicts previous studies that reported increased immunoproteasome protein levels in AD brains (*Mishto et al., 2006; Nijholt et al., 2011; Orre et al.,2013*). It has been proposed that the stimulation of the immunoproteasome might 'hijack' the action of the constitutive proteasome, resulting in its malfunction (*Nguyen et al., 2010*). However, because we are evaluating immunoproteasome levels throughout the whole cellular population, it is still possible that the immunoproteasome is increased in certain cell types, such as neurons. To completely comprehend the connection between immunoproteasome, neuroinflammation, and AD, further research will undoubtedly be required.

The microtubule-associated protein tau is a crucial factor in the development of Alzheimer's disease (*Wang and Mandelkow,2012*). Any disruption of the UPS may block tau protein degradation and, in conjunction with hyperphosphorylation, increase tau tangle development (*Brunello et al.,2019*). To confirm the relevance of P2X7R in AD-related UPS impairment, we investigated the effects of pharmacological inhibition of this receptor in

P301S mice, a well-characterized AD mouse model (*Yoshiyama et al., 2007*). These mice express 5-fold more tau protein and show an onset of phospho-tau pathology at around 6 months of age in the absence of amyloid-β plaques (*Yoshiyama et al., 2007*). As we previously observed in AD pathology, our western blots and immunohistochemical analysis revealed that hippocampi of 9-months-old heterozygous P301S mice display a substantial accumulation of polyubiquitin conjugates. Moreover, P301S mice showed a decrease in CT-L and PG-L activities, which was related to lower protein expression of the constitutive catalytic subunits β5 and β1 and reduced mRNA levels of *psmb5 (*β5) and β1 and *pmsb6* (β1) compared to WT mice. In P301S mice, pharmacological P2X7R blockage induced by the selective antagonist GSK 1482160A (GSK, 100 mg/kg, i.p) reversed those proteasomal changes, enhancing CT-L and PG-L activities and restoring protein and messenger levels of the constitutive proteasomal subunits β5 and β1. Those findings are consistent with earlier research, which found that *in vivo* pharmacological blockage or genetic deletion of P2X7 improves both symptomatology and neuropathology in several APP mice models (*Chen et al., 2014; Diaz-Hernandez et al., 2012; Martin et al., 2019; Ryu and McLarnon, 2008*). It is widely recognized that a disruption in proteasome activity, in addition to causing abnormal protein aggregation or accumulation, eventually results in cell death (*Goldberg, 2003; Sherman & Goldberg, 2001*). It has previously been observed that P301S mice had fewer neurons crossing the pyramidal cell layer of the hippocampus CA3 area (*Yoshiyama et al., 2007*). The generation of a double transgenic mouse, Ub^{G76V}-GFP;P301S (expressing the UPS reporter and a mutant form of human microtubule-associated protein tau) allowed us to see that the CA3 area of the hippocampus is the most sensible to tau-induced UPS impairment and cellular death. In line with previous findings that P2X7 inhibition reduces neuronal death associated with various neurodegenerative diseases, including Huntington disease (HD) and AD (*Diaz-Hernandez et al., 2012; Diaz-Hernandez et al., 2009*), we discovered that *in vivo* P2X7R blockade decreased cellular death associated with P301S mice. Furthermore, we observed that inhibiting P2X7R with A43 recovers cellular death induced by the proteasome inhibitor MG132 in N2a cells. Overall, our results show that P2X7 blockage reverted UPS impairment associated with Tauopathies and improved neuronal survival, adding credence to targeting P2X7R as a potential treatment approach.

A century after Dr. Alzheimer discovered tangles in a patient's brain for the first time, we still lack a preventative or therapeutic therapy for AD that may successfully lower the risk

or postpone the clinical phase of the condition. It is to be mentioned that the US Food and Drug Administration (FDA) recently authorized Aducanumab (Biogen), a human monoclonal antibody that increases clearance of beta-amyloid plaques from the brain (*Sevigny et al.,2016; Selkoe., 2019; Mullard., 2021*), for patients with early AD, providing new hope for the future. Rather than this, a proposed treatment method for reducing protein aggregates, which are common in AD, is to target the UPS system to accelerate the breakdown of such aggregates. Numerous ubiquitination enzymes are potential targets for AD treatments since they regulate not just Aβ metabolism but also the UPS in Alzheimer's brains. E2-25K is one of the unique Ub-conjugating enzymes that is elevated and contributes to Aβ toxicity (*Song et al.,2008*). Moreover, Lonskaya et al. discovered that the Ub E3 ligase parkin could repair intracellular Aβ accumulation and impair proteasome function (*Lonskaya et al. 2013a; Lonskaya et al. 2013b*). Furthermore, parkin has the capability to defend neurons against diverse insults, which leads to the prevention of AD. DUBs can also be potential targets when a specific role for these enzymes in AD is recognized. Small chemical control of particular DUBs enhances deubiquitylation of poly-Ub chains of mutant UBB + 1, which may be an additional possibility because these chains inhibit proteasomes in AD brains (*Lam et al. 2000*). Even though most of the therapeutic strategies developed are designed to remove and reduce Aβ from the brain, such techniques should be approached with caution since, in addition to its presence in neuritic plaques, Aβ has a broad distribution throughout the brain and body, even in cognitively normal persons. Instead of being a toxic peptide, evidence suggests that soluble Aβ serves a variety of physiological functions, including synaptic function modulation (*Kamenetz et al.,2003; Ikegaya et al., 2002*), facilitation of neuronal growth and survival (*Wujek et al.,1996; DeWitt et al.,1996; Bishop et al.,2003)* and protection against oxidative stress (*Kontush et al.,2001*). These physiological processes must be considered while developing methods to decrease Aβ burden in Alzheimer's disease.

Because abnormal protein accumulation and proteasome suppression are common features of AD and other neurodegenerative diseases, improving proteasome activity with the use of small molecules may be an efficient way to remove the accumulations that aggregate in the AD brain (*Upadhya and Hegde 2005*). A synthetic peptide known as proteasome-activating peptide 1 (PAP1) has been shown to enhance CT-L proteasome activity via a gate opening mechanism in the 20S core particle's ring (*Dal Vechio et al., 2014*). Moreover, the phosphorylation of the proteasome Rpt6 subunit by cAMP-dependent protein

kinase A (PKA) has been reported to upregulate 26S assembly and proteasome activity *in vitro* and *in vivo (Zhang et al.,2007; Asai et al.,2009)*. Rolipram, a small molecule inhibitor of phosphodiesterase type 4 (PDE4) (*Park et al.,2012*) was found to raise the level of cAMP in the brain of mice, activated PKA and presumably increased proteasome activity through subunit (Rpt6) phosphorylation. Most importantly, rolipram promoted the clearance of abnormal tau and improved cognition in mouse model of AD (*Myeku et al.,2016*). Pioneer work involving upregulation of proteasome activity through genetic manipulation were first illustrated by Gaczynska et al. (*Gaczynska et al.,1994; Gaczynska et al.,1996*). In these studies, overexpression of β5i (LMP7) subunit in lymphoblasts and HeLa cells resulted in increased CT-L and T-L activities, while overexpression of β1i (LMP2) only elevated the T-L like activity. Therefore, increasing the activity of the immunoproteasome could also increase antigen processing thereby boosting immune response. A recent study also revealed that activation of Nrf2 with antioxidant 3H-1,2-dithiole-3-thione (D3T) significantly improved cognitive deficits in a mouse model of AD and dramatically reduced the level of insoluble Aβ, as well as oxidative stress (*Cui et al.,2018*). Here, we believe that the selective inhibition of P2X7 receptor might have a therapeutic impact, helping to regulate the UPS impairment associated with neurogenerative disease in the early stages, restoring cellular proteostasis. Taking into account all of the aforementioned factors concerning the function of P2X7R in AD, it is reinforced that blocking the P2X7 receptor might be a suitable therapeutic strategy for the treatment of neurogenerative diseases.

5.3 The P2X7 Receptor appears to control UPS malfunction during *status epilepticus*

Epilepsy is a complicated set of chronic neurological disorders defined by recurring seizures, with a 1% prevalence and approximately 60 million individuals globally suffering from the condition (*Moshé et al., 2015*). P2X7R has been proposed as a novel drug target for a host of neurological conditions, including epilepsy (*Engel et al., 2016; Rassendren and Audinat, 2016*). Changes in P2X7 expression have been seen in both experimental mouse models and humans following prolonged seizures (*status epilepticus*). In the intra-amygdala (i.a.) KA mouse model of epilepsy, P2X7 protein levels were shown to be increased in the hippocampus and cortex (*Jimenez-Pacheco et al., 2013; Jimenez-Pacheco et al., 2016*). Furthermore, Jimenez-Pacheco et al. discovered increased P2X7R levels in the surgically

resected hippocampus and neocortex of drug-resistant individuals with temporal lobe epilepsy (TLE) (*Jimenez-Pacheco et al., 2016*). Thanks to the use of transgenic *P2rx7*-GFP reporter mice, Engel et al., described cell-specific patterns of *P2rx7* transcription during epilepsy (*Engel et al., 2012; Jimenez-Pacheco et al., 2016*). In the i.a. KA model, cortical and hippocampal *P2rx7* induction was mainly restricted to neurons and microglia; GFP was most prominent in the hippocampal subfield CA1 followed by the dentate gyrus and subfield CA3 (*Jimenez-Pacheco et al., 2016*). Epilepsy and seizures are potentially derived from neuronal injury and/or loss, synaptic remodeling (mossy fiber sprouting), brain blood barrier collapse, lymphocyte buildup, and angiogenesis, which are all linked with an increase in P2X7 activity (*Friedman and Dingledine, 2011; Sperlágh and Illes, 2014*). Previous studies have reported that UPS function is altered in epilepsy both *in vitro* and *in vivo* (*Caldeira et al.,2013; Engel et al.,2017*). Notably, seizures are a common co-morbidity of many neurodegenerative disorders, including Alzheimer's and Huntington's (*Vossel et al.,2013; Cepeda-Prado et al.,2012*), and may contribute to the accumulation of polyubiquitinated aggregates during these disorders. As previously stated, P2X7R levels are altered during seizures, and since we previously established P2X7R's modulatory role in UPS impairment AD-associated, we wanted to explore if the receptor was also involved in the UPS's regulating activity following seizures. Accordingly to a previous study, in this Doctoral thesis, we demonstrate an increased accumulation of polyubiquitinated proteins in the hippocampus after prolonged seizures, suggesting a seizure-induced inhibition of the UPS (*Engel et al.,2017*). Importantly, after *status epilepticus*, we observed a substantial decrease in the accumulation of those polyubiquitin conjugates and an increase in CT-L activity in the hippocampus of P2X7 -/- mice, validating our theory about the role of P2X7R in the regulation of UPS activity after KA-induced seizures. Later, we demonstrated that there is an impairment in UPS function following protracted seizures caused by an intraperitoneal injection of KA using UPS green-fluorescent protein (GFP)-reporter mice, corroborating previous findings (*Engel et al.,2017*). Furthermore, we discovered that protracted seizures disrupt the UPS in cells expressing P2X7R, notably in the CA1 subfield of the hippocampus, which is one of the areas most affected following KA injection (*Mouri et al.,2008*). The generation of double transgenic mice expressing the UPS reporter and lacking the P2X7 receptor (Ub^{G76V}-GFP / P2X7 -/-) similarly resulted in a significant decrease in cells displaying UPS failure following status epilepticus induced by an intraperitoneal KA injection (25mg/Kg). Moreover, those mice showed an

improvement in neuronal survival, particularly in the hippocampal subfield CA1, a brain region vulnerable to ischemia-induced cell death (*Bartsch et al.,2015*). Overall, our findings indicate that P2X7R plays an essential role in altering the balance of cellular proteostasis produced by KA injection, lending credence to the notion that inhibiting or depleting P2X7R may be therapeutic. Engel et al. produced one of the first evidence for P2X7R antagonist's antiepileptogenic ability, demonstrating that targeting P2X7R might be useful as supplementary treatment, boosting the seizure-suppressive effects of lorazepam (*Engel et al.,2012).* Later, in a rat epileptogenic model, another research found that treatment with the selective antagonist BBG significantly reduced the mean kindling score and restored behavioral deficits such as cognition and motor coordination (*Soni et al., 2015*). Furthermore, employing JNJ-47965567 and AFC-5128, two powerful and brain-permeable P2X7 antagonists, dramatically delayed kindling development in the same rat model. This effect lasted a long time, but the drugs couldn't prevent or reverse epileptogenesis (*Fischer et al., 2016*). Importantly, another study found that increasing P2X7R expression with a particular microRNA (microRNA-22) resulted in a more severe epileptic phenotype (*Jimenez-Mateos et al., 2015*). A recent study discovered that, rather than reducing seizures, the P2X7 antagonist JNJ-42253432 induces a shift in seizure severity, resulting in a milder epileptic phenotype (*Amhaoul et al., 2016*). The most impressive findings, however, were achieved by Jimenez-Pacheco et al, who found that treatment with JNJ-47965567 therapy reduced the overall frequency of epileptic episodes by more than 50% while the severity of the seizures remained unchanged (*Jimenez-Pacheco et al., 2016*). Despite the fact that it is still unclear whether blocking P2X7 has an anticonvulsive or neuroprotective effect, and that it appears to depend on the type of epileptic mouse models used, further research will be worthwhile in order to solve unanswered questions and accelerate progress toward possible future clinical use.

5.4 P2X7 Receptor is released into the circulation and might be considered as a blood biomarker for Alzheimer's patients

In the last part of this Doctoral Thesis, we evaluated the possibility of considering blood purines, in particular the P2X7 receptor, as a novel diagnostic for AD. A biomarker, in broad terms, is a quantifiable indicator of a biological state or pathological condition. In AD, there

have been validated cerebrospinal fluid (CSF) biomarkers for Aβ plaques, as well as neuronal tau mis metabolism that is associated with neurodegeneration and tangle pathology (increased secretion of both total tau [T-tau] and phosphorylated tau [P-tau] into the CSF) (*Zetterberg, 2017*). Interestingly, these biomarkers are 85–95% sensitive and specific for AD in both mild cognitive impairment (MCI) and dementia stages of the disease (*Zetterberg.,2019*). Although CSF biomarkers have been included in the clinical criteria for AD (*Dubois et al., 2014*), CSF sampling (through lumbar puncture) is a very invasive technique. Undoubtedly, a blood biomarker would be simpler. It has proven challenging to develop reliable blood biomarkers for Aβ pathology. Although Aβ proteins may be detected in plasma, their relationship with cerebral amyloidosis is either nonexistent or poor when the latter is evaluated immunochemically (*Olsson et al., 2016*). Plasma Aβ concentrations are most likely regulated by platelet and other extracerebral tissue releases (*Zetterberg, 2015*). Plasma tau has also emerged as a potential blood test for AD diagnosis, with some research focusing on tau quantification in AD, Mild Cognitive Impairment (MCI), and normal groups. They discovered increased t-tau levels in plasma from AD patients compared to control or MCI patients, but no difference was detected between MCI patients who developed AD and stable MCI patients (*Zetterberg et al.,2013*). As previously mentioned, P2X7R is an extracellular ATP-activated key inflammatory receptor that is abundantly expressed by immune and tumor cells (*Di Virgilio et al.,2017; Di Virgilio et al.,2018b)*. In the immune system, the P2X7R has been shown to drive NLRP3 inflammasome activation and IL-1β release (*Orioli et al.,2017; Giuliani et al.,2017*). Furthermore, a recent study found that P2X7R is released in the blood in conjunction with microvesicles/microparticles, and its levels are higher in diseased subjects (*Giuliani et al.,2019*). Based on the well-known relationship between P2X7R, AD, and inflammation, we hypothesized that P2X7R levels might be utilized as a blood biomarker for AD. According to ELISA measurements, MCI and AD plasma samples had higher P2X7R concentrations ranging from 26,6 to 295,5 pg/mL compared to control subjects. The difference in plasma levels of P2X7R was more pronounced in female MCI participants compared to female controls than in male subjects. We do not know what the reasons for the observed differences are. It could be related to the fact that inflammation is a risk factor for AD that differs by gender (*Hanamsagar et al.,2016*), with inflammatory dysregulation being stronger in females (*Hall., et al 2013*). We also discovered a link between high levels of P2X7R in the blood and the Total Mini-Mental Status Examination (MMSETOT), which is used to

assess the risk of dementia development in MCI patients, indicating that P2X7R might be a specific disease biomarker. The longitudinal research that we conducted on MCI and control subjects was inconclusive since the MCI group did not demonstrate any significant difference in P2X7R plasma levels at the start of the trial compared to control individuals. The fact that the sample size was very small, even though the cohort was adequately defined as an MCI cohort, most likely influenced the generalizability of the findings. In conclusion, we have shown that the P2X7R is released into the blood and that its levels are significantly higher in MCI and AD patients. Although blood P2X7R levels are not utilized in laboratory practice, they may be useful in identifying an inflammatory state, which is frequent in many neurodegenerative disorders. P2X7R data might be coupled with measurements of other inflammatory players, such as IL-1β, to supplement and reinforce the diagnostic relevance of inflammatory diseases (*Giuliani et al.,2019*). Furthermore, such biomarkers could be employed in longitudinal studies to track the temporal development of different pathologies during neurodegenerative disease progression and to assess how their interactions lead to clinical symptoms.

Conclusions

The following conclusions arose from the experiments conducted in this doctoral thesis to evaluate the role of the P2X7 receptor in UPS impairment associated with neurological diseases and the potential of blocking it as a novel treatment strategy:

- Overall, our findings imply that P2X7R activation impairs UPS both *in vitro* and *in vivo*, by decreasing the expression and avoiding the correct assembly of the 20S proteasome catalytic subunits β5 and β1 through the signaling pathway Akt/GSK3/Nrf2.
- The activation of P2X7 receptor plays a key role in the alteration of cellular proteostasis, particularly in neurons and microglia cells.
- *In vivo* pharmacological blockage of P2X7R reverses proteasomal alterations in a Tauopathy mouse model, and it may prevent UPS impairment-induced cellular death.
- P2X7R is critical in changing the equilibrium of cellular proteostasis following prolonged seizures that disrupt the UPS.
- P2X7R might be a promising target for a potential therapeutic approach to treat neuroinflammatory diseases associated with UPS dysfunction, such as Alzheimer's disease and Epilepsy.
- P2X7 Receptor plasma levels are considerably higher in MCI and AD patients, implying that P2X7R measurement might be used as a diagnostic biomarker to follow the course of neurodegenerative disease.

Conclusiones

Las siguientes conclusiones surgieron de los experimentos llevados a cabo en la presente tesis doctoral para evaluar el papel del receptor P2X7 en el deterioro del UPS asociado a enfermedades neurológicas y al potencial de bloqueo de dicho receptor como una nueva estrategia de tratamiento:

- En general, la activación de P2X7R regula la actividad del UPS tanto *in vitro* como *in vivo*, disminuyendo la expresión y el correcto ensamblaje de las subunidades catalíticas del proteasoma β5 y β1 a través de la ruta de señalización Akt/GSK3/Nrf2.
- La activación del receptor P2X7 juega un papel clave en la alteración de la proteostasis celular, particularmente en neuronas y células microgliales.
- El bloqueo farmacológico de P2X7R *in vivo* revierte las alteraciones proteasómicas en un modelo de ratón de tauopatía y puede prevenir la muerte celular inducida por el deterioro del UPS.
- P2X7R es fundamental para cambiar el equilibrio de la proteostasis celular después de convulsiones prolongadas que desregulan el UPS.
- P2X7R podría ser un objetivo viable para una nueva estrategia terapéutica para tratar los trastornos neuroinflamatorios relacionados con la disfunción del UPS, como la enfermedad de Alzheimer y epilepsia.
- Los niveles plasmáticos del receptor P2X7 son considerablemente más altos en pacientes con DCL y EA, lo que implica que la medición de P2X7R podría usarse como un biomarcador de diagnóstico para seguir el curso de las enfermedades neurodegenerativas.

Bibliography

Abdi, M. H., P. J. Beswick, A. Billinton, L. J. Chambers, A. Charlton, S. D. Collins, K. L. Collis, D. K. Dean, E. Fonfria, R. J. Gleave, C. L. Lejeune, D. G. Livermore, S. J. Medhurst, A. D. Michel, A. P. Moses, L. Page, S. Patel, S. A. Roman, S. Senger, B. Slingsby, J. G. Steadman, A. J. Stevens, and D. S. Walter. "Discovery and Structure-Activity Relationships of a Series of Pyroglutamic Acid Amide Antagonists of the P2x7 Receptor." *Bioorg Med Chem Lett* 20, no. 17 (Sep 1 2010): 5080-4. https://dx.doi.org/10.1016/j.bmcl.2010.07.033.

Adinolfi, E., M. Cirillo, R. Woltersdorf, S. Falzoni, P. Chiozzi, P. Pellegatti, M. G. Callegari, D. Sandonà, F. Markwardt, G. Schmalzing, and F. Di Virgilio. "Trophic Activity of a Naturally Occurring Truncated Isoform of the P2x7 Receptor." *Faseb j* 24, no. 9 (Sep 2010): 3393-404. https://dx.doi.org/10.1096/fj.09-153601.

Adinolfi, E., A. L. Giuliani, E. De Marchi, A. Pegoraro, E. Orioli, and F. Di Virgilio. "The P2x7 Receptor: A Main Player in Inflammation." *Biochem Pharmacol* 151 (May 2018): 234-44. https://dx.doi.org/10.1016/j.bcp.2017.12.021.

Adinolfi, E., L. Raffaghello, A. L. Giuliani, L. Cavazzini, M. Capece, P. Chiozzi, G. Bianchi, G. Kroemer, V. Pistoia, and F. Di Virgilio. "Expression of P2x7 Receptor Increases in Vivo Tumor Growth." *Cancer Res* 72, no. 12 (Jun 15 2012): 2957-69. https://dx.doi.org/10.1158/0008-5472.Can-11-1947.

Aga, M., C. J. Johnson, A. P. Hart, A. G. Guadarrama, M. Suresh, J. Svaren, P. J. Bertics, and B. J. Darien. "Modulation of Monocyte Signaling and Pore Formation in Response to Agonists of the Nucleotide Receptor P2x(7)." *J Leukoc Biol* 72, no. 1 (Jul 2002): 222-32.

Ajit, D., L. T. Woods, J. M. Camden, C. N. Thebeau, F. G. El-Sayed, G. W. Greeson, L. Erb, M. J. Petris, D. C. Miller, G. Y. Sun, and G. A. Weisman. "Loss of $P2y_2$ Nucleotide Receptors Enhances Early Pathology in the Tgcrnd8 Mouse Model of Alzheimer's Disease." *Mol Neurobiol* 49, no. 2 (Apr 2014): 1031-42. https://dx.doi.org/10.1007/s12035-013-8577-5.

Albers, D. S., and M. F. Beal. "Mitochondrial Dysfunction and Oxidative Stress in Aging and Neurodegenerative Disease." *J Neural Transm Suppl* 59 (2000): 133-54. https://dx.doi.org/10.1007/978-3-7091-6781-6_16.

Amhaoul, H., I. Ali, M. Mola, A. Van Eetveldt, K. Szewczyk, S. Missault, K. Bielen, S. Kumar-Singh, J. Rech, B. Lord, M. Ceusters, A. Bhattacharya, and S. Dedeurwaerdere. "P2x7 Receptor Antagonism Reduces the Severity of Spontaneous Seizures in a Chronic Model of Temporal Lobe Epilepsy." *Neuropharmacology* 105 (Jun 2016): 175-85. https://dx.doi.org/10.1016/j.neuropharm.2016.01.018.

Armstrong, J. N., T. B. Brust, R. G. Lewis, and B. A. MacVicar. "Activation of Presynaptic P2x7-Like Receptors Depresses Mossy Fiber-Ca3 Synaptic Transmission through P38 Mitogen-Activated Protein Kinase." *J Neurosci* 22, no. 14 (Jul 15 2002): 5938-45. https://dx.doi.org/10.1523/jneurosci.22-14-05938.2002.

Atkinson, L., T. F. Batten, T. S. Moores, H. Varoqui, J. D. Erickson, and J. Deuchars. "Differential Co-Localisation of the P2x7 Receptor Subunit with Vesicular Glutamate Transporters Vglut1 and Vglut2 in Rat Cns." *Neuroscience* 123, no. 3 (2004): 761-8. https://dx.doi.org/10.1016/j.neuroscience.2003.08.065.

Aubin-Tam, M. E., A. O. Olivares, R. T. Sauer, T. A. Baker, and M. J. Lang. "Single-Molecule Protein Unfolding and Translocation by an Atp-Fueled Proteolytic Machine." *Cell* 145, no. 2 (Apr 15 2011): 257-67. https://dx.doi.org/10.1016/j.cell.2011.03.036.

Avanzini, G., and S. Franceschetti. "Cellular Biology of Epileptogenesis." *Lancet Neurol* 2, no. 1 (Jan 2003): 33-42. https://dx.doi.org/10.1016/s1474-4422(03)00265-5.

Bankston, L. A., and G. Guidotti. "Characterization of Atp Transport into Chromaffin Granule Ghosts. Synergy of Atp and Serotonin Accumulation in Chromaffin Granule Ghosts." *J Biol Chem* 271, no. 29 (Jul 19 1996): 17132-8. https://dx.doi.org/10.1074/jbc.271.29.17132.

Bao, L., F. Sachs, and G. Dahl. "Connexins Are Mechanosensitive." *Am J Physiol Cell Physiol* 287, no. 5 (Nov 2004): C1389-95. https://dx.doi.org/10.1152/ajpcell.00220.2004.

Barberà-Cremades, M., A. Baroja-Mazo, A. I. Gomez, F. Machado, F. Di Virgilio, and P. Pelegrín. "P2x7 Receptor-Stimulation Causes Fever Via Pge2 and Il-1β Release." *Faseb j* 26, no. 7 (Jul 2012): 2951-62. https://dx.doi.org/10.1096/fj.12-205765.

Barberà-Cremades, M., A. I. Gómez, A. Baroja-Mazo, L. Martínez-Alarcón, C. M. Martínez, C. de Torre-Minguela, and P. Pelegrín. "P2x7 Receptor Induces Tumor Necrosis Factor-A Converting Enzyme Activation and Release to Boost Tnf-A Production." *Front Immunol* 8 (2017): 862. https://dx.doi.org/10.3389/fimmu.2017.00862.

Baroja-Mazo, A., M. Barberà-Cremades, and P. Pelegrín. "The Participation of Plasma Membrane Hemichannels to Purinergic Signaling." *Biochim Biophys Acta* 1828, no. 1 (Jan 2013): 79-93. https://dx.doi.org/10.1016/j.bbamem.2012.01.002.

Bartsch, T., J. Döhring, S. Reuter, C. Finke, A. Rohr, H. Brauer, G. Deuschl, and O. Jansen. "Selective Neuronal Vulnerability of Human Hippocampal Ca1 Neurons: Lesion Evolution, Temporal Course, and Pattern of Hippocampal Damage in Diffusion-Weighted Mr Imaging." *J Cereb Blood Flow Metab* 35, no. 11 (Nov 2015): 1836-45. https://dx.doi.org/10.1038/jcbfm.2015.137.

Bateman, R. J., C. Xiong, T. L. Benzinger, A. M. Fagan, A. Goate, N. C. Fox, D. S. Marcus, N. J. Cairns, X. Xie, T. M. Blazey, D. M. Holtzman, A. Santacruz, V. Buckles, A. Oliver, K. Moulder, P. S. Aisen, B. Ghetti, W. E. Klunk, E. McDade, R. N. Martins, C. L. Masters, R. Mayeux, J. M. Ringman, M. N. Rossor, P. R. Schofield, R. A. Sperling, S. Salloway, and J. C. Morris. "Clinical and Biomarker Changes in Dominantly Inherited Alzheimer's Disease." *N Engl J Med* 367, no. 9 (Aug 30 2012): 795-804. https://dx.doi.org/10.1056/NEJMoa1202753.

Beigi, R. D., S. B. Kertesy, G. Aquilina, and G. R. Dubyak. "Oxidized Atp (Oatp) Attenuates Proinflammatory Signaling Via P2 Receptor-Independent Mechanisms." *Br J Pharmacol* 140, no. 3 (Oct 2003): 507-19. https://dx.doi.org/10.1038/sj.bjp.0705470.

Benzinger, T. L., T. Blazey, C. R. Jack, Jr., R. A. Koeppe, Y. Su, C. Xiong, M. E. Raichle, A. Z. Snyder, B. M. Ances, R. J. Bateman, N. J. Cairns, A. M. Fagan, A. Goate, D. S. Marcus, P. S. Aisen, J. J. Christensen, L. Ercole, R. C. Hornbeck, A. M. Farrar, P. Aldea, M. S. Jasielec, C. J. Owen, X. Xie, R. Mayeux, A. Brickman, E. McDade, W. Klunk, C. A. Mathis, J. Ringman, P. M. Thompson, B. Ghetti, A. J. Saykin, R. A. Sperling, K. A. Johnson, S. Salloway, S. Correia, P. R. Schofield, C. L. Masters, C. Rowe, V. L. Villemagne, R. Martins, S. Ourselin, M. N. Rossor, N. C. Fox, D. M. Cash, M. W. Weiner, D. M. Holtzman, V. D. Buckles, K. Moulder, and J. C. Morris. "Regional Variability of Imaging Biomarkers in Autosomal Dominant Alzheimer's Disease." *Proc Natl Acad Sci U S A* 110, no. 47 (Nov 19 2013): E4502-9. https://dx.doi.org/10.1073/pnas.1317918110.

Bhattacharya, A., and K. Biber. "The Microglial Atp-Gated Ion Channel P2x7 as a Cns Drug Target." *Glia* 64, no. 10 (Oct 2016): 1772-87. https://dx.doi.org/10.1002/glia.23001.

Bhattacharya, A., N. C. Derecki, T. W. Lovenberg, and W. C. Drevets. "Role of Neuro-Immunological Factors in the Pathophysiology of Mood Disorders." *Psychopharmacology (Berl)* 233, no. 9 (May 2016): 1623-36. https://dx.doi.org/10.1007/s00213-016-4214-0.

Bhattacharya, A., Q. Wang, H. Ao, J. R. Shoblock, B. Lord, L. Aluisio, I. Fraser, D. Nepomuceno, R. A. Neff, N. Welty, T. W. Lovenberg, P. Bonaventure, A. D. Wickenden, and M. A. Letavic. "Pharmacological Characterization of a Novel Centrally Permeable P2x7 Receptor Antagonist: Jnj-47965567." *Br J Pharmacol* 170, no. 3 (Oct 2013): 624-40. https://dx.doi.org/10.1111/bph.12314.

Bhattacharyya, S., H. Yu, C. Mim, and A. Matouschek. "Regulated Protein Turnover: Snapshots of the Proteasome in Action." *Nat Rev Mol Cell Biol* 15, no. 2 (Feb 2014): 122-33. https://dx.doi.org/10.1038/nrm3741.

Bin, A., V. Caputi, M. Bistoletti, M. Montopoli, R. Colucci, L. Antonioli, S. De Martin, I. Castagliuolo, G. Orso, C. Giaroni, P. Debetto, and M. C. Giron. "The Ecto-Enzymes Cd73 and Adenosine Deaminase Modulate 5'-Amp-Derived Adenosine in Myofibroblasts of the Rat Small Intestine." *Purinergic Signal* 14, no. 4 (Dec 2018): 409-21. https://dx.doi.org/10.1007/s11302-018-9623-6.

Bishop GM, Robinson SR. Human Aβ1–42 reduces iron-induced toxicity in rat cerebral cortex. J Neurosci Res 2003; 73(3): 316–23

Bodin, P., and G. Burnstock. "Purinergic Signalling: Atp Release." *Neurochem Res* 26, no. 8-9 (Sep 2001): 959-69. https://dx.doi.org/10.1023/a:1012388618693.

Boumechache, M., M. Masin, J. M. Edwardson, D. C. Górecki, and R. Murrell-Lagnado. "Analysis of Assembly and Trafficking of Native P2x4 and P2x7 Receptor Complexes in Rodent Immune

Cells." *J Biol Chem* 284, no. 20 (May 15 2009): 13446-54. https://dx.doi.org/10.1074/jbc.M901255200.

Brunello, C. A., M. Merezhko, R. L. Uronen, and H. J. Huttunen. "Mechanisms of Secretion and Spreading of Pathological Tau Protein." *Cell Mol Life Sci* 77, no. 9 (May 2020): 1721-44. https://dx.doi.org/10.1007/s00018-019-03349-1.

Buell, G. N., F. Talabot, A. Gos, J. Lorenz, E. Lai, M. A. Morris, and S. E. Antonarakis. "Gene Structure and Chromosomal Localization of the Human P2x7 Receptor." *Recept Channels* 5, no. 6 (1998): 347-54.

Buisman, H. P., T. H. Steinberg, J. Fischbarg, S. C. Silverstein, S. A. Vogelzang, C. Ince, D. L. Ypey, and P. C. Leijh. "Extracellular Atp Induces a Large Nonselective Conductance in Macrophage Plasma Membranes." *Proc Natl Acad Sci U S A* 85, no. 21 (Nov 1988): 7988-92. https://dx.doi.org/10.1073/pnas.85.21.7988.

Burnstock, G. "Purinergic Nerves." *Pharmacol Rev* 24, no. 3 (Sep 1972): 509-81.

Burnstock, G. "Purinergic Receptors." *J Theor Biol* 62, no. 2 (Oct 21 1976): 491-503. https://dx.doi.org/10.1016/0022-5193(76)90133-8.

Burnstock, G. "Physiology and Pathophysiology of Purinergic Neurotransmission." *Physiol Rev* 87, no. 2 (Apr 2007): 659-797. https://dx.doi.org/10.1152/physrev.00043.2006.

Burnstock, G. "Non-Synaptic Transmission at Autonomic Neuroeffector Junctions." *Neurochem Int* 52, no. 1-2 (Jan 2008): 14-25. https://dx.doi.org/10.1016/j.neuint.2007.03.007.

Burnstock, G. "Purinergic Signalling in Endocrine Organs." *Purinergic Signal* 10, no. 1 (Mar 2014): 189-231. https://dx.doi.org/10.1007/s11302-013-9396-x.

Burnstock, G. "The Therapeutic Potential of Purinergic Signalling." *Biochem Pharmacol* 151 (May 2018): 157-65. https://dx.doi.org/10.1016/j.bcp.2017.07.016.

Burnstock, G. "Purine and Purinergic Receptors." *Brain Neurosci Adv* 2 (Jan-Dec 2018): 2398212818817494. https://dx.doi.org/10.1177/2398212818817494.

Burnstock, G., G. Campbell, D. Satchell, and A. Smythe. "Evidence That Adenosine Triphosphate or a Related Nucleotide Is the Transmitter Substance Released by Non-Adrenergic Inhibitory Nerves in the Gut." *Br J Pharmacol* 40, no. 4 (Dec 1970): 668-88. https://dx.doi.org/10.1111/j.1476-5381.1970.tb10646.x.

Burnstock, G., and C. Kennedy. "Is There a Basis for Distinguishing Two Types of P2-Purinoceptor?", *Gen Pharmacol* 16, no. 5 (1985): 433-40. https://dx.doi.org/10.1016/0306-3623(85)90001-1.

Burnstock, G., and G. E. Knight. "Cellular Distribution and Functions of P2 Receptor Subtypes in Different Systems." *Int Rev Cytol* 240 (2004): 31-304. https://dx.doi.org/10.1016/s0074-7696(04)40002-3.

Caldeira, M. V., I. L. Salazar, M. Curcio, L. M. Canzoniero, and C. B. Duarte. "Role of the Ubiquitin-Proteasome System in Brain Ischemia: Friend or Foe?", *Prog Neurobiol* 112 (Jan 2014): 50-69. https://dx.doi.org/10.1016/j.pneurobio.2013.10.003.

Camden, J. M., A. M. Schrader, R. E. Camden, F. A. González, L. Erb, C. I. Seye, and G. A. Weisman. "P2y2 Nucleotide Receptors Enhance Alpha-Secretase-Dependent Amyloid Precursor Protein Processing." *J Biol Chem* 280, no. 19 (May 13 2005): 18696-702. https://dx.doi.org/10.1074/jbc.M500219200.

Caporali, F., P. L. Capecchi, A. Gamberucci, P. E. Lazzerini, G. Pompella, M. Natale, S. Lorenzini, E. Selvi, M. Galeazzi, and F. Laghi Pasini. "Human Rheumatoid Synoviocytes Express Functional P2x7 Receptors." *J Mol Med (Berl)* 86, no. 8 (Aug 2008): 937-49. https://dx.doi.org/10.1007/s00109-008-0365-8.

Carrasquero, L. M., E. G. Delicado, L. Sánchez-Ruiloba, T. Iglesias, and M. T. Miras-Portugal. "Mechanisms of Protein Kinase D Activation in Response to P2y(2) and P2x7 Receptors in Primary Astrocytes." *Glia* 58, no. 8 (Jun 2010): 984-95. https://dx.doi.org/10.1002/glia.20980.

Cepeda-Prado, E., S. Popp, U. Khan, D. Stefanov, J. Rodríguez, L. B. Menalled, D. Dow-Edwards, S. A. Small, and H. Moreno. "R6/2 Huntington's Disease Mice Develop Early and Progressive Abnormal Brain Metabolism and Seizures." *J Neurosci* 32, no. 19 (May 9 2012): 6456-67. https://dx.doi.org/10.1523/jneurosci.0388-12.2012.

Cepeda-Prado, E., S. Popp, U. Khan, D. Stefanov, J. Rodríguez, L. B. Menalled, D. Dow-Edwards, S. A. Small, and H. Moreno. "R6/2 Huntington's Disease Mice Develop Early and Progressive Abnormal Brain Metabolism and Seizures." *J Neurosci* 32, no. 19 (May 9 2012): 6456-67. https://dx.doi.org/10.1523/jneurosci.0388-12.2012.

Chen, M., H. K. Lee, L. Moo, E. Hanlon, T. Stein, and W. Xia. "Common Proteomic Profiles of Induced Pluripotent Stem Cell-Derived Three-Dimensional Neurons and Brain Tissue from Alzheimer Patients." *J Proteomics* 182 (Jun 30 2018): 21-33. https://dx.doi.org/10.1016/j.jprot.2018.04.032.

Chen, S., Q. Ma, P. R. Krafft, Q. Hu, W. Rolland, 2nd, P. Sherchan, J. Zhang, J. Tang, and J. H. Zhang. "P2x7r/Cryopyrin Inflammasome Axis Inhibition Reduces Neuroinflammation after Sah." *Neurobiol Dis* 58 (Oct 2013): 296-307. https://dx.doi.org/10.1016/j.nbd.2013.06.011.

Chen, X., J. Hu, L. Jiang, S. Xu, B. Zheng, C. Wang, J. Zhang, X. Wei, L. Chang, and Q. Wang. "Brilliant Blue G Improves Cognition in an Animal Model of Alzheimer's Disease and Inhibits Amyloid-B-Induced Loss of Filopodia and Dendrite Spines in Hippocampal Neurons." *Neuroscience* 279 (Oct 24 2014): 94-101. https://dx.doi.org/10.1016/j.neuroscience.2014.08.036.

Chessell, I. P., J. P. Hatcher, C. Bountra, A. D. Michel, J. P. Hughes, P. Green, J. Egerton, M. Murfin, J. Richardson, W. L. Peck, C. B. A. Grahames, M. A. Casula, Y. Yiangou, R. Birch, P. Anand, and G. N. Buell. "Disruption of the P2x7 Purinoceptor Gene Abolishes Chronic Inflammatory and

Neuropathic Pain." *Pain* 114, no. 3 (Apr 2005): 386-96. https://dx.doi.org/10.1016/j.pain.2005.01.002.

Chiozzi, P., A. C. Sarti, J. M. Sanz, A. L. Giuliani, E. Adinolfi, V. Vultaggio-Poma, S. Falzoni, and F. Di Virgilio. "Amyloid B-Dependent Mitochondrial Toxicity in Mouse Microglia Requires P2x7 Receptor Expression and Is Prevented by Nimodipine." *Sci Rep* 9, no. 1 (Apr 24 2019): 6475. https://dx.doi.org/10.1038/s41598-019-42931-2.

Chowdhry, S., Y. Zhang, M. McMahon, C. Sutherland, A. Cuadrado, and J. D. Hayes. "Nrf2 Is Controlled by Two Distinct B-Trcp Recognition Motifs in Its Neh6 Domain, One of Which Can Be Modulated by Gsk-3 Activity." *Oncogene* 32, no. 32 (Aug 8 2013): 3765-81. https://dx.doi.org/10.1038/onc.2012.388.

Chrovian, C. C., A. Soyode-Johnson, A. A. Peterson, C. F. Gelin, X. Deng, C. A. Dvorak, N. I. Carruthers, B. Lord, I. Fraser, L. Aluisio, K. J. Coe, B. Scott, T. Koudriakova, F. Schoetens, K. Sepassi, D. J. Gallacher, A. Bhattacharya, and M. A. Letavic. "A Dipolar Cycloaddition Reaction to Access 6-Methyl-4,5,6,7-Tetrahydro-1h-[1,2,3]Triazolo[4,5-C]Pyridines Enables the Discovery Synthesis and Preclinical Profiling of a P2x7 Antagonist Clinical Candidate." *J Med Chem* 61, no. 1 (Jan 11 2018): 207-23. https://dx.doi.org/10.1021/acs.jmedchem.7b01279.

Ciechanover, A., S. Elias, H. Heller, and A. Hershko. ""Covalent Affinity" Purification of Ubiquitin-Activating Enzyme." *J Biol Chem* 257, no. 5 (Mar 10 1982): 2537-42.

Ciechanover, A., and Y. T. Kwon. "Degradation of Misfolded Proteins in Neurodegenerative Diseases: Therapeutic Targets and Strategies." *Exp Mol Med* 47, no. 3 (Mar 13 2015): e147. https://dx.doi.org/10.1038/emm.2014.117.

Coco, S., F. Calegari, E. Pravettoni, D. Pozzi, E. Taverna, P. Rosa, M. Matteoli, and C. Verderio. "Storage and Release of Atp from Astrocytes in Culture." *J Biol Chem* 278, no. 2 (Jan 10 2003): 1354-62. https://dx.doi.org/10.1074/jbc.M209454200.

Dagvadorj, J., K. Shimada, S. Chen, H. D. Jones, G. Tumurkhuu, W. Zhang, K. A. Wawrowsky, T. R. Crother, and M. Arditi. "Lipopolysaccharide Induces Alveolar Macrophage Necrosis Via Cd14 and the P2x7 Receptor Leading to Interleukin-1α Release." *Immunity* 42, no. 4 (Apr 21 2015): 640-53. https://dx.doi.org/10.1016/j.immuni.2015.03.007.

Dahlmann, B. "Mammalian Proteasome Subtypes: Their Diversity in Structure and Function." *Arch Biochem Biophys* 591 (Feb 1 2016): 132-40. https://dx.doi.org/10.1016/j.abb.2015.12.012.

Dantuma, N. P., and K. Lindsten. "Stressing the Ubiquitin-Proteasome System." *Cardiovasc Res* 85, no. 2 (Jan 15 2010): 263-71. https://dx.doi.org/10.1093/cvr/cvp255.

Dantuma, N. P., K. Lindsten, R. Glas, M. Jellne, and M. G. Masucci. "Short-Lived Green Fluorescent Proteins for Quantifying Ubiquitin/Proteasome-Dependent Proteolysis in Living Cells." *Nat Biotechnol* 18, no. 5 (May 2000): 538-43. https://dx.doi.org/10.1038/75406.

Darmellah, A., A. Rayah, R. Auger, M. H. Cuif, M. Prigent, M. Arpin, A. Alcover, C. Delarasse, and J. M. Kanellopoulos. "Ezrin/Radixin/Moesin Are Required for the Purinergic P2x7 Receptor (P2x7r)-Dependent Processing of the Amyloid Precursor Protein." *J Biol Chem* 287, no. 41 (Oct 5 2012): 34583-95. https://dx.doi.org/10.1074/jbc.M112.400010.

Das, S., and N. P. Bhattacharyya. "Transcription Regulation of Hypk by Heat Shock Factor 1." *PLoS One* 9, no. 1 (2014): e85552. https://dx.doi.org/10.1371/journal.pone.0085552.

David, D. C., R. Layfield, L. Serpell, Y. Narain, M. Goedert, and M. G. Spillantini. "Proteasomal Degradation of Tau Protein." *J Neurochem* 83, no. 1 (Oct 2002): 176-85. https://dx.doi.org/10.1046/j.1471-4159.2002.01137.x.

de Diego García, L., Á Sebastián-Serrano, I. H. Hernández, J. Pintor, J. J. Lucas, and M. Díaz-Hernández. "The Regulation of Proteostasis in Glial Cells by Nucleotide Receptors Is Key in Acute Neuroinflammation." *Faseb j* 32, no. 6 (Jun 2018): 3020-32. https://dx.doi.org/10.1096/fj.201701064RR.

de Diego-García, L., M. Ramírez-Escudero, Á Sebastián-Serrano, J. I. Diaz-Hernández, J. Pintor, J. J. Lucas, and M. Díaz-Hernández. "Regulation of Proteasome Activity by P2y(2) Receptor Underlies the Neuroprotective Effects of Extracellular Nucleotides." *Biochim Biophys Acta Mol Basis Dis* 1863, no. 1 (Jan 2017): 43-51. https://dx.doi.org/10.1016/j.bbadis.2016.10.012.

De Marchi, E., E. Orioli, A. Pegoraro, S. Sangaletti, P. Portararo, A. Curti, M. P. Colombo, F. Di Virgilio, and E. Adinolfi. "The P2x7 Receptor Modulates Immune Cells Infiltration, Ectonucleotidases Expression and Extracellular Atp Levels in the Tumor Microenvironment." *Oncogene* 38, no. 19 (May 2019): 3636-50. https://dx.doi.org/10.1038/s41388-019-0684-y.

de Vrij, F. M., D. F. Fischer, F. W. van Leeuwen, and E. M. Hol. "Protein Quality Control in Alzheimer's Disease by the Ubiquitin Proteasome System." *Prog Neurobiol* 74, no. 5 (Dec 2004): 249-70. https://dx.doi.org/10.1016/j.pneurobio.2004.10.001.

.

DeWitt DA, Silver J. Regenerative failure: a potential mechanism for neuritic dystrophy in Alzheimer's disease. Exp Neurol 1996; 142(1): 103–10

Delarasse, C., R. Auger, P. Gonnord, B. Fontaine, and J. M. Kanellopoulos. "The Purinergic Receptor P2x7 Triggers Alpha-Secretase-Dependent Processing of the Amyloid Precursor Protein." *J Biol Chem* 286, no. 4 (Jan 28 2011): 2596-606. https://dx.doi.org/10.1074/jbc.M110.200618.

DeMartino, G. N., and C. A. Slaughter. "The Proteasome, a Novel Protease Regulated by Multiple Mechanisms." *J Biol Chem* 274, no. 32 (Aug 6 1999): 22123-6. https://dx.doi.org/10.1074/jbc.274.32.22123.

Deshaies, R. J., and C. A. Joazeiro. "Ring Domain E3 Ubiquitin Ligases." *Annu Rev Biochem* 78 (2009): 399-434. https://dx.doi.org/10.1146/annurev.biochem.78.101807.093809.

DeTure, M. A., and D. W. Dickson. "The Neuropathological Diagnosis of Alzheimer's Disease." *Mol Neurodegener* 14, no. 1 (Aug 2 2019): 32. https://dx.doi.org/10.1186/s13024-019-0333-5.

DeTure, M. A., and D. W. Dickson. "The Neuropathological Diagnosis of Alzheimer's Disease." *Mol Neurodegener* 14, no. 1 (Aug 2 2019): 32. https://dx.doi.org/10.1186/s13024-019-0333-5.

Deuchars, S. A., L. Atkinson, R. E. Brooke, H. Musa, C. J. Milligan, T. F. Batten, N. J. Buckley, S. H. Parson, and J. Deuchars. "Neuronal P2x7 Receptors Are Targeted to Presynaptic Terminals in the Central and Peripheral Nervous Systems." *J Neurosci* 21, no. 18 (Sep 15 2001): 7143-52. https://dx.doi.org/10.1523/jneurosci.21-18-07143.2001.

Di Virgilio, F. "The P2z Purinoceptor: An Intriguing Role in Immunity, Inflammation and Cell Death." *Immunol Today* 16, no. 11 (Nov 1995): 524-8. https://dx.doi.org/10.1016/0167-5699(95)80045-x.

Di Virgilio, F. "Novel Data Point to a Broader Mechanism of Action of Oxidized Atp: The P2x7 Receptor Is Not the Only Target." *Br J Pharmacol* 140, no. 3 (Oct 2003): 441-3. https://dx.doi.org/10.1038/sj.bjp.0705469.

Di Virgilio, F. "Liaisons Dangereuses: P2x(7) and the Inflammasome." *Trends Pharmacol Sci* 28, no. 9 (Sep 2007): 465-72. https://dx.doi.org/10.1016/j.tips.2007.07.002.

Di Virgilio, F. "P2rx7: A Receptor with a Split Personality in Inflammation and Cancer." *Mol Cell Oncol* 3, no. 2 (Mar 2016): e1010937. https://dx.doi.org/10.1080/23723556.2015.1010937.

Di Virgilio, F., D. Dal Ben, A. C. Sarti, A. L. Giuliani, and S. Falzoni. "The P2x7 Receptor in Infection and Inflammation." *Immunity* 47, no. 1 (Jul 18 2017): 15-31. https://dx.doi.org/10.1016/j.immuni.2017.06.020.

Di Virgilio, F., A. L. Giuliani, V. Vultaggio-Poma, S. Falzoni, and A. C. Sarti. "Non-Nucleotide Agonists Triggering P2x7 Receptor Activation and Pore Formation." *Front Pharmacol* 9 (2018): 39. https://dx.doi.org/10.3389/fphar.2018.00039.

Di Virgilio, F., A. C. Sarti, and R. Coutinho-Silva. "Purinergic Signaling, Damps, and Inflammation." *Am J Physiol Cell Physiol* 318, no. 5 (May 1 2020): C832-c35. https://dx.doi.org/10.1152/ajpcell.00053.2020.

Di Virgilio, F., A. C. Sarti, S. Falzoni, E. De Marchi, and E. Adinolfi. "Extracellular Atp and P2 Purinergic Signalling in the Tumour Microenvironment." *Nat Rev Cancer* 18, no. 10 (Oct 2018): 601-18. https://dx.doi.org/10.1038/s41568-018-0037-0.

Di Virgilio, F., G. Schmalzing, and F. Markwardt. "The Elusive P2x7 Macropore." *Trends Cell Biol* 28, no. 5 (May 2018): 392-404. https://dx.doi.org/10.1016/j.tcb.2018.01.005.

Dias, V., E. Junn, and M. M. Mouradian. "The Role of Oxidative Stress in Parkinson's Disease." *J Parkinsons Dis* 3, no. 4 (2013): 461-91. https://dx.doi.org/10.3233/jpd-130230.

Diaz-Hernandez, J. I., R. Gomez-Villafuertes, M. León-Otegui, L. Hontecillas-Prieto, A. Del Puerto, J. L. Trejo, J. J. Lucas, J. J. Garrido, J. Gualix, M. T. Miras-Portugal, and M. Diaz-Hernandez. "In Vivo

P2x7 Inhibition Reduces Amyloid Plaques in Alzheimer's Disease through Gsk3β and Secretases." *Neurobiol Aging* 33, no. 8 (Aug 2012): 1816-28. https://dx.doi.org/10.1016/j.neurobiolaging.2011.09.040.

Díaz-Hernandez, M., A. del Puerto, J. I. Díaz-Hernandez, M. Diez-Zaera, J. J. Lucas, J. J. Garrido, and M. T. Miras-Portugal. "Inhibition of the Atp-Gated P2x7 Receptor Promotes Axonal Growth and Branching in Cultured Hippocampal Neurons." *J Cell Sci* 121, no. Pt 22 (Nov 15 2008): 3717-28. https://dx.doi.org/10.1242/jcs.034082.

Díaz-Hernández, M., M. Díez-Zaera, J. Sánchez-Nogueiro, R. Gómez-Villafuertes, J. M. Canals, J. Alberch, M. T. Miras-Portugal, and J. J. Lucas. "Altered P2x7-Receptor Level and Function in Mouse Models of Huntington's Disease and Therapeutic Efficacy of Antagonist Administration." *Faseb j* 23, no. 6 (Jun 2009): 1893-906. https://dx.doi.org/10.1096/fj.08-122275.

Díaz-Hernández, M., J. Pintor, E. Castro, and M. T. Miras-Portugal. "Co-Localisation of Functional Nicotinic and Ionotropic Nucleotide Receptors in Isolated Cholinergic Synaptic Terminals." *Neuropharmacology* 42, no. 1 (Jan 2002): 20-33. https://dx.doi.org/10.1016/s0028-3908(01)00157-5.

Díaz-Hernández, M., J. Sánchez-Nogueiro, and M. T. Miras-Portugal. "Role of Cacmkii in the Cross Talk between Ionotropic Nucleotide and Nicotinic Receptors in Individual Cholinergic Terminals." *J Mol Neurosci* 30, no. 1-2 (2006): 177-80. https://dx.doi.org/10.1385/jmn:30:1:177.

Díaz-Hernández, M., A. G. Valera, M. A. Morán, P. Gómez-Ramos, B. Alvarez-Castelao, J. G. Castaño, F. Hernández, and J. J. Lucas. "Inhibition of 26s Proteasome Activity by Huntingtin Filaments but Not Inclusion Bodies Isolated from Mouse and Human Brain." *J Neurochem* 98, no. 5 (Sep 2006): 1585-96. https://dx.doi.org/10.1111/j.1471-4159.2006.03968.x.

Dikic, I., S. Wakatsuki, and K. J. Walters. "Ubiquitin-Binding Domains - from Structures to Functions." *Nat Rev Mol Cell Biol* 10, no. 10 (Oct 2009): 659-71. https://dx.doi.org/10.1038/nrm2767.

Donnelly-Roberts, D. L., M. T. Namovic, P. Han, and M. F. Jarvis. "Mammalian P2x7 Receptor Pharmacology: Comparison of Recombinant Mouse, Rat and Human P2x7 Receptors." *Br J Pharmacol* 157, no. 7 (Aug 2009): 1203-14. https://dx.doi.org/10.1111/j.1476-5381.2009.00233.x.

Donoso, M. V., R. Miranda, R. Briones, M. J. Irarrázaval, and J. P. Huidobro-Toro. "Release and Functional Role of Neuropeptide Y as a Sympathetic Modulator in Human Saphenous Vein Biopsies." *Peptides* 25, no. 1 (Jan 2004): 53-64. https://dx.doi.org/10.1016/j.peptides.2003.11.001.

Drury, A. N., and A. Szent-Györgyi. "The Physiological Activity of Adenine Compounds with Especial Reference to Their Action Upon the Mammalian Heart." *J Physiol* 68, no. 3 (Nov 25 1929): 213-37. https://dx.doi.org/10.1113/jphysiol.1929.sp002608.

Dubois, B., H. H. Feldman, C. Jacova, H. Hampel, J. L. Molinuevo, K. Blennow, S. T. DeKosky, S. Gauthier, D. Selkoe, R. Bateman, S. Cappa, S. Crutch, S. Engelborghs, G. B. Frisoni, N. C. Fox, D. Galasko, M. O. Habert, G. A. Jicha, A. Nordberg, F. Pasquier, G. Rabinovici, P. Robert, C. Rowe, S. Salloway, M. Sarazin, S. Epelbaum, L. C. de Souza, B. Vellas, P. J. Visser, L. Schneider, Y. Stern, P. Scheltens, and J. L. Cummings. "Advancing Research Diagnostic Criteria for Alzheimer's Disease: The Iwg-2 Criteria." *Lancet Neurol* 13, no. 6 (Jun 2014): 614-29. https://dx.doi.org/10.1016/s1474-4422(14)70090-0.

Dutta, A. K., R. Z. Sabirov, H. Uramoto, and Y. Okada. "Role of Atp-Conductive Anion Channel in Atp Release from Neonatal Rat Cardiomyocytes in Ischaemic or Hypoxic Conditions." *J Physiol* 559, no. Pt 3 (Sep 15 2004): 799-812. https://dx.doi.org/10.1113/jphysiol.2004.069245.

Ebstein, F., P. M. Kloetzel, E. Krüger, and U. Seifert. "Emerging Roles of Immunoproteasomes Beyond Mhc Class I Antigen Processing." *Cell Mol Life Sci* 69, no. 15 (Aug 2012): 2543-58. https://dx.doi.org/10.1007/s00018-012-0938-0.

Egan, T. M., and B. S. Khakh. "Contribution of Calcium Ions to P2x Channel Responses." *J Neurosci* 24, no. 13 (Mar 31 2004): 3413-20. https://dx.doi.org/10.1523/jneurosci.5429-03.2004.

Ekberg, J., F. Schuetz, N. A. Boase, S. J. Conroy, J. Manning, S. Kumar, P. Poronnik, and D. J. Adams. "Regulation of the Voltage-Gated K(+) Channels Kcnq2/3 and Kcnq3/5 by Ubiquitination. Novel Role for Nedd4-2." *J Biol Chem* 282, no. 16 (Apr 20 2007): 12135-42. https://dx.doi.org/10.1074/jbc.M609385200.

Engel, T., M. Alves, C. Sheedy, and D. C. Henshall. "Atpergic Signalling During Seizures and Epilepsy." *Neuropharmacology* 104 (May 2016): 140-53. https://dx.doi.org/10.1016/j.neuropharm.2015.11.001.

Engel, T., R. Gomez-Villafuertes, K. Tanaka, G. Mesuret, A. Sanz-Rodriguez, P. Garcia-Huerta, M. T. Miras-Portugal, D. C. Henshall, and M. Diaz-Hernandez. "Seizure Suppression and Neuroprotection by Targeting the Purinergic P2x7 Receptor During Status Epilepticus in Mice." *Faseb j* 26, no. 4 (Apr 2012): 1616-28. https://dx.doi.org/10.1096/fj.11-196089.

Engel, T., and D. C. Henshall. "Apoptosis, Bcl-2 Family Proteins and Caspases: The Abcs of Seizure-Damage and Epileptogenesis?", *Int J Physiol Pathophysiol Pharmacol* 1, no. 2 (Mar 30 2009): 97-115.

Engel, T., A. Jimenez-Pacheco, M. T. Miras-Portugal, M. Diaz-Hernandez, and D. C. Henshall. "P2x7 Receptor in Epilepsy; Role in Pathophysiology and Potential Targeting for Seizure Control." *Int J Physiol Pathophysiol Pharmacol* 4, no. 4 (2012): 174-87.

Engel, T., J. Martinez-Villarreal, C. Henke, E. M. Jimenez-Mateos, A. Sanz-Rodriguez, M. Alves, Y. Hernandez-Santana, G. P. Brennan, A. Kenny, A. Campbell, J. J. Lucas, and D. C. Henshall. "Spatiotemporal Progression of Ubiquitin-Proteasome System Inhibition after Status Epilepticus Suggests Protective Adaptation against Hippocampal Injury." *Mol Neurodegener* 12, no. 1 (Feb 24 2017): 21. https://dx.doi.org/10.1186/s13024-017-0163-2.

Erb, L., Z. Liao, C. I. Seye, and G. A. Weisman. "P2 Receptors: Intracellular Signaling." *Pflugers Arch* 452, no. 5 (Aug 2006): 552-62. https://dx.doi.org/10.1007/s00424-006-0069-2.

Evans, R. J., C. Lewis, G. Buell, S. Valera, R. A. North, and A. Surprenant. "Pharmacological Characterization of Heterologously Expressed Atp-Gated Cation Channels (P2x Purinoceptors)." *Mol Pharmacol* 48, no. 2 (Aug 1995): 178-83.

Faliti, C. E., R. Gualtierotti, E. Rottoli, M. Gerosa, L. Perruzza, A. Romagnani, G. Pellegrini, B. De Ponte Conti, R. L. Rossi, M. Idzko, E. M. C. Mazza, S. Bicciato, E. Traggiai, P. L. Meroni, and F. Grassi. "P2x7 Receptor Restrains Pathogenic Tfh Cell Generation in Systemic Lupus Erythematosus." *J Exp Med* 216, no. 2 (Feb 4 2019): 317-36. https://dx.doi.org/10.1084/jem.20171976.

Ferrari, D., S. Wesselborg, M. K. Bauer, and K. Schulze-Osthoff. "Extracellular Atp Activates Transcription Factor Nf-Kappab through the P2z Purinoreceptor by Selectively Targeting Nf-Kappab P65." *J Cell Biol* 139, no. 7 (Dec 29 1997): 1635-43. https://dx.doi.org/10.1083/jcb.139.7.1635.

Ferrington, D. A., and D. S. Gregerson. "Immunoproteasomes: Structure, Function, and Antigen Presentation." *Prog Mol Biol Transl Sci* 109 (2012): 75-112. https://dx.doi.org/10.1016/b978-0-12-397863-9.00003-1.

Fischer, D. F., R. A. De Vos, R. Van Dijk, F. M. De Vrij, E. A. Proper, M. A. Sonnemans, M. C. Verhage, J. A. Sluijs, B. Hobo, M. Zouambia, E. N. Steur, W. Kamphorst, E. M. Hol, and F. W. Van Leeuwen. "Disease-Specific Accumulation of Mutant Ubiquitin as a Marker for Proteasomal Dysfunction in the Brain." *Faseb j* 17, no. 14 (Nov 2003): 2014-24. https://dx.doi.org/10.1096/fj.03-0205com.

Fischer, W., H. Franke, U. Krügel, H. Müller, K. Dinkel, B. Lord, M. A. Letavic, D. C. Henshall, and T. Engel. "Critical Evaluation of P2x7 Receptor Antagonists in Selected Seizure Models." *PLoS One* 11, no. 6 (2016): e0156468. https://dx.doi.org/10.1371/journal.pone.0156468.

Fisher, R. S., W. van Emde Boas, W. Blume, C. Elger, P. Genton, P. Lee, and J. Engel, Jr. "Epileptic Seizures and Epilepsy: Definitions Proposed by the International League against Epilepsy (Ilae) and the International Bureau for Epilepsy (Ibe)." *Epilepsia* 46, no. 4 (Apr 2005): 470-2. https://dx.doi.org/10.1111/j.0013-9580.2005.66104.x.

Folstein, M. F., S. E. Folstein, and P. R. McHugh. ""Mini-Mental State". A Practical Method for Grading the Cognitive State of Patients for the Clinician." *J Psychiatr Res* 12, no. 3 (Nov 1975): 189-98. https://dx.doi.org/10.1016/0022-3956(75)90026-6.

Franceschini, A., M. Capece, P. Chiozzi, S. Falzoni, J. M. Sanz, A. C. Sarti, M. Bonora, P. Pinton, and F. Di Virgilio. "The P2x7 Receptor Directly Interacts with the Nlrp3 Inflammasome Scaffold Protein." *Faseb j* 29, no. 6 (Jun 2015): 2450-61. https://dx.doi.org/10.1096/fj.14-268714.

Francistiová, L., C. Bianchi, C. Di Lauro, Á Sebastián-Serrano, L. de Diego-García, J. Kobolák, A. Dinnyés, and M. Díaz-Hernández. "The Role of P2x7 Receptor in Alzheimer's Disease." *Front Mol Neurosci* 13 (2020): 94. https://dx.doi.org/10.3389/fnmol.2020.00094.

Friedman, A., and R. Dingledine. "Molecular Cascades That Mediate the Influence of Inflammation on Epilepsy." *Epilepsia* 52 Suppl 3, no. 0 3 (May 2011): 33-9. https://dx.doi.org/10.1111/j.1528-1167.2011.03034.x.

Gadhave, K., N. Bolshette, A. Ahire, R. Pardeshi, K. Thakur, C. Trandafir, A. Istrate, S. Ahmed, M. Lahkar, D. F. Muresanu, and M. Balea. "The Ubiquitin Proteasomal System: A Potential Target for the Management of Alzheimer's Disease." *J Cell Mol Med* 20, no. 7 (Jul 2016): 1392-407. https://dx.doi.org/10.1111/jcmm.12817.

Gaitán-Peñas, H., A. Gradogna, L. Laparra-Cuervo, C. Solsona, V. Fernández-Dueñas, A. Barrallo-Gimeno, F. Ciruela, M. Lakadamyali, M. Pusch, and R. Estévez. "Investigation of Lrrc8-Mediated Volume-Regulated Anion Currents in Xenopus Oocytes." *Biophys J* 111, no. 7 (Oct 4 2016): 1429-43. https://dx.doi.org/10.1016/j.bpj.2016.08.030.

Gargett, C. E., and J. S. Wiley. "The Isoquinoline Derivative Kn-62 a Potent Antagonist of the P2z-Receptor of Human Lymphocytes." *Br J Pharmacol* 120, no. 8 (Apr 1997): 1483-90. https://dx.doi.org/10.1038/sj.bjp.0701081.

Gentry, M. S., C. A. Worby, and J. E. Dixon. "Insights into Lafora Disease: Malin Is an E3 Ubiquitin Ligase That Ubiquitinates and Promotes the Degradation of Laforin." *Proc Natl Acad Sci U S A* 102, no. 24 (Jun 14 2005): 8501-6. https://dx.doi.org/10.1073/pnas.0503285102.

Gever, Joel R., Debra A. Cockayne, Michael P. Dillon, Geoffrey Burnstock, and Anthony P. D. W. Ford. "Pharmacology of P2x Channels." *Pflügers Archiv* 452, no. 5 (2006/08/01 2006): 513-37. https://dx.doi.org/10.1007/s00424-006-0070-9.

Ghiringhelli, F., L. Apetoh, A. Tesniere, L. Aymeric, Y. Ma, C. Ortiz, K. Vermaelen, T. Panaretakis, G. Mignot, E. Ullrich, J. L. Perfettini, F. Schlemmer, E. Tasdemir, M. Uhl, P. Génin, A. Civas, B. Ryffel, J. Kanellopoulos, J. Tschopp, F. André, R. Lidereau, N. M. McLaughlin, N. M. Haynes, M. J. Smyth, G. Kroemer, and L. Zitvogel. "Activation of the Nlrp3 Inflammasome in Dendritic Cells Induces Il-1beta-Dependent Adaptive Immunity against Tumors." *Nat Med* 15, no. 10 (Oct 2009): 1170-8. https://dx.doi.org/10.1038/nm.2028.

Gilon, T., O. Chomsky, and R. G. Kulka. "Degradation Signals for Ubiquitin System Proteolysis in Saccharomyces Cerevisiae." *Embo j* 17, no. 10 (May 15 1998): 2759-66. https://dx.doi.org/10.1093/emboj/17.10.2759.

Giuliani, A. L., M. Berchan, J. M. Sanz, A. Passaro, S. Pizzicotti, V. Vultaggio-Poma, A. C. Sarti, and F. Di Virgilio. "The P2x7 Receptor Is Shed into Circulation: Correlation with C-Reactive Protein Levels." *Front Immunol* 10 (2019): 793. https://dx.doi.org/10.3389/fimmu.2019.00793.

Giuliani, A. L., A. C. Sarti, S. Falzoni, and F. Di Virgilio. "The P2x7 Receptor-Interleukin-1 Liaison." *Front Pharmacol* 8 (2017): 123. https://dx.doi.org/10.3389/fphar.2017.00123.

Glaser, T., S. L. de Oliveira, A. Cheffer, R. Beco, P. Martins, M. Fornazari, C. Lameu, H. M. Junior, R. Coutinho-Silva, and H. Ulrich. "Modulation of Mouse Embryonic Stem Cell Proliferation and

Neural Differentiation by the P2x7 Receptor." *PLoS One* 9, no. 5 (2014): e96281. https://dx.doi.org/10.1371/journal.pone.0096281.

Glickman, M. H., and A. Ciechanover. "The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction." *Physiol Rev* 82, no. 2 (Apr 2002): 373-428. https://dx.doi.org/10.1152/physrev.00027.2001.

Glickman, M. H., and D. Raveh. "Proteasome Plasticity." *FEBS Lett* 579, no. 15 (Jun 13 2005): 3214-23. https://dx.doi.org/10.1016/j.febslet.2005.04.048.

Glickman, M. H., D. M. Rubin, O. Coux, I. Wefes, G. Pfeifer, Z. Cjeka, W. Baumeister, V. A. Fried, and D. Finley. "A Subcomplex of the Proteasome Regulatory Particle Required for Ubiquitin-Conjugate Degradation and Related to the Cop9-Signalosome and Eif3." *Cell* 94, no. 5 (Sep 4 1998): 615-23. https://dx.doi.org/10.1016/s0092-8674(00)81603-7.

Goate, A., M. C. Chartier-Harlin, M. Mullan, J. Brown, F. Crawford, L. Fidani, L. Giuffra, A. Haynes, N. Irving, L. James, and et al. "Segregation of a Missense Mutation in the Amyloid Precursor Protein Gene with Familial Alzheimer's Disease." *Nature* 349, no. 6311 (Feb 21 1991): 704-6. https://dx.doi.org/10.1038/349704a0.

Goding, J. W., B. Grobben, and H. Slegers. "Physiological and Pathophysiological Functions of the Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase Family." *Biochim Biophys Acta* 1638, no. 1 (May 20 2003): 1-19. https://dx.doi.org/10.1016/s0925-4439(03)00058-9.

Goldberg, A. L. "Protein Degradation and Protection against Misfolded or Damaged Proteins." *Nature* 426, no. 6968 (Dec 18 2003): 895-9. https://dx.doi.org/10.1038/nature02263.

Goldstein, G., M. Scheid, U. Hammerling, D. H. Schlesinger, H. D. Niall, and E. A. Boyse. "Isolation of a Polypeptide That Has Lymphocyte-Differentiating Properties and Is Probably Represented Universally in Living Cells." *Proc Natl Acad Sci U S A* 72, no. 1 (Jan 1975): 11-5. https://dx.doi.org/10.1073/pnas.72.1.11.

Gómez-Villafuertes, R., A. del Puerto, M. Díaz-Hernández, D. Bustillo, J. I. Díaz-Hernández, P. G. Huerta, A. R. Artalejo, J. J. Garrido, and M. T. Miras-Portugal. "Ca2+/Calmodulin-Dependent Kinase Ii Signalling Cascade Mediates P2x7 Receptor-Dependent Inhibition of Neuritogenesis in Neuroblastoma Cells." *Febs j* 276, no. 18 (Sep 2009): 5307-25. https://dx.doi.org/10.1111/j.1742-4658.2009.07228.x.

Gómez-Villafuertes, R., P. García-Huerta, J. I. Díaz-Hernández, and M. T. Miras-Portugal. "Pi3k/Akt Signaling Pathway Triggers P2x7 Receptor Expression as a Pro-Survival Factor of Neuroblastoma Cells under Limiting Growth Conditions." *Sci Rep* 5 (Dec 21 2015): 18417. https://dx.doi.org/10.1038/srep18417.

Gómez-Villafuertes, R., J. Gualix, and M. T. Miras-Portugal. "Single Gabaergic Synaptic Terminals from Rat Midbrain Exhibit Functional P2x and Dinucleotide Receptors, Able to Induce Gaba Secretion." *J Neurochem* 77, no. 1 (Apr 2001): 84-93. https://dx.doi.org/10.1046/j.1471-4159.2001.t01-1-00228.x.

Gonçalves, F. Q., J. P. Lopes, H. B. Silva, C. Lemos, A. C. Silva, N. Gonçalves, R. Tomé Â, S. G. Ferreira, P. M. Canas, D. Rial, P. Agostinho, and R. A. Cunha. "Synaptic and Memory Dysfunction in a B-Amyloid Model of Early Alzheimer's Disease Depends on Increased Formation of Atp-Derived Extracellular Adenosine." *Neurobiol Dis* 132 (Dec 2019): 104570. https://dx.doi.org/10.1016/j.nbd.2019.104570.

Grice, G. L., I. T. Lobb, M. P. Weekes, S. P. Gygi, R. Antrobus, and J. A. Nathan. "The Proteasome Distinguishes between Heterotypic and Homotypic Lysine-11-Linked Polyubiquitin Chains." *Cell Rep* 12, no. 4 (Jul 28 2015): 545-53. https://dx.doi.org/10.1016/j.celrep.2015.06.061.

Groettrup, M., S. Khan, K. Schwarz, and G. Schmidtke. "Interferon-Gamma Inducible Exchanges of 20s Proteasome Active Site Subunits: Why?", *Biochimie* 83, no. 3-4 (Mar-Apr 2001): 367-72. https://dx.doi.org/10.1016/s0300-9084(01)01251-2.

Groettrup, M., C. J. Kirk, and M. Basler. "Proteasomes in Immune Cells: More Than Peptide Producers?", *Nat Rev Immunol* 10, no. 1 (Jan 2010): 73-8. https://dx.doi.org/10.1038/nri2687.

Groll, M., M. Bajorek, A. Köhler, L. Moroder, D. M. Rubin, R. Huber, M. H. Glickman, and D. Finley. "A Gated Channel into the Proteasome Core Particle." *Nat Struct Biol* 7, no. 11 (Nov 2000): 1062-7. https://dx.doi.org/10.1038/80992.

Groll, M., L. Ditzel, J. Löwe, D. Stock, M. Bochtler, H. D. Bartunik, and R. Huber. "Structure of 20s Proteasome from Yeast at 2.4 a Resolution." *Nature* 386, no. 6624 (Apr 3 1997): 463-71. https://dx.doi.org/10.1038/386463a0.

Grune, T., D. Botzen, M. Engels, P. Voss, B. Kaiser, T. Jung, S. Grimm, G. Ermak, and K. J. Davies. "Tau Protein Degradation Is Catalyzed by the Atp/Ubiquitin-Independent 20s Proteasome under Normal Cell Conditions." *Arch Biochem Biophys* 500, no. 2 (Aug 15 2010): 181-8. https://dx.doi.org/10.1016/j.abb.2010.05.008.

Grune, T., D. Botzen, M. Engels, P. Voss, B. Kaiser, T. Jung, S. Grimm, G. Ermak, and K. J. Davies. "Tau Protein Degradation Is Catalyzed by the Atp/Ubiquitin-Independent 20s Proteasome under Normal Cell Conditions." *Arch Biochem Biophys* 500, no. 2 (Aug 15 2010): 181-8. https://dx.doi.org/10.1016/j.abb.2010.05.008.

Gu, B. J., R. Sluyter, K. K. Skarratt, A. N. Shemon, L. P. Dao-Ung, S. J. Fuller, J. A. Barden, A. L. Clarke, S. Petrou, and J. S. Wiley. "An Arg307 to Gln Polymorphism within the Atp-Binding Site Causes Loss of Function of the Human P2x7 Receptor." *J Biol Chem* 279, no. 30 (Jul 23 2004): 31287-95. https://dx.doi.org/10.1074/jbc.M313902200.

Gu, B. J., W. Y. Zhang, L. J. Bendall, I. P. Chessell, G. N. Buell, and J. S. Wiley. "Expression of P2x(7) Purinoceptors on Human Lymphocytes and Monocytes: Evidence for Nonfunctional P2x(7) Receptors." *Am J Physiol Cell Physiol* 279, no. 4 (Oct 2000): C1189-97. https://dx.doi.org/10.1152/ajpcell.2000.279.4.C1189.

Gualix, J., R. Gómez-Villafuertes, M. Díaz-Hernández, and M. T. Miras-Portugal. "Presence of Functional Atp and Dinucleotide Receptors in Glutamatergic Synaptic Terminals from Rat Midbrain." *J Neurochem* 87, no. 1 (Oct 2003): 160-71. https://dx.doi.org/10.1046/j.1471-4159.2003.01975.x.

Gutiérrez-Martín, Y., D. Bustillo, R. Gómez-Villafuertes, J. Sánchez-Nogueiro, C. Torregrosa-Hetland, T. Binz, L. M. Gutiérrez, M. T. Miras-Portugal, and A. R. Artalejo. "P2x7 Receptors Trigger Atp Exocytosis and Modify Secretory Vesicle Dynamics in Neuroblastoma Cells." *J Biol Chem* 286, no. 13 (Apr 1 2011): 11370-81. https://dx.doi.org/10.1074/jbc.M110.139410.

Haanes, K. A., and I. Novak. "Atp Storage and Uptake by Isolated Pancreatic Zymogen Granules." *Biochem J* 429, no. 2 (Jul 15 2010): 303-11. https://dx.doi.org/10.1042/bj20091337.

Haas, A. L., and I. A. Rose. "The Mechanism of Ubiquitin Activating Enzyme. A Kinetic and Equilibrium Analysis." *J Biol Chem* 257, no. 17 (Sep 10 1982): 10329-37.

Haas, A. L., J. V. Warms, A. Hershko, and I. A. Rose. "Ubiquitin-Activating Enzyme. Mechanism and Role in Protein-Ubiquitin Conjugation." *J Biol Chem* 257, no. 5 (Mar 10 1982): 2543-8.

Hall, J. R., A. R. Wiechmann, L. A. Johnson, M. Edwards, R. C. Barber, A. S. Winter, M. Singh, and S. E. O'Bryant. "Biomarkers of Vascular Risk, Systemic Inflammation, and Microvascular Pathology and Neuropsychiatric Symptoms in Alzheimer's Disease." *J Alzheimers Dis* 35, no. 2 (2013): 363-71. https://dx.doi.org/10.3233/jad-122359.

Hall, M. B. H., I. A. Nissen, E. C. W. van Straaten, P. L. Furlong, C. Witton, E. Foley, S. Seri, and A. Hillebrand. "An Evaluation of Kurtosis Beamforming in Magnetoencephalography to Localize the Epileptogenic Zone in Drug Resistant Epilepsy Patients." *Clin Neurophysiol* 129, no. 6 (Jun 2018): 1221-29. https://dx.doi.org/10.1016/j.clinph.2017.12.040.

Hanamsagar, R., and S. D. Bilbo. "Sex Differences in Neurodevelopmental and Neurodegenerative Disorders: Focus on Microglial Function and Neuroinflammation During Development." *J Steroid Biochem Mol Biol* 160 (Jun 2016): 127-33. https://dx.doi.org/10.1016/j.jsbmb.2015.09.039.

Hanseeuw, B. J., R. A. Betensky, H. I. L. Jacobs, A. P. Schultz, J. Sepulcre, J. A. Becker, D. M. O. Cosio, M. Farrell, Y. T. Quiroz, E. C. Mormino, R. F. Buckley, K. V. Papp, R. A. Amariglio, I. Dewachter, A. Ivanoiu, W. Huijbers, T. Hedden, G. A. Marshall, J. P. Chhatwal, D. M. Rentz, R. A. Sperling, and K. Johnson. "Association of Amyloid and Tau with Cognition in Preclinical Alzheimer Disease: A Longitudinal Study." *JAMA Neurol* 76, no. 8 (Aug 1 2019): 915-24. https://dx.doi.org/10.1001/jamaneurol.2019.1424.

He, F., X. Ru, and T. Wen. "Nrf2, a Transcription Factor for Stress Response and Beyond." *Int J Mol Sci* 21, no. 13 (Jul 6 2020). https://dx.doi.org/10.3390/ijms21134777.

Hegde, A. N., and S. C. Upadhya. "The Ubiquitin-Proteasome Pathway in Health and Disease of the Nervous System." *Trends Neurosci* 30, no. 11 (Nov 2007): 587-95. https://dx.doi.org/10.1016/j.tins.2007.08.005.

Heneka, M. T., M. P. Kummer, A. Stutz, A. Delekate, S. Schwartz, A. Vieira-Saecker, A. Griep, D. Axt, A. Remus, T. C. Tzeng, E. Gelpi, A. Halle, M. Korte, E. Latz, and D. T. Golenbock. "Nlrp3 Is Activated in Alzheimer's Disease and Contributes to Pathology in App/Ps1 Mice." *Nature* 493, no. 7434 (Jan 31 2013): 674-8. https://dx.doi.org/10.1038/nature11729.

Hernández, F., M. Díaz-Hernández, J. Avila, and J. J. Lucas. "Testing the Ubiquitin-Proteasome Hypothesis of Neurodegeneration in Vivo." *Trends Neurosci* 27, no. 2 (Feb 2004): 66-9. https://dx.doi.org/10.1016/j.tins.2003.12.002.

Hershko, A., and A. Ciechanover. "The Ubiquitin System." *Annu Rev Biochem* 67 (1998): 425-79. https://dx.doi.org/10.1146/annurev.biochem.67.1.425.

Hochrainer, K., and J. Lipp. "Ubiquitylation within Signaling Pathways in- and Outside of Inflammation." *Thromb Haemost* 97, no. 3 (Mar 2007): 370-7.

Höhn, A., A. Tramutola, and R. Cascella. "Proteostasis Failure in Neurodegenerative Diseases: Focus on Oxidative Stress." *Oxid Med Cell Longev* 2020 (2020): 5497046. https://dx.doi.org/10.1155/2020/5497046.

Holmes, B. B., J. L. Furman, T. E. Mahan, T. R. Yamasaki, H. Mirbaha, W. C. Eades, L. Belaygorod, N. J. Cairns, D. M. Holtzman, and M. I. Diamond. "Proteopathic Tau Seeding Predicts Tauopathy in Vivo." *Proc Natl Acad Sci U S A* 111, no. 41 (Oct 14 2014): E4376-85. https://dx.doi.org/10.1073/pnas.1411649111.

Hong, L., H. C. Huang, and Z. F. Jiang. "Relationship between Amyloid-Beta and the Ubiquitin-Proteasome System in Alzheimer's Disease." *Neurol Res* 36, no. 3 (Mar 2014): 276-82. https://dx.doi.org/10.1179/1743132813y.0000000288.

Honore, P., D. Donnelly-Roberts, M. T. Namovic, G. Hsieh, C. Z. Zhu, J. P. Mikusa, G. Hernandez, C. Zhong, D. M. Gauvin, P. Chandran, R. Harris, A. P. Medrano, W. Carroll, K. Marsh, J. P. Sullivan, C. R. Faltynek, and M. F. Jarvis. "A-740003 [N-(1-{[(Cyanoimino)(5-Quinolinylamino) Methyl]Amino}-2,2-Dimethylpropyl)-2-(3,4-Dimethoxyphenyl)Acetamide], a Novel and Selective P2x7 Receptor Antagonist, Dose-Dependently Reduces Neuropathic Pain in the Rat." *J Pharmacol Exp Ther* 319, no. 3 (Dec 2006): 1376-85. https://dx.doi.org/10.1124/jpet.106.111559.

Humphreys, B. D., and G. R. Dubyak. "Induction of the P2z/P2x7 Nucleotide Receptor and Associated Phospholipase D Activity by Lipopolysaccharide and Ifn-Gamma in the Human Thp-1 Monocytic Cell Line." *J Immunol* 157, no. 12 (Dec 15 1996): 5627-37.

Hyde, S. C., P. Emsley, M. J. Hartshorn, M. M. Mimmack, U. Gileadi, S. R. Pearce, M. P. Gallagher, D. R. Gill, R. E. Hubbard, and C. F. Higgins. "Structural Model of Atp-Binding Proteins Associated with Cystic Fibrosis, Multidrug Resistance and Bacterial Transport." *Nature* 346, no. 6282 (Jul 26 1990): 362-5. https://dx.doi.org/10.1038/346362a0.

Illes, P., and J. Alexandre Ribeiro. "Molecular Physiology of P2 Receptors in the Central Nervous System." *Eur J Pharmacol* 483, no. 1 (Jan 1 2004): 5-17. https://dx.doi.org/10.1016/j.ejphar.2003.10.030.

Illes, P., T. M. Khan, and P. Rubini. "Neuronal P2x7 Receptors Revisited: Do They Really Exist?", *J Neurosci* 37, no. 30 (Jul 26 2017): 7049-62. https://dx.doi.org/10.1523/jneurosci.3103-16.2017.

Irwin, J. A., A. Erisir, and I. Kwon. "Oral Triphenylmethane Food Dye Analog, Brilliant Blue G, Prevents Neuronal Loss in Appswdi/Nos2-/- Mouse Model." *Curr Alzheimer Res* 13, no. 6 (2016): 663-77. https://dx.doi.org/10.2174/1567205013666160208142456 8.

Iwata, M., K. T. Ota, X. Y. Li, F. Sakaue, N. Li, S. Dutheil, M. Banasr, V. Duric, T. Yamanashi, K. Kaneko, K. Rasmussen, A. Glasebrook, A. Koester, D. Song, K. A. Jones, S. Zorn, G. Smagin, and R. S. Duman. "Psychological Stress Activates the Inflammasome Via Release of Adenosine Triphosphate and Stimulation of the Purinergic Type 2x7 Receptor." *Biol Psychiatry* 80, no. 1 (Jul 1 2016): 12-22. https://dx.doi.org/10.1016/j.biopsych.2015.11.026.

Jacobson, K. A., S. Costanzi, B. V. Joshi, P. Besada, D. H. Shin, H. Ko, A. A. Ivanov, and L. Mamedova. "Agonists and Antagonists for P2 Receptors." *Novartis Found Symp* 276 (2006): 58-68; discussion 68-72, 107-12, 275-81. https://dx.doi.org/10.1002/9780470032244.ch6.

Jacobson, K. A., M. F. Jarvis, and M. Williams. "Purine and Pyrimidine (P2) Receptors as Drug Targets." *J Med Chem* 45, no. 19 (Sep 12 2002): 4057-93. https://dx.doi.org/10.1021/jm020046y.

Jamart, C., A. V. Gomes, S. Dewey, L. Deldicque, J. M. Raymackers, and M. Francaux. "Regulation of Ubiquitin-Proteasome and Autophagy Pathways after Acute Lps and Epoxomicin Administration in Mice." *BMC Musculoskelet Disord* 15 (May 22 2014): 166. https://dx.doi.org/10.1186/1471-2474-15-166.

Jankowsky, J. L., D. J. Fadale, J. Anderson, G. M. Xu, V. Gonzales, N. A. Jenkins, N. G. Copeland, M. K. Lee, L. H. Younkin, S. L. Wagner, S. G. Younkin, and D. R. Borchelt. "Mutant Presenilins Specifically Elevate the Levels of the 42 Residue Beta-Amyloid Peptide in Vivo: Evidence for Augmentation of a 42-Specific Gamma Secretase." *Hum Mol Genet* 13, no. 2 (Jan 15 2004): 159-70. https://dx.doi.org/10.1093/hmg/ddh019.

Janks, L., C. V. R. Sharma, and T. M. Egan. "A Central Role for P2x7 Receptors in Human Microglia." *J Neuroinflammation* 15, no. 1 (Nov 21 2018): 325. https://dx.doi.org/10.1186/s12974-018-1353-8.

Jaramillo, M. C., and D. D. Zhang. "The Emerging Role of the Nrf2-Keap1 Signaling Pathway in Cancer." *Genes Dev* 27, no. 20 (Oct 15 2013): 2179-91. https://dx.doi.org/10.1101/gad.225680.113.

Jiang, L. H., J. M. Baldwin, S. Roger, and S. A. Baldwin. "Insights into the Molecular Mechanisms Underlying Mammalian P2x7 Receptor Functions and Contributions in Diseases, Revealed by Structural Modeling and Single Nucleotide Polymorphisms." *Front Pharmacol* 4 (2013): 55. https://dx.doi.org/10.3389/fphar.2013.00055.

Jimenez-Mateos, E. M., M. Arribas-Blazquez, A. Sanz-Rodriguez, C. Concannon, L. A. Olivos-Ore, C. R. Reschke, C. M. Mooney, C. Mooney, E. Lugara, J. Morgan, E. Langa, A. Jimenez-Pacheco, L. F. Silva, G. Mesuret, D. Boison, M. T. Miras-Portugal, M. Letavic, A. R. Artalejo, A. Bhattacharya, M. Diaz-Hernandez, D. C. Henshall, and T. Engel. "Microrna Targeting of the P2x7 Purinoceptor Opposes a Contralateral Epileptogenic Focus in the Hippocampus." *Sci Rep* 5 (Dec 3 2015): 17486. https://dx.doi.org/10.1038/srep17486.

Jimenez-Pacheco, A., M. Diaz-Hernandez, M. Arribas-Blázquez, A. Sanz-Rodriguez, L. A. Olivos-Oré, A. R. Artalejo, M. Alves, M. Letavic, M. T. Miras-Portugal, R. M. Conroy, N. Delanty, M. A. Farrell, D. F. O'Brien, A. Bhattacharya, T. Engel, and D. C. Henshall. "Transient P2x7 Receptor Antagonism Produces Lasting Reductions in Spontaneous Seizures and Gliosis in Experimental Temporal Lobe Epilepsy." *J Neurosci* 36, no. 22 (Jun 1 2016): 5920-32. https://dx.doi.org/10.1523/jneurosci.4009-15.2016.

Jimenez-Pacheco, A., G. Mesuret, A. Sanz-Rodriguez, K. Tanaka, C. Mooney, R. Conroy, M. T. Miras-Portugal, M. Diaz-Hernandez, D. C. Henshall, and T. Engel. "Increased Neocortical Expression of the P2x7 Receptor after Status Epilepticus and Anticonvulsant Effect of P2x7 Receptor Antagonist a-438079." *Epilepsia* 54, no. 9 (Sep 2013): 1551-61. https://dx.doi.org/10.1111/epi.12257.

Jin, H., J. Han, D. Resing, H. Liu, X. Yue, R. L. Miller, K. M. Schoch, T. M. Miller, J. S. Perlmutter, T. M. Egan, and Z. Tu. "Synthesis and in Vitro Characterization of a P2x7 Radioligand [(123)I]Tz6019 and Its Response to Neuroinflammation in a Mouse Model of Alzheimer Disease." *Eur J Pharmacol* 820 (Feb 5 2018): 8-17. https://dx.doi.org/10.1016/j.ejphar.2017.12.006.

Kalia, M. "Dysphagia and Aspiration Pneumonia in Patients with Alzheimer's Disease." *Metabolism* 52, no. 10 Suppl 2 (Oct 2003): 36-8. https://dx.doi.org/10.1016/s0026-0495(03)00300-7.

Kamenetz F, Tomita T, Hsieh H, et al. “APP processing and synaptic function”. Neuron 2003; 37(6): 925–37

Karasawa, A., and T. Kawate. "Structural Basis for Subtype-Specific Inhibition of the P2x7 Receptor." *Elife* 5 (Dec 9 2016). https://dx.doi.org/10.7554/eLife.22153.

Karasawa, A., K. Michalski, P. Mikhelzon, and T. Kawate. "The P2x7 Receptor Forms a Dye-Permeable Pore Independent of Its Intracellular Domain but Dependent on Membrane Lipid Composition." *Elife* 6 (Sep 18 2017). https://dx.doi.org/10.7554/eLife.31186.

Kaushik, S., and A. M. Cuervo. "Proteostasis and Aging." *Nat Med* 21, no. 12 (Dec 2015): 1406-15. https://dx.doi.org/10.1038/nm.4001.

Kawakami, A., T. Nakashima, H. Sakai, A. Hida, S. Urayama, S. Yamasaki, H. Nakamura, H. Ida, Y. Ichinose, T. Aoyagi, I. Furuichi, M. Nakashima, K. Migita, Y. Kawabe, and K. Eguchi. "Regulation of Synovial Cell Apoptosis by Proteasome Inhibitor." *Arthritis Rheum* 42, no. 11 (Nov 1999): 2440-8. https://dx.doi.org/10.1002/1529-0131(199911)42:11<2440::Aid-anr23>3.0.Co;2-0.

Kawate, T., J. C. Michel, W. T. Birdsong, and E. Gouaux. "Crystal Structure of the Atp-Gated P2x(4) Ion Channel in the Closed State." *Nature* 460, no. 7255 (Jul 30 2009): 592-8. https://dx.doi.org/10.1038/nature08198.

Keck, S., R. Nitsch, T. Grune, and O. Ullrich. "Proteasome Inhibition by Paired Helical Filament-Tau in Brains of Patients with Alzheimer's Disease." *J Neurochem* 85, no. 1 (Apr 2003): 115-22. https://dx.doi.org/10.1046/j.1471-4159.2003.01642.x.

Keller, J. N., K. B. Hanni, and W. R. Markesbery. "Impaired Proteasome Function in Alzheimer's Disease." *J Neurochem* 75, no. 1 (Jul 2000): 436-9. https://dx.doi.org/10.1046/j.1471-4159.2000.0750436.x.

Kennedy, C., and G. Burnstock. "Evidence for Two Types of P2-Purinoceptor in Longitudinal Muscle of the Rabbit Portal Vein." *Eur J Pharmacol* 111, no. 1 (Apr 23 1985): 49-56. https://dx.doi.org/10.1016/0014-2999(85)90112-8.

Kessler, B., X. Hong, J. Petrovic, A. Borodovsky, N. P. Dantuma, M. Bogyo, H. S. Overkleeft, H. Ploegh, and R. Glas. "Pathways Accessory to Proteasomal Proteolysis Are Less Efficient in Major Histocompatibility Complex Class I Antigen Production." *J Biol Chem* 278, no. 12 (Mar 21 2003): 10013-21. https://dx.doi.org/10.1074/jbc.M211221200.

Khakh, B. S., and G. Burnstock. "The Double Life of Atp." *Sci Am* 301, no. 6 (Dec 2009): 84-90, 92. https://dx.doi.org/10.1038/scientificamerican1209-84.

Khakh, B. S., and R. A. North. "P2x Receptors as Cell-Surface Atp Sensors in Health and Disease." *Nature* 442, no. 7102 (Aug 3 2006): 527-32. https://dx.doi.org/10.1038/nature04886.

Kim, M., Y. S. Kim, D. H. Kim, T. W. Yang, and O. Y. Kwon. "Major Depressive Disorder in Epilepsy Clinics: A Meta-Analysis." *Epilepsy Behav* 84 (Jul 2018): 56-69. https://dx.doi.org/10.1016/j.yebeh.2018.04.015.

Kim, S. Y., J. H. Moon, H. G. Lee, S. U. Kim, and Y. B. Lee. "Atp Released from Beta-Amyloid-Stimulated Microglia Induces Reactive Oxygen Species Production in an Autocrine Fashion." *Exp Mol Med* 39, no. 6 (Dec 31 2007): 820-7. https://dx.doi.org/10.1038/emm.2007.89.

Knapp, K., M. Zebisch, J. Pippel, A. El-Tayeb, C. E. Müller, and N. Sträter. "Crystal Structure of the Human Ecto-5'-Nucleotidase (Cd73): Insights into the Regulation of Purinergic Signaling." *Structure* 20, no. 12 (Dec 5 2012): 2161-73. https://dx.doi.org/10.1016/j.str.2012.10.001.

Kniepert, A., and M. Groettrup. "The Unique Functions of Tissue-Specific Proteasomes." *Trends Biochem Sci* 39, no. 1 (Jan 2014): 17-24. https://dx.doi.org/10.1016/j.tibs.2013.10.004.

Koegl, M., T. Hoppe, S. Schlenker, H. D. Ulrich, T. U. Mayer, and S. Jentsch. "A Novel Ubiquitination Factor, E4, Is Involved in Multiubiquitin Chain Assembly." *Cell* 96, no. 5 (Mar 5 1999): 635-44. https://dx.doi.org/10.1016/s0092-8674(00)80574-7.

Koeller, H. B., M. E. Ross, and S. B. Glickstein. "Cyclin D1 in Excitatory Neurons of the Adult Brain Enhances Kainate-Induced Neurotoxicity." *Neurobiol Dis* 31, no. 2 (Aug 2008): 230-41. https://dx.doi.org/10.1016/j.nbd.2008.04.010.

Komander, D. "The Emerging Complexity of Protein Ubiquitination." *Biochem Soc Trans* 37, no. Pt 5 (Oct 2009): 937-53. https://dx.doi.org/10.1042/bst0370937.

Kong, Q., T. S. Peterson, O. Baker, E. Stanley, J. Camden, C. I. Seye, L. Erb, A. Simonyi, W. G. Wood, G. Y. Sun, and G. A. Weisman. "Interleukin-1beta Enhances Nucleotide-Induced and Alpha-Secretase-Dependent Amyloid Precursor Protein Processing in Rat Primary Cortical Neurons Via up-Regulation of the P2y(2) Receptor." *J Neurochem* 109, no. 5 (Jun 2009): 1300-10. https://dx.doi.org/10.1111/j.1471-4159.2009.06048.x.

Kontush A, Berndt C, Weber W, et al. Amyloid-β is an antioxidant for lipoproteins in cerebrospinal fluid and plasma. Free Radic Biol Med 2001; 30(1): 119–28

Kudo, T., K. Iqbal, R. Ravid, D. F. Swaab, and I. Grundke-Iqbal. "Alzheimer Disease: Correlation of Cerebro-Spinal Fluid and Brain Ubiquitin Levels." *Brain Res* 639, no. 1 (Mar 7 1994): 1-7. https://dx.doi.org/10.1016/0006-8993(94)91757-4.

Kuhlbrodt, K., J. Mouysset, and T. Hoppe. "Orchestra for Assembly and Fate of Polyubiquitin Chains." *Essays Biochem* 41 (2005): 1-14. https://dx.doi.org/10.1042/eb0410001.

Kukley, M., J. A. Barden, C. Steinhäuser, and R. Jabs. "Distribution of P2x Receptors on Astrocytes in Juvenile Rat Hippocampus." *Glia* 36, no. 1 (Oct 2001): 11-21. https://dx.doi.org/10.1002/glia.1091.

Kwak, M. K., J. M. Cho, B. Huang, S. Shin, and T. W. Kensler. "Role of Increased Expression of the Proteasome in the Protective Effects of Sulforaphane against Hydrogen Peroxide-Mediated Cytotoxicity in Murine Neuroblastoma Cells." *Free Radic Biol Med* 43, no. 5 (Sep 1 2007): 809-17. https://dx.doi.org/10.1016/j.freeradbiomed.2007.05.029.

Kwak, M. K., and T. W. Kensler. "Induction of 26s Proteasome Subunit Psmb5 by the Bifunctional Inducer 3-Methylcholanthrene through the Nrf2-Are, but Not the Ahr/Arnt-Xre, Pathway." *Biochem Biophys Res Commun* 345, no. 4 (Jul 14 2006): 1350-7. https://dx.doi.org/10.1016/j.bbrc.2006.05.043.

Kwak, M. K., N. Wakabayashi, J. L. Greenlaw, M. Yamamoto, and T. W. Kensler. "Antioxidants Enhance Mammalian Proteasome Expression through the Keap1-Nrf2 Signaling Pathway." *Mol Cell Biol* 23, no. 23 (Dec 2003): 8786-94. https://dx.doi.org/10.1128/mcb.23.23.8786-8794.2003.

Lai, M. K., M. G. Tan, S. Kirvell, C. Hobbs, J. Lee, M. M. Esiri, C. P. Chen, and P. T. Francis. "Selective Loss of P2y2 Nucleotide Receptor Immunoreactivity Is Associated with Alzheimer's Disease Neuropathology." *J Neural Transm (Vienna)* 115, no. 8 (Aug 2008): 1165-72. https://dx.doi.org/10.1007/s00702-008-0067-y.

Lam, Y. A., C. M. Pickart, A. Alban, M. Landon, C. Jamieson, R. Ramage, R. J. Mayer, and R. Layfield. "Inhibition of the Ubiquitin-Proteasome System in Alzheimer's Disease." *Proc Natl Acad Sci U S A* 97, no. 18 (Aug 29 2000): 9902-6. https://dx.doi.org/10.1073/pnas.170173897.

Lam, Y. A., W. Xu, G. N. DeMartino, and R. E. Cohen. "Editing of Ubiquitin Conjugates by an Isopeptidase in the 26s Proteasome." *Nature* 385, no. 6618 (Feb 20 1997): 737-40. https://dx.doi.org/10.1038/385737a0.

Lander, G. C., E. Estrin, M. E. Matyskiela, C. Bashore, E. Nogales, and A. Martin. "Complete Subunit Architecture of the Proteasome Regulatory Particle." *Nature* 482, no. 7384 (Jan 11 2012): 186-91. https://dx.doi.org/10.1038/nature10774.

Lanz, T. A., D. B. Carter, and K. M. Merchant. "Dendritic Spine Loss in the Hippocampus of Young Pdapp and Tg2576 Mice and Its Prevention by the Apoe2 Genotype." *Neurobiol Dis* 13, no. 3 (Aug 2003): 246-53. https://dx.doi.org/10.1016/s0969-9961(03)00079-2.

Lauter, H. "[on the Clinical Study and Psychopathology of Alzheimer's Disease. Demonstration of 203 Pathologically-Anatomically Verified Cases]." *Psychiatr Clin (Basel)* 1, no. 2 (1968): 85-108. Zur Klinik und Psychopatholgie der Alzheimerschen Krankheit. Erhebungen an 203 pathologisch-anatomisch verifizierten Fällen.

Lazarowski, E. R. "Vesicular and Conductive Mechanisms of Nucleotide Release." *Purinergic Signal* 8, no. 3 (Sep 2012): 359-73. https://dx.doi.org/10.1007/s11302-012-9304-9.

Lazarowski, E. R., R. C. Boucher, and T. K. Harden. "Mechanisms of Release of Nucleotides and Integration of Their Action as P2x- and P2y-Receptor Activating Molecules." *Mol Pharmacol* 64, no. 4 (Oct 2003): 785-95. https://dx.doi.org/10.1124/mol.64.4.785.

Lazarowski, E. R., W. C. Watt, M. J. Stutts, R. C. Boucher, and T. K. Harden. "Pharmacological Selectivity of the Cloned Human P2u-Purinoceptor: Potent Activation by Diadenosine Tetraphosphate." *Br J Pharmacol* 116, no. 1 (Sep 1995): 1619-27. https://dx.doi.org/10.1111/j.1476-5381.1995.tb16382.x.

Ledent, C., J. M. Vaugeois, S. N. Schiffmann, T. Pedrazzini, M. El Yacoubi, J. J. Vanderhaeghen, J. Costentin, J. K. Heath, G. Vassart, and M. Parmentier. "Aggressiveness, Hypoalgesia and High Blood Pressure in Mice Lacking the Adenosine A2a Receptor." *Nature* 388, no. 6643 (Aug 14 1997): 674-8. https://dx.doi.org/10.1038/41771.

Lee, H. G., S. M. Won, B. J. Gwag, and Y. B. Lee. "Microglial $P2x_7$ Receptor Expression Is Accompanied by Neuronal Damage in the Cerebral Cortex of the Appswe/Ps1de9 Mouse Model of Alzheimer's Disease." *Exp Mol Med* 43, no. 1 (Jan 31 2011): 7-14. https://dx.doi.org/10.3858/emm.2011.43.1.001.

Leigh, P. N., H. Whitwell, O. Garofalo, J. Buller, M. Swash, J. E. Martin, J. M. Gallo, R. O. Weller, and B. H. Anderton. "Ubiquitin-Immunoreactive Intraneuronal Inclusions in Amyotrophic Lateral Sclerosis. Morphology, Distribution, and Specificity." *Brain* 114 (Pt 2) (Apr 1991): 775-88. https://dx.doi.org/10.1093/brain/114.2.775.

León, D., C. Hervás, and M. T. Miras-Portugal. "P2y1 and P2x7 Receptors Induce Calcium/Calmodulin-Dependent Protein Kinase Ii Phosphorylation in Cerebellar Granule Neurons." *Eur J Neurosci* 23, no. 11 (Jun 2006): 2999-3013. https://dx.doi.org/10.1111/j.1460-9568.2006.04832.x.

León-Otegui, M., R. Gómez-Villafuertes, J. I. Díaz-Hernández, M. Díaz-Hernández, M. T. Miras-Portugal, and J. Gualix. "Opposite Effects of P2x7 and P2y2 Nucleotide Receptors on A-Secretase-Dependent App Processing in Neuro-2a Cells." *FEBS Lett* 585, no. 14 (Jul 21 2011): 2255-62. https://dx.doi.org/10.1016/j.febslet.2011.05.048.

Letavic, M. A., B. M. Savall, B. D. Allison, L. Aluisio, J. I. Andres, M. De Angelis, H. Ao, D. A. Beauchamp, P. Bonaventure, S. Bryant, N. I. Carruthers, M. Ceusters, K. J. Coe, C. A. Dvorak, I. C. Fraser, C. F. Gelin, T. Koudriakova, J. Liang, B. Lord, T. W. Lovenberg, M. A. Otieno, F. Schoetens, D. M. Swanson, Q. Wang, A. D. Wickenden, and A. Bhattacharya. "4-Methyl-6,7-Dihydro-4h-Triazolo[4,5-C]Pyridine-Based P2x7 Receptor Antagonists: Optimization of Pharmacokinetic Properties Leading to the Identification of a Clinical Candidate." *J Med Chem* 60, no. 11 (Jun 8 2017): 4559-72. https://dx.doi.org/10.1021/acs.jmedchem.7b00408.

Levy-Lahad, E., A. Lahad, E. M. Wijsman, T. D. Bird, and G. D. Schellenberg. "Apolipoprotein E Genotypes and Age of Onset in Early-Onset Familial Alzheimer's Disease." *Ann Neurol* 38, no. 4 (Oct 1995): 678-80. https://dx.doi.org/10.1002/ana.410380420.

Lindsten, K., and N. P. Dantuma. "Monitoring the Ubiquitin/Proteasome System in Conformational Diseases." *Ageing Res Rev* 2, no. 4 (Oct 2003): 433-49. https://dx.doi.org/10.1016/s1568-1637(03)00031-x.

Lindsten, K., F. M. de Vrij, L. G. Verhoef, D. F. Fischer, F. W. van Leeuwen, E. M. Hol, M. G. Masucci, and N. P. Dantuma. "Mutant Ubiquitin Found in Neurodegenerative Disorders Is a Ubiquitin Fusion Degradation Substrate That Blocks Proteasomal Degradation." *J Cell Biol* 157, no. 3 (Apr 29 2002): 417-27. https://dx.doi.org/10.1083/jcb.200111034.

Lioi, A. B., B. M. Ferrari, G. R. Dubyak, A. Weinberg, and S. F. Sieg. "Human B Defensin-3 Increases Cd86 Expression on Monocytes by Activating the Atp-Gated Channel P2x7." *J Immunol* 195, no. 9 (Nov 1 2015): 4438-45. https://dx.doi.org/10.4049/jimmunol.1401319.

Liu, J., Z. Liao, J. Camden, K. D. Griffin, R. C. Garrad, L. I. Santiago-Pérez, F. A. González, C. I. Seye, G. A. Weisman, and L. Erb. "Src Homology 3 Binding Sites in the P2y2 Nucleotide Receptor Interact with Src and Regulate Activities of Src, Proline-Rich Tyrosine Kinase 2, and Growth Factor Receptors." *J Biol Chem* 279, no. 9 (Feb 27 2004): 8212-8. https://dx.doi.org/10.1074/jbc.M312230200.

Liu, Y., C. L. Hettinger, D. Zhang, K. Rezvani, X. Wang, and H. Wang. "Sulforaphane Enhances Proteasomal and Autophagic Activities in Mice and Is a Potential Therapeutic Reagent for Huntington's Disease." *J Neurochem* 129, no. 3 (May 2014): 539-47. https://dx.doi.org/10.1111/jnc.12647.

Lonskaya, I., N. M. Desforges, M. L. Hebron, and C. E. Moussa. "Ubiquitination Increases Parkin Activity to Promote Autophagic A-Synuclein Clearance." *PLoS One* 8, no. 12 (2013): e83914. https://dx.doi.org/10.1371/journal.pone.0083914.

López Salon, M., L. Morelli, E. M. Castaño, E. F. Soto, and J. M. Pasquini. "Defective Ubiquitination of Cerebral Proteins in Alzheimer's Disease." *J Neurosci Res* 62, no. 2 (Oct 15 2000): 302-10. https://dx.doi.org/10.1002/1097-4547(20001015)62:2<302::Aid-jnr15>3.0.Co;2-l.

Lopez Salon, M., L. Pasquini, M. Besio Moreno, J. M. Pasquini, and E. Soto. "Relationship between Beta-Amyloid Degradation and the 26s Proteasome in Neural Cells." *Exp Neurol* 180, no. 2 (Apr 2003): 131-43. https://dx.doi.org/10.1016/s0014-4886(02)00060-2.

Lustig, K. D., A. K. Shiau, A. J. Brake, and D. Julius. "Expression Cloning of an Atp Receptor from Mouse Neuroblastoma Cells." *Proc Natl Acad Sci U S A* 90, no. 11 (Jun 1 1993): 5113-7. https://dx.doi.org/10.1073/pnas.90.11.5113.

Lustig, K. D., G. A. Weisman, J. T. Turner, R. Garrad, A. K. Shiau, and L. Erb. "P2u Purinoceptors: Cdna Cloning, Signal Transduction Mechanisms and Structure-Function Analysis." *Ciba Found Symp* 198 (1996): 193-204; discussion 04-7. https://dx.doi.org/10.1002/9780470514900.ch11.

Macdonald, R. L., and J. Q. Kang. "Mrna Surveillance and Endoplasmic Reticulum Quality Control Processes Alter Biogenesis of Mutant Gabaa Receptor Subunits Associated with Genetic Epilepsies." *Epilepsia* 53 Suppl 9, no. 0 9 (Dec 2012): 59-70. https://dx.doi.org/10.1111/epi.12035.

Marathe, S., S. Liu, E. Brai, M. Kaczarowski, and L. Alberi. "Notch Signaling in Response to Excitotoxicity Induces Neurodegeneration Via Erroneous Cell Cycle Reentry." *Cell Death Differ* 22, no. 11 (Nov 2015): 1775-84. https://dx.doi.org/10.1038/cdd.2015.23.

Margolis, S. S., G. L. Sell, M. A. Zbinden, and L. M. Bird. "Angelman Syndrome." *Neurotherapeutics* 12, no. 3 (Jul 2015): 641-50. https://dx.doi.org/10.1007/s13311-015-0361-y.

Martin, E., M. Amar, C. Dalle, I. Youssef, C. Boucher, C. Le Duigou, M. Brückner, A. Prigent, V. Sazdovitch, A. Halle, J. M. Kanellopoulos, B. Fontaine, B. Delatour, and C. Delarasse. "New Role of P2x7 Receptor in an Alzheimer's Disease Mouse Model." *Mol Psychiatry* 24, no. 1 (Jan 2019): 108-25. https://dx.doi.org/10.1038/s41380-018-0108-3.

Martìn-Clemente, B., B. Alvarez-Castelao, I. Mayo, A. B. Sierra, V. Dìaz, M. Milán, I. Fariñas, T. Gómez-Isla, I. Ferrer, and J. G. Castaño. "Alpha-Synuclein Expression Levels Do Not Significantly Affect Proteasome Function and Expression in Mice and Stably Transfected Pc12 Cell Lines." *J Biol Chem* 279, no. 51 (Dec 17 2004): 52984-90. https://dx.doi.org/10.1074/jbc.M409028200.

Martínez-Frailes, C., C. Di Lauro, C. Bianchi, L. de Diego-García, Á Sebastián-Serrano, L. Boscá, and M. Díaz-Hernández. "Amyloid Peptide Induced Neuroinflammation Increases the P2x7 Receptor Expression in Microglial Cells, Impacting on Its Functionality." *Front Cell Neurosci* 13 (2019): 143. https://dx.doi.org/10.3389/fncel.2019.00143.

McLarnon, J. G., J. K. Ryu, D. G. Walker, and H. B. Choi. "Upregulated Expression of Purinergic P2x(7) Receptor in Alzheimer Disease and Amyloid-Beta Peptide-Treated Microglia and in Peptide-Injected Rat Hippocampus." *J Neuropathol Exp Neurol* 65, no. 11 (Nov 2006): 1090-7. https://dx.doi.org/10.1097/01.jnen.0000240470.97295.d3.

McNamara, J. O. "Emerging Insights into the Genesis of Epilepsy." *Nature* 399, no. 6738 Suppl (Jun 24 1999): A15-22. https://dx.doi.org/10.1038/399a015.

Mehta, N., M. Kaur, M. Singh, S. Chand, B. Vyas, P. Silakari, M. S. Bahia, and O. Silakari. "Purinergic Receptor $P2x_7$: A Novel Target for Anti-Inflammatory Therapy." *Bioorg Med Chem* 22, no. 1 (Jan 1 2014): 54-88. https://dx.doi.org/10.1016/j.bmc.2013.10.054.

Messemer, N., C. Kunert, M. Grohmann, H. Sobottka, K. Nieber, H. Zimmermann, H. Franke, W. Nörenberg, I. Straub, M. Schaefer, T. Riedel, P. Illes, and P. Rubini. "P2x7 Receptors at Adult Neural Progenitor Cells of the Mouse Subventricular Zone." *Neuropharmacology* 73 (Oct 2013): 122-37. https://dx.doi.org/10.1016/j.neuropharm.2013.05.017.

Metzger, M. W., S. M. Walser, F. Aprile-Garcia, N. Dedic, A. Chen, F. Holsboer, E. Arzt, W. Wurst, and J. M. Deussing. "Genetically Dissecting P2rx7 Expression within the Central Nervous System Using Conditional Humanized Mice." *Purinergic Signal* 13, no. 2 (Jun 2017): 153-70. https://dx.doi.org/10.1007/s11302-016-9546-z.

Michel, A. D., L. J. Chambers, W. C. Clay, J. P. Condreay, D. S. Walter, and I. P. Chessell. "Direct Labelling of the Human P2x7 Receptor and Identification of Positive and Negative Cooperativity of Binding." *Br J Pharmacol* 151, no. 1 (May 2007): 103-14. https://dx.doi.org/10.1038/sj.bjp.0707196.

Millán, J. L. "Alkaline Phosphatases : Structure, Substrate Specificity and Functional Relatedness to Other Members of a Large Superfamily of Enzymes." *Purinergic Signal* 2, no. 2 (Jun 2006): 335-41. https://dx.doi.org/10.1007/s11302-005-5435-6.

Miras-Portugal, M. T., M. Díaz-Hernández, L. Giráldez, C. Hervás, R. Gómez-Villafuertes, R. P. Sen, J. Gualix, and J. Pintor. "P2x7 Receptors in Rat Brain: Presence in Synaptic Terminals and Granule Cells." *Neurochem Res* 28, no. 10 (Oct 2003): 1597-605. https://dx.doi.org/10.1023/a:1025690913206.

Miras-Portugal, M. T., Á Sebastián-Serrano, L. de Diego García, and M. Díaz-Hernández. "Neuronal P2x7 Receptor: Involvement in Neuronal Physiology and Pathology." *J Neurosci* 37, no. 30 (Jul 26 2017): 7063-72. https://dx.doi.org/10.1523/jneurosci.3104-16.2017.

Mishto, M., E. Bellavista, A. Santoro, A. Stolzing, C. Ligorio, B. Nacmias, L. Spazzafumo, M. Chiappelli, F. Licastro, S. Sorbi, A. Pession, T. Ohm, T. Grune, and C. Franceschi. "Immunoproteasome and Lmp2 Polymorphism in Aged and Alzheimer's Disease Brains." *Neurobiol Aging* 27, no. 1 (Jan 2006): 54-66. https://dx.doi.org/10.1016/j.neurobiolaging.2004.12.004.

Mishto, M., C. Ligorio, E. Bellavista, M. Martucci, A. Santoro, M. Giulioni, G. Marucci, and C. Franceschi. "Immunoproteasome Expression Is Induced in Mesial Temporal Lobe Epilepsy."

Biochem Biophys Res Commun 408, no. 1 (Apr 29 2011): 65-70. https://dx.doi.org/10.1016/j.bbrc.2011.03.117.

Monif, M., G. Burnstock, and D. A. Williams. "Microglia: Proliferation and Activation Driven by the P2x7 Receptor." *Int J Biochem Cell Biol* 42, no. 11 (Nov 2010): 1753-6. https://dx.doi.org/10.1016/j.biocel.2010.06.021.

Morawe, T., C. Hiebel, A. Kern, and C. Behl. "Protein Homeostasis, Aging and Alzheimer's Disease." *Mol Neurobiol* 46, no. 1 (Aug 2012): 41-54. https://dx.doi.org/10.1007/s12035-012-8246-0.

Moreira-Souza, A. C. A., C. L. C. Almeida-da-Silva, T. P. Rangel, G. D. C. Rocha, M. Bellio, D. S. Zamboni, R. C. Vommaro, and R. Coutinho-Silva. "The P2x7 Receptor Mediates Toxoplasma Gondii Control in Macrophages through Canonical Nlrp3 Inflammasome Activation and Reactive Oxygen Species Production." *Front Immunol* 8 (2017): 1257. https://dx.doi.org/10.3389/fimmu.2017.01257.

Moshé, S. L., E. Perucca, P. Ryvlin, and T. Tomson. "Epilepsy: New Advances." *Lancet* 385, no. 9971 (Mar 7 2015): 884-98. https://dx.doi.org/10.1016/s0140-6736(14)60456-6.

Mouri, G., E. Jimenez-Mateos, T. Engel, M. Dunleavy, S. Hatazaki, A. Paucard, S. Matsushima, W. Taki, and D. C. Henshall. "Unilateral Hippocampal Ca3-Predominant Damage and Short Latency Epileptogenesis after Intra-Amygdala Microinjection of Kainic Acid in Mice." *Brain Res* 1213 (Jun 5 2008): 140-51. https://dx.doi.org/10.1016/j.brainres.2008.03.061.

Mukhopadhyay, D., and H. Riezman. "Proteasome-Independent Functions of Ubiquitin in Endocytosis and Signaling." *Science* 315, no. 5809 (Jan 12 2007): 201-5. https://dx.doi.org/10.1126/science.1127085.

Mullard, A. "2020 Fda Drug Approvals." *Nat Rev Drug Discov* 20, no. 2 (Feb 2021): 85-90. https://dx.doi.org/10.1038/d41573-021-00002-0.

Munoz, F. M., R. Gao, Y. Tian, B. A. Henstenburg, J. E. Barrett, and H. Hu. "Neuronal P2x7 Receptor-Induced Reactive Oxygen Species Production Contributes to Nociceptive Behavior in Mice." *Sci Rep* 7, no. 1 (Jun 14 2017): 3539. https://dx.doi.org/10.1038/s41598-017-03813-7.

Murrell-Lagnado, R. D., and O. S. Qureshi. "Assembly and Trafficking of P2x Purinergic Receptors (Review)." *Mol Membr Biol* 25, no. 4 (May 2008): 321-31. https://dx.doi.org/10.1080/09687680802050385.

Myeku, N., C. L. Clelland, S. Emrani, N. V. Kukushkin, W. H. Yu, A. L. Goldberg, and K. E. Duff. "Tau-Driven 26s Proteasome Impairment and Cognitive Dysfunction Can Be Prevented Early in Disease by Activating Camp-Pka Signaling." *Nat Med* 22, no. 1 (Jan 2016): 46-53. https://dx.doi.org/10.1038/nm.4011.

Nejime, N., S. Kagota, Y. Tada, K. Nakamura, M. Hashimoto, M. Kunitomo, and K. Shinozuka. "Possible Participation of Chloride Ion Channels in Atp Release from Cancer Cells in Suspension." *Clin*

Exp Pharmacol Physiol 36, no. 3 (Mar 2009): 278-82. https://dx.doi.org/10.1111/j.1440-1681.2008.05060.x.

Nelson, D. W., R. J. Gregg, M. E. Kort, A. Perez-Medrano, E. A. Voight, Y. Wang, G. Grayson, M. T. Namovic, D. L. Donnelly-Roberts, W. Niforatos, P. Honore, M. F. Jarvis, C. R. Faltynek, and W. A. Carroll. "Structure-Activity Relationship Studies on a Series of Novel, Substituted 1-Benzyl-5-Phenyltetrazole P2x7 Antagonists." *J Med Chem* 49, no. 12 (Jun 15 2006): 3659-66. https://dx.doi.org/10.1021/jm051202e.

Neumann, M., D. M. Sampathu, L. K. Kwong, A. C. Truax, M. C. Micsenyi, T. T. Chou, J. Bruce, T. Schuck, M. Grossman, C. M. Clark, L. F. McCluskey, B. L. Miller, E. Masliah, I. R. Mackenzie, H. Feldman, W. Feiden, H. A. Kretzschmar, J. Q. Trojanowski, and V. M. Lee. "Ubiquitinated Tdp-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis." *Science* 314, no. 5796 (Oct 6 2006): 130-3. https://dx.doi.org/10.1126/science.1134108.

Neumann, M., D. M. Sampathu, L. K. Kwong, A. C. Truax, M. C. Micsenyi, T. T. Chou, J. Bruce, T. Schuck, M. Grossman, C. M. Clark, L. F. McCluskey, B. L. Miller, E. Masliah, I. R. Mackenzie, H. Feldman, W. Feiden, H. A. Kretzschmar, J. Q. Trojanowski, and V. M. Lee. "Ubiquitinated Tdp-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis." *Science* 314, no. 5796 (Oct 6 2006): 130-3. https://dx.doi.org/10.1126/science.1134108.

Nguyen, T. P., V. M. Soukup, and B. B. Gelman. "Persistent Hijacking of Brain Proteasomes in Hiv-Associated Dementia." *Am J Pathol* 176, no. 2 (Feb 2010): 893-902. https://dx.doi.org/10.2353/ajpath.2010.090390.

Nijholt, D. A., L. De Kimpe, H. L. Elfrink, J. J. Hoozemans, and W. Scheper. "Removing Protein Aggregates: The Role of Proteolysis in Neurodegeneration." *Curr Med Chem* 18, no. 16 (2011): 2459-76. https://dx.doi.org/10.2174/092986711795843236.

Nilius, B., J. Eggermont, T. Voets, G. Buyse, V. Manolopoulos, and G. Droogmans. "Properties of Volume-Regulated Anion Channels in Mammalian Cells." *Prog Biophys Mol Biol* 68, no. 1 (1997): 69-119. https://dx.doi.org/10.1016/s0079-6107(97)00021-7.

Nörenberg, W., and P. Illes. "Neuronal P2x Receptors: Localisation and Functional Properties." *Naunyn Schmiedebergs Arch Pharmacol* 362, no. 4-5 (Nov 2000): 324-39. https://dx.doi.org/10.1007/s002100000311.

North, R. A. "P2x Receptors." *Philos Trans R Soc Lond B Biol Sci* 371, no. 1700 (Aug 5 2016). https://dx.doi.org/10.1098/rstb.2015.0427.

North, R. A., and A. Verkhratsky. "Purinergic Transmission in the Central Nervous System." *Pflugers Arch* 452, no. 5 (Aug 2006): 479-85. https://dx.doi.org/10.1007/s00424-006-0060-y.

Nuttle, L. C., and G. R. Dubyak. "Differential Activation of Cation Channels and Non-Selective Pores by Macrophage P2z Purinergic Receptors Expressed in Xenopus Oocytes." *J Biol Chem* 269, no. 19 (May 13 1994): 13988-96.

Oddo, S. "The Ubiquitin-Proteasome System in Alzheimer's Disease." *J Cell Mol Med* 12, no. 2 (Apr 2008): 363-73. https://dx.doi.org/10.1111/j.1582-4934.2008.00276.x.

Oddo, S. "The Ubiquitin-Proteasome System in Alzheimer's Disease." *J Cell Mol Med* 12, no. 2 (Apr 2008): 363-73. https://dx.doi.org/10.1111/j.1582-4934.2008.00276.x.

Ohishi, A., Y. Keno, A. Marumiya, Y. Sudo, Y. Uda, K. Matsuda, Y. Morita, T. Furuta, K. Nishida, and K. Nagasawa. "Expression Level of P2x7 Receptor Is a Determinant of Atp-Induced Death of Mouse Cultured Neurons." *Neuroscience* 319 (Apr 5 2016): 35-45. https://dx.doi.org/10.1016/j.neuroscience.2016.01.048.

Okada, S. F., W. K. O'Neal, P. Huang, R. A. Nicholas, L. E. Ostrowski, W. J. Craigen, E. R. Lazarowski, and R. C. Boucher. "Voltage-Dependent Anion Channel-1 (Vdac-1) Contributes to Atp Release and Cell Volume Regulation in Murine Cells." *J Gen Physiol* 124, no. 5 (Nov 2004): 513-26. https://dx.doi.org/10.1085/jgp.200409154.

Okada, Y., T. Okada, M. R. Islam, and R. Z. Sabirov. "Molecular Identities and Atp Release Activities of Two Types of Volume-Regulatory Anion Channels, Vsor and Maxi-Cl." *Curr Top Membr* 81 (2018): 125-76. https://dx.doi.org/10.1016/bs.ctm.2018.07.004.

Olsson, B., R. Lautner, U. Andreasson, A. Öhrfelt, E. Portelius, M. Bjerke, M. Hölttä, C. Rosén, C. Olsson, G. Strobel, E. Wu, K. Dakin, M. Petzold, K. Blennow, and H. Zetterberg. "Csf and Blood Biomarkers for the Diagnosis of Alzheimer's Disease: A Systematic Review and Meta-Analysis." *Lancet Neurol* 15, no. 7 (Jun 2016): 673-84. https://dx.doi.org/10.1016/s1474-4422(16)00070-3.

Olteanu, D., M. B. Hovater, and E. M. Schwiebert. "Intraluminal Autocrine Purinergic Signaling within Cysts: Implications for the Progression of Diseases That Involve Encapsulated Cyst Formation." *Am J Physiol Renal Physiol* 292, no. 1 (Jan 2007): F11-4. https://dx.doi.org/10.1152/ajprenal.00291.2006.

Orioli, E., E. De Marchi, A. L. Giuliani, and E. Adinolfi. "P2x7 Receptor Orchestrates Multiple Signalling Pathways Triggering Inflammation, Autophagy and Metabolic/Trophic Responses." *Curr Med Chem* 24, no. 21 (2017): 2261-75. https://dx.doi.org/10.2174/0929867324666170303161659.

Orre, M., W. Kamphuis, S. Dooves, L. Kooijman, E. T. Chan, C. J. Kirk, V. Dimayuga Smith, S. Koot, C. Mamber, A. H. Jansen, H. Ovaa, and E. M. Hol. "Reactive Glia Show Increased Immunoproteasome Activity in Alzheimer's Disease." *Brain* 136, no. Pt 5 (May 2013): 1415-31. https://dx.doi.org/10.1093/brain/awt083.

Ortega, F., R. Pérez-Sen, E. G. Delicado, and M. T. Miras-Portugal. "P2x7 Nucleotide Receptor Is Coupled to Gsk-3 Inhibition and Neuroprotection in Cerebellar Granule Neurons." *Neurotox Res* 15, no. 3 (Apr 2009): 193-204. https://dx.doi.org/10.1007/s12640-009-9020-6.

Osmulski, P. A., M. Hochstrasser, and M. Gaczynska. "A Tetrahedral Transition State at the Active Sites of the 20s Proteasome Is Coupled to Opening of the Alpha-Ring Channel." *Structure* 17, no. 8 (Aug 12 2009): 1137-47. https://dx.doi.org/10.1016/j.str.2009.06.011.

Pajares, M., A. Cuadrado, and A. I. Rojo. "Modulation of Proteostasis by Transcription Factor Nrf2 and Impact in Neurodegenerative Diseases." *Redox Biol* 11 (Apr 2017): 543-53. https://dx.doi.org/10.1016/j.redox.2017.01.006.

Pankratov, Y., U. Lalo, A. Verkhratsky, and R. A. North. "Vesicular Release of Atp at Central Synapses." *Pflugers Arch* 452, no. 5 (Aug 2006): 589-97. https://dx.doi.org/10.1007/s00424-006-0061-x.

Parr, C. E., D. M. Sullivan, A. M. Paradiso, E. R. Lazarowski, L. H. Burch, J. C. Olsen, L. Erb, G. A. Weisman, R. C. Boucher, and J. T. Turner. "Cloning and Expression of a Human P2u Nucleotide Receptor, a Target for Cystic Fibrosis Pharmacotherapy." *Proc Natl Acad Sci U S A* 91, no. 8 (Apr 12 1994): 3275-9. https://dx.doi.org/10.1073/pnas.91.8.3275.

Parvathenani, L. K., S. Tertyshnikova, C. R. Greco, S. B. Roberts, B. Robertson, and R. Posmantur. "P2x7 Mediates Superoxide Production in Primary Microglia and Is up-Regulated in a Transgenic Mouse Model of Alzheimer's Disease." *J Biol Chem* 278, no. 15 (Apr 11 2003): 13309-17. https://dx.doi.org/10.1074/jbc.M209478200.

Pelegrin, P., and A. Surprenant. "Pannexin-1 Mediates Large Pore Formation and Interleukin-1beta Release by the Atp-Gated P2x7 Receptor." *Embo j* 25, no. 21 (Nov 1 2006): 5071-82. https://dx.doi.org/10.1038/sj.emboj.7601378.

Pelegrin, P., and A. Surprenant. "The P2x(7) Receptor-Pannexin Connection to Dye Uptake and Il-1beta Release." *Purinergic Signal* 5, no. 2 (Jun 2009): 129-37. https://dx.doi.org/10.1007/s11302-009-9141-7.

Peng, Z., Y. Shen, S. Feng, X. Wang, B. N. Chitteti, R. D. Vierstra, and X. W. Deng. "Evidence for a Physical Association of the Cop9 Signalosome, the Proteasome, and Specific Scf E3 Ligases in Vivo." *Curr Biol* 13, no. 13 (Jul 1 2003): R504-5. https://dx.doi.org/10.1016/s0960-9822(03)00439-1.

Petersen, R. C., R. Doody, A. Kurz, R. C. Mohs, J. C. Morris, P. V. Rabins, K. Ritchie, M. Rossor, L. Thal, and B. Winblad. "Current Concepts in Mild Cognitive Impairment." *Arch Neurol* 58, no. 12 (Dec 2001): 1985-92. https://dx.doi.org/10.1001/archneur.58.12.1985.

Piccini, A., S. Carta, S. Tassi, D. Lasiglié, G. Fossati, and A. Rubartelli. "Atp Is Released by Monocytes Stimulated with Pathogen-Sensing Receptor Ligands and Induces Il-1beta and Il-18 Secretion in an Autocrine Way." *Proc Natl Acad Sci U S A* 105, no. 23 (Jun 10 2008): 8067-72. https://dx.doi.org/10.1073/pnas.0709684105.

Pickart, C. M. "Mechanisms Underlying Ubiquitination." *Annu Rev Biochem* 70 (2001): 503-33. https://dx.doi.org/10.1146/annurev.biochem.70.1.503.

Pickart, C. M., and R. E. Cohen. "Proteasomes and Their Kin: Proteases in the Machine Age." *Nat Rev Mol Cell Biol* 5, no. 3 (Mar 2004): 177-87. https://dx.doi.org/10.1038/nrm1336.

Pickart, C. M., and I. A. Rose. "Functional Heterogeneity of Ubiquitin Carrier Proteins." *J Biol Chem* 260, no. 3 (Feb 10 1985): 1573-81.

Pintado, C., M. P. Gavilán, E. Gavilán, L. García-Cuervo, A. Gutiérrez, J. Vitorica, A. Castaño, R. M. Ríos, and D. Ruano. "Lipopolysaccharide-Induced Neuroinflammation Leads to the Accumulation of Ubiquitinated Proteins and Increases Susceptibility to Neurodegeneration Induced by Proteasome Inhibition in Rat Hippocampus." *J Neuroinflammation* 9 (May 4 2012): 87. https://dx.doi.org/10.1186/1742-2094-9-87.

Pitkänen, A., and K. Lukasiuk. "Mechanisms of Epileptogenesis and Potential Treatment Targets." *Lancet Neurol* 10, no. 2 (Feb 2011): 173-86. https://dx.doi.org/10.1016/s1474-4422(10)70310-0.

Pupovac, A., N. J. Geraghty, D. Watson, and R. Sluyter. "Activation of the P2x7 Receptor Induces the Rapid Shedding of Cd23 from Human and Murine B Cells." *Immunol Cell Biol* 93, no. 1 (Jan 2015): 77-85. https://dx.doi.org/10.1038/icb.2014.69.

Rada, P., A. I. Rojo, A. Offergeld, G. J. Feng, J. P. Velasco-Martín, J. M. González-Sancho, M. Valverde Á, T. Dale, J. Regadera, and A. Cuadrado. "Wnt-3a Regulates an Axin1/Nrf2 Complex That Regulates Antioxidant Metabolism in Hepatocytes." *Antioxid Redox Signal* 22, no. 7 (Mar 1 2015): 555-71. https://dx.doi.org/10.1089/ars.2014.6040.

Raiborg, C., and H. Stenmark. "The Escrt Machinery in Endosomal Sorting of Ubiquitylated Membrane Proteins." *Nature* 458, no. 7237 (Mar 26 2009): 445-52. https://dx.doi.org/10.1038/nature07961.

Ralevic, V., and G. Burnstock. "Receptors for Purines and Pyrimidines." *Pharmacol Rev* 50, no. 3 (Sep 1998): 413-92.

Rao, S. N., J. Sharma, R. Maity, and N. R. Jana. "Co-Chaperone Chip Stabilizes Aggregate-Prone Malin, a Ubiquitin Ligase Mutated in Lafora Disease." *J Biol Chem* 285, no. 2 (Jan 8 2010): 1404-13. https://dx.doi.org/10.1074/jbc.M109.006312.

Rassendren, F., and E. Audinat. "Purinergic Signaling in Epilepsy." *J Neurosci Res* 94, no. 9 (Sep 2016): 781-93. https://dx.doi.org/10.1002/jnr.23770.

Rassendren, F., G. Buell, A. Newbolt, R. A. North, and A. Surprenant. "Identification of Amino Acid Residues Contributing to the Pore of a P2x Receptor." *Embo j* 16, no. 12 (Jun 16 1997): 3446-54. https://dx.doi.org/10.1093/emboj/16.12.3446.

Retamal, M. A. "Connexin and Pannexin Hemichannels Are Regulated by Redox Potential." *Front Physiol* 5 (2014): 80. https://dx.doi.org/10.3389/fphys.2014.00080.

Robson, S. C., J. Sévigny, and H. Zimmermann. "The E-Ntpdase Family of Ectonucleotidases: Structure Function Relationships and Pathophysiological Significance." *Purinergic Signal* 2, no. 2 (Jun 2006): 409-30. https://dx.doi.org/10.1007/s11302-006-9003-5.

Rock, K. L., and A. L. Goldberg. "Degradation of Cell Proteins and the Generation of Mhc Class I-Presented Peptides." *Annu Rev Immunol* 17 (1999): 739-79. https://dx.doi.org/10.1146/annurev.immunol.17.1.739.

Rodriguez-Vilariño, S., J. Arribas, P. Arizti, and J. G. Castaño. "Proteolytic Processing and Assembly of the C5 Subunit into the Proteasome Complex." *J Biol Chem* 275, no. 9 (Mar 3 2000): 6592-9. https://dx.doi.org/10.1074/jbc.275.9.6592.

Rojo, A. I., M. R. Sagarra, and A. Cuadrado. "Gsk-3beta Down-Regulates the Transcription Factor Nrf2 after Oxidant Damage: Relevance to Exposure of Neuronal Cells to Oxidative Stress." *J Neurochem* 105, no. 1 (Apr 2008): 192-202. https://dx.doi.org/10.1111/j.1471-4159.2007.05124.x.

Ruan, Z., J. C. Delpech, S. Venkatesan Kalavai, A. A. Van Enoo, J. Hu, S. Ikezu, and T. Ikezu. "P2rx7 Inhibitor Suppresses Exosome Secretion and Disease Phenotype in P301s Tau Transgenic Mice." *Mol Neurodegener* 15, no. 1 (Aug 18 2020): 47. https://dx.doi.org/10.1186/s13024-020-00396-2.

Ryu, J. K., and J. G. McLarnon. "Block of Purinergic P2x(7) Receptor Is Neuroprotective in an Animal Model of Alzheimer's Disease." *Neuroreport* 19, no. 17 (Nov 19 2008): 1715-9. https://dx.doi.org/10.1097/WNR.0b013e3283179333.

Ryu, J. K., and J. G. McLarnon. "A Leaky Blood-Brain Barrier, Fibrinogen Infiltration and Microglial Reactivity in Inflamed Alzheimer's Disease Brain." *J Cell Mol Med* 13, no. 9a (Sep 2009): 2911-25. https://dx.doi.org/10.1111/j.1582-4934.2008.00434.x.

Sáez-Orellana, F., M. C. Fuentes-Fuentes, P. A. Godoy, T. Silva-Grecchi, J. D. Panes, L. Guzmán, G. E. Yévenes, J. Gavilán, T. M. Egan, L. G. Aguayo, and J. Fuentealba. "P2x Receptor Overexpression Induced by Soluble Oligomers of Amyloid Beta Peptide Potentiates Synaptic Failure and Neuronal Dyshomeostasis in Cellular Models of Alzheimer's Disease." *Neuropharmacology* 128 (Jan 2018): 366-78. https://dx.doi.org/10.1016/j.neuropharm.2017.10.027.

Sáez-Orellana, F., P. A. Godoy, C. Y. Bastidas, T. Silva-Grecchi, L. Guzmán, L. G. Aguayo, and J. Fuentealba. "Atp Leakage Induces P2xr Activation and Contributes to Acute Synaptic Excitotoxicity Induced by Soluble Oligomers of B-Amyloid Peptide in Hippocampal Neurons." *Neuropharmacology* 100 (Jan 2016): 116-23. https://dx.doi.org/10.1016/j.neuropharm.2015.04.005.

Saidi, L. J., M. Polydoro, K. R. Kay, L. Sanchez, E. M. Mandelkow, B. T. Hyman, and T. L. Spires-Jones. "Carboxy Terminus Heat Shock Protein 70 Interacting Protein Reduces Tau-Associated Degenerative Changes." *J Alzheimers Dis* 44, no. 3 (2015): 937-47. https://dx.doi.org/10.3233/jad-142094.

Salazar, M., A. I. Rojo, D. Velasco, R. M. de Sagarra, and A. Cuadrado. "Glycogen Synthase Kinase-3beta Inhibits the Xenobiotic and Antioxidant Cell Response by Direct Phosphorylation and Nuclear Exclusion of the Transcription Factor Nrf2." *J Biol Chem* 281, no. 21 (May 26 2006): 14841-51. https://dx.doi.org/10.1074/jbc.M513737200.

Sanz, J. M., P. Chiozzi, D. Ferrari, M. Colaianna, M. Idzko, S. Falzoni, R. Fellin, L. Trabace, and F. Di Virgilio. "Activation of Microglia by Amyloid {Beta} Requires P2x7 Receptor Expression." *J Immunol* 182, no. 7 (Apr 1 2009): 4378-85. https://dx.doi.org/10.4049/jimmunol.0803612.

Sanz, J. M., S. Falzoni, R. Rizzo, F. Cipollone, G. Zuliani, and F. Di Virgilio. "Possible Protective Role of the 489c>T P2x7r Polymorphism in Alzheimer's Disease." *Exp Gerontol* 60 (Dec 2014): 117-9. https://dx.doi.org/10.1016/j.exger.2014.10.009.

Sawada, K., N. Echigo, N. Juge, T. Miyaji, M. Otsuka, H. Omote, A. Yamamoto, and Y. Moriyama. "Identification of a Vesicular Nucleotide Transporter." *Proc Natl Acad Sci U S A* 105, no. 15 (Apr 15 2008): 5683-6. https://dx.doi.org/10.1073/pnas.0800141105.

Schnell, J. D., and L. Hicke. "Non-Traditional Functions of Ubiquitin and Ubiquitin-Binding Proteins." *J Biol Chem* 278, no. 38 (Sep 19 2003): 35857-60. https://dx.doi.org/10.1074/jbc.R300018200.

Schwiebert, E. M. "Abc Transporter-Facilitated Atp Conductive Transport." *Am J Physiol* 276, no. 1 (Jan 1999): C1-8. https://dx.doi.org/10.1152/ajpcell.1999.276.1.C1.

Sebastián-Serrano, Á, L. de Diego-García, C. di Lauro, C. Bianchi, and M. Díaz-Hernández. "Nucleotides Regulate the Common Molecular Mechanisms That Underlie Neurodegenerative Diseases; Therapeutic Implications." *Brain Res Bull* 151 (Sep 2019): 84-91. https://dx.doi.org/10.1016/j.brainresbull.2019.01.031.

Sebastián-Serrano, Á, T. Engel, L. de Diego-García, L. A. Olivos-Oré, M. Arribas-Blázquez, C. Martínez-Frailes, C. Pérez-Díaz, J. L. Millán, A. R. Artalejo, M. T. Miras-Portugal, D. C. Henshall, and M. Díaz-Hernández. "Neurodevelopmental Alterations and Seizures Developed by Mouse Model of Infantile Hypophosphatasia Are Associated with Purinergic Signalling Deregulation." *Hum Mol Genet* 25, no. 19 (Oct 1 2016): 4143-56. https://dx.doi.org/10.1093/hmg/ddw248.

Selkoe, D. J. "Alzheimer Disease and Aducanumab: Adjusting Our Approach." *Nat Rev Neurol* 15, no. 7 (Jul 2019): 365-66. https://dx.doi.org/10.1038/s41582-019-0205-1.

Sevigny, J., P. Chiao, T. Bussière, P. H. Weinreb, L. Williams, M. Maier, R. Dunstan, S. Salloway, T. Chen, Y. Ling, J. O'Gorman, F. Qian, M. Arastu, M. Li, S. Chollate, M. S. Brennan, O. Quintero-Monzon, R. H. Scannevin, H. M. Arnold, T. Engber, K. Rhodes, J. Ferrero, Y. Hang, A. Mikulskis, J. Grimm, C. Hock, R. M. Nitsch, and A. Sandrock. "The Antibody Aducanumab Reduces Aβ Plaques in Alzheimer's Disease." *Nature* 537, no. 7618 (Sep 1 2016): 50-6. https://dx.doi.org/10.1038/nature19323.

Seye, C. I., M. W. Knaapen, D. Daret, C. Desgranges, A. G. Herman, M. M. Kockx, and H. Bult. "7-Ketocholesterol Induces Reversible Cytochrome C Release in Smooth Muscle Cells in Absence

of Mitochondrial Swelling." *Cardiovasc Res* 64, no. 1 (Oct 1 2004): 144-53. https://dx.doi.org/10.1016/j.cardiores.2004.05.016.

Sherman, M. Y., and A. L. Goldberg. "Cellular Defenses against Unfolded Proteins: A Cell Biologist Thinks About Neurodegenerative Diseases." *Neuron* 29, no. 1 (Jan 2001): 15-32. https://dx.doi.org/10.1016/s0896-6273(01)00177-5.

Sherrington, R., E. I. Rogaev, Y. Liang, E. A. Rogaeva, G. Levesque, M. Ikeda, H. Chi, C. Lin, G. Li, K. Holman, T. Tsuda, L. Mar, J. F. Foncin, A. C. Bruni, M. P. Montesi, S. Sorbi, I. Rainero, L. Pinessi, L. Nee, I. Chumakov, D. Pollen, A. Brookes, P. Sanseau, R. J. Polinsky, W. Wasco, H. A. Da Silva, J. L. Haines, M. A. Perkicak-Vance, R. E. Tanzi, A. D. Roses, P. E. Fraser, J. M. Rommens, and P. H. St George-Hyslop. "Cloning of a Gene Bearing Missense Mutations in Early-Onset Familial Alzheimer's Disease." *Nature* 375, no. 6534 (Jun 29 1995): 754-60. https://dx.doi.org/10.1038/375754a0.

Shimura, H., D. Schwartz, S. P. Gygi, and K. S. Kosik. "Chip-Hsc70 Complex Ubiquitinates Phosphorylated Tau and Enhances Cell Survival." *J Biol Chem* 279, no. 6 (Feb 6 2004): 4869-76. https://dx.doi.org/10.1074/jbc.M305838200.

Siebert, A. P., Z. Ma, J. D. Grevet, A. Demuro, I. Parker, and J. K. Foskett. "Structural and Functional Similarities of Calcium Homeostasis Modulator 1 (Calhm1) Ion Channel with Connexins, Pannexins, and Innexins." *J Biol Chem* 288, no. 9 (Mar 1 2013): 6140-53. https://dx.doi.org/10.1074/jbc.M112.409789.

Sluyter, R. "The P2x7 Receptor." *Adv Exp Med Biol* 1051 (2017): 17-53. https://dx.doi.org/10.1007/5584_2017_59.

Sluyter, R., and J. S. Wiley. "Extracellular Adenosine 5'-Triphosphate Induces a Loss of Cd23 from Human Dendritic Cells Via Activation of P2x7 Receptors." *Int Immunol* 14, no. 12 (Dec 2002): 1415-21. https://dx.doi.org/10.1093/intimm/dxf111.

Soltoff, S. P. "Related Adhesion Focal Tyrosine Kinase and the Epidermal Growth Factor Receptor Mediate the Stimulation of Mitogen-Activated Protein Kinase by the G-Protein-Coupled P2y2 Receptor. Phorbol Ester or [Ca2+]I Elevation Can Substitute for Receptor Activation." *J Biol Chem* 273, no. 36 (Sep 4 1998): 23110-7. https://dx.doi.org/10.1074/jbc.273.36.23110.

Song, S., S. Y. Kim, Y. M. Hong, D. G. Jo, J. Y. Lee, S. M. Shim, C. W. Chung, S. J. Seo, Y. J. Yoo, J. Y. Koh, M. C. Lee, A. J. Yates, H. Ichijo, and Y. K. Jung. "Essential Role of E2-25k/Hip-2 in Mediating Amyloid-Beta Neurotoxicity." *Mol Cell* 12, no. 3 (Sep 2003): 553-63. https://dx.doi.org/10.1016/j.molcel.2003.08.005.

Song, S., H. Lee, T. I. Kam, M. L. Tai, J. Y. Lee, J. Y. Noh, S. M. Shim, S. J. Seo, Y. Y. Kong, T. Nakagawa, C. W. Chung, D. Y. Choi, H. Oubrahim, and Y. K. Jung. "E2-25k/Hip-2 Regulates Caspase-12 in Er Stress-Mediated Abeta Neurotoxicity." *J Cell Biol* 182, no. 4 (Aug 25 2008): 675-84. https://dx.doi.org/10.1083/jcb.200711066.

Soni, N., P. Koushal, B. V. Reddy, R. Deshmukh, and P. Kumar. "Effect of Glt-1 Modulator and P2x7 Antagonists Alone and in Combination in the Kindling Model of Epilepsy in Rats." *Epilepsy Behav* 48 (Jul 2015): 4-14. https://dx.doi.org/10.1016/j.yebeh.2015.04.056.

Sperlágh, B., and P. Illes. "P2x7 Receptor: An Emerging Target in Central Nervous System Diseases." *Trends Pharmacol Sci* 35, no. 10 (Oct 2014): 537-47. https://dx.doi.org/10.1016/j.tips.2014.08.002.

Sperlágh, B., A. Köfalvi, J. Deuchars, L. Atkinson, C. J. Milligan, N. J. Buckley, and E. S. Vizi. "Involvement of P2x7 Receptors in the Regulation of Neurotransmitter Release in the Rat Hippocampus." *J Neurochem* 81, no. 6 (Jun 2002): 1196-211. https://dx.doi.org/10.1046/j.1471-4159.2002.00920.x.

Stadtmueller, B. M., and C. P. Hill. "Proteasome Activators." *Mol Cell* 41, no. 1 (Jan 7 2011): 8-19. https://dx.doi.org/10.1016/j.molcel.2010.12.020.

Sugiyama, T., H. Oku, M. Shibata, M. Fukuhara, H. Yoshida, and T. Ikeda. "Involvement of P2x7 Receptors in the Hypoxia-Induced Death of Rat Retinal Neurons." *Invest Ophthalmol Vis Sci* 51, no. 6 (Jun 2010): 3236-43. https://dx.doi.org/10.1167/iovs.09-4192.

Surprenant, A. "Functional Properties of Native and Cloned P2x Receptors." *Ciba Found Symp* 198 (1996): 208-19; discussion 19-22. https://dx.doi.org/10.1002/9780470514900.ch12.

Surprenant, A., and R. A. North. "Signaling at Purinergic P2x Receptors." *Annu Rev Physiol* 71 (2009): 333-59. https://dx.doi.org/10.1146/annurev.physiol.70.113006.100630.

Sutterwala, F. S., Y. Ogura, M. Szczepanik, M. Lara-Tejero, G. S. Lichtenberger, E. P. Grant, J. Bertin, A. J. Coyle, J. E. Galán, P. W. Askenase, and R. A. Flavell. "Critical Role for Nalp3/Cias1/Cryopyrin in Innate and Adaptive Immunity through Its Regulation of Caspase-1." *Immunity* 24, no. 3 (Mar 2006): 317-27. https://dx.doi.org/10.1016/j.immuni.2006.02.004.

Tai, H. C., A. Serrano-Pozo, T. Hashimoto, M. P. Frosch, T. L. Spires-Jones, and B. T. Hyman. "The Synaptic Accumulation of Hyperphosphorylated Tau Oligomers in Alzheimer Disease Is Associated with Dysfunction of the Ubiquitin-Proteasome System." *Am J Pathol* 181, no. 4 (Oct 2012): 1426-35. https://dx.doi.org/10.1016/j.ajpath.2012.06.033.

Takenouchi, T., K. Sekiyama, A. Sekigawa, M. Fujita, M. Waragai, S. Sugama, Y. Iwamaru, H. Kitani, and M. Hashimoto. "P2x7 Receptor Signaling Pathway as a Therapeutic Target for Neurodegenerative Diseases." *Arch Immunol Ther Exp (Warsz)* 58, no. 2 (Apr 2010): 91-6. https://dx.doi.org/10.1007/s00005-010-0069-y.

Tan, Z., X. Sun, F. S. Hou, H. W. Oh, L. G. Hilgenberg, E. M. Hol, F. W. van Leeuwen, M. A. Smith, D. K. O'Dowd, and S. S. Schreiber. "Mutant Ubiquitin Found in Alzheimer's Disease Causes Neuritic Beading of Mitochondria in Association with Neuronal Degeneration." *Cell Death Differ* 14, no. 10 (Oct 2007): 1721-32. https://dx.doi.org/10.1038/sj.cdd.4402180.

Tanaka, K., and M. Kasahara. "The Mhc Class I Ligand-Generating System: Roles of Immunoproteasomes and the Interferon-Gamma-Inducible Proteasome Activator Pa28." *Immunol Rev* 163 (Jun 1998): 161-76. https://dx.doi.org/10.1111/j.1600-065x.1998.tb01195.x.

Tarawneh, R., and D. M. Holtzman. "The Clinical Problem of Symptomatic Alzheimer Disease and Mild Cognitive Impairment." *Cold Spring Harb Perspect Med* 2, no. 5 (May 2012): a006148. https://dx.doi.org/10.1101/cshperspect.a006148.

Thijs, R. D., R. Surges, T. J. O'Brien, and J. W. Sander. "Epilepsy in Adults." *Lancet* 393, no. 10172 (Feb 16 2019): 689-701. https://dx.doi.org/10.1016/s0140-6736(18)32596-0.

Tramutola, A., C. Lanzillotta, M. Perluigi, and D. A. Butterfield. "Oxidative Stress, Protein Modification and Alzheimer Disease." *Brain Res Bull* 133 (Jul 2017): 88-96. https://dx.doi.org/10.1016/j.brainresbull.2016.06.005.

Tsao, H. K., P. H. Chiu, and S. H. Sun. "Pkc-Dependent Erk Phosphorylation Is Essential for P2x7 Receptor-Mediated Neuronal Differentiation of Neural Progenitor Cells." *Cell Death Dis* 4, no. 8 (Aug 1 2013): e751. https://dx.doi.org/10.1038/cddis.2013.274.

Tseng, B. P., K. N. Green, J. L. Chan, M. Blurton-Jones, and F. M. LaFerla. "Abeta Inhibits the Proteasome and Enhances Amyloid and Tau Accumulation." *Neurobiol Aging* 29, no. 11 (Nov 2008): 1607-18. https://dx.doi.org/10.1016/j.neurobiolaging.2007.04.014.

Ulrich, H. D., and H. Walden. "Ubiquitin Signalling in DNA Replication and Repair." *Nat Rev Mol Cell Biol* 11, no. 7 (Jul 2010): 479-89. https://dx.doi.org/10.1038/nrm2921.

Upadhya, S. C., and A. N. Hegde. "Ubiquitin-Proteasome Pathway Components as Therapeutic Targets for Cns Maladies." *Curr Pharm Des* 11, no. 29 (2005): 3807-28. https://dx.doi.org/10.2174/138161205774580651.

Upadhya, S. C., and A. N. Hegde. "Role of the Ubiquitin Proteasome System in Alzheimer's Disease." *BMC Biochem* 8 Suppl 1, no. Suppl 1 (Nov 22 2007): S12. https://dx.doi.org/10.1186/1471-2091-8-s1-s12.

van Leeuwen, F. W., D. P. de Kleijn, H. H. van den Hurk, A. Neubauer, M. A. Sonnemans, J. A. Sluijs, S. Köycü, R. D. Ramdjielal, A. Salehi, G. J. Martens, F. G. Grosveld, J. Peter, H. Burbach, and E. M. Hol. "Frameshift Mutants of Beta Amyloid Precursor Protein and Ubiquitin-B in Alzheimer's and Down Patients." *Science* 279, no. 5348 (Jan 9 1998): 242-7. https://dx.doi.org/10.1126/science.279.5348.242.

Verma, R., L. Aravind, R. Oania, W. H. McDonald, J. R. Yates, 3rd, E. V. Koonin, and R. J. Deshaies. "Role of Rpn11 Metalloprotease in Deubiquitination and Degradation by the 26s Proteasome." *Science* 298, no. 5593 (Oct 18 2002): 611-5. https://dx.doi.org/10.1126/science.1075898.

Vollmayer, P., T. Clair, J. W. Goding, K. Sano, J. Servos, and H. Zimmermann. "Hydrolysis of Diadenosine Polyphosphates by Nucleotide Pyrophosphatases/Phosphodiesterases." *Eur J Biochem* 270, no. 14 (Jul 2003): 2971-8. https://dx.doi.org/10.1046/j.1432-1033.2003.03674.x.

Vossel, K. A., A. J. Beagle, G. D. Rabinovici, H. Shu, S. E. Lee, G. Naasan, M. Hegde, S. B. Cornes, M. L. Henry, A. B. Nelson, W. W. Seeley, M. D. Geschwind, M. L. Gorno-Tempini, T. Shih, H. E. Kirsch, P. A. Garcia, B. L. Miller, and L. Mucke. "Seizures and Epileptiform Activity in the Early Stages of Alzheimer Disease." *JAMA Neurol* 70, no. 9 (Sep 1 2013): 1158-66. https://dx.doi.org/10.1001/jamaneurol.2013.136.

Vossel, K. A., A. J. Beagle, G. D. Rabinovici, H. Shu, S. E. Lee, G. Naasan, M. Hegde, S. B. Cornes, M. L. Henry, A. B. Nelson, W. W. Seeley, M. D. Geschwind, M. L. Gorno-Tempini, T. Shih, H. E. Kirsch, P. A. Garcia, B. L. Miller, and L. Mucke. "Seizures and Epileptiform Activity in the Early Stages of Alzheimer Disease." *JAMA Neurol* 70, no. 9 (Sep 1 2013): 1158-66. https://dx.doi.org/10.1001/jamaneurol.2013.136.

Vultaggio-Poma, V., A. C. Sarti, and F. Di Virgilio. "Extracellular Atp: A Feasible Target for Cancer Therapy." *Cells* 9, no. 11 (Nov 17 2020). https://dx.doi.org/10.3390/cells9112496.

Wang, Y., and E. Mandelkow. "Degradation of Tau Protein by Autophagy and Proteasomal Pathways." *Biochem Soc Trans* 40, no. 4 (Aug 2012): 644-52. https://dx.doi.org/10.1042/bst20120071.

Weisman, G. A., D. Ajit, R. Garrad, T. S. Peterson, L. T. Woods, C. Thebeau, J. M. Camden, and L. Erb. "Neuroprotective Roles of the P2y(2) Receptor." *Purinergic Signal* 8, no. 3 (Sep 2012): 559-78. https://dx.doi.org/10.1007/s11302-012-9307-6.

Welch, B. D., N. G. Carlson, H. Shi, L. Myatt, and B. K. Kishore. "P2y2 Receptor-Stimulated Release of Prostaglandin E2 by Rat Inner Medullary Collecting Duct Preparations." *Am J Physiol Renal Physiol* 285, no. 4 (Oct 2003): F711-21. https://dx.doi.org/10.1152/ajprenal.00096.2003.

Widagdo, J., S. Guntupalli, S. E. Jang, and V. Anggono. "Regulation of Ampa Receptor Trafficking by Protein Ubiquitination." *Front Mol Neurosci* 10 (2017): 347. https://dx.doi.org/10.3389/fnmol.2017.00347.

Wu, P. Y., Y. C. Lin, C. L. Chang, H. T. Lu, C. H. Chin, T. T. Hsu, D. Chu, and S. H. Sun. "Functional Decreases in P2x7 Receptors Are Associated with Retinoic Acid-Induced Neuronal Differentiation of Neuro-2a Neuroblastoma Cells." *Cell Signal* 21, no. 6 (Jun 2009): 881-91. https://dx.doi.org/10.1016/j.cellsig.2009.01.036.

Wujek JR, Dority MD, Frederickson RC, et al. Deposits of Aβ fibrils are not toxic to cortical and hippocampal neurons in vitro. Neurobiol Aging 1996; 17(1): 107–13

Xiang, Z., and G. Burnstock. "Expression of P2x Receptors on Rat Microglial Cells During Early Development." *Glia* 52, no. 2 (Nov 1 2005): 119-26. https://dx.doi.org/10.1002/glia.20227.

Xu, H., T. W. Rösler, T. Carlsson, A. de Andrade, O. Fiala, M. Hollerhage, W. H. Oertel, M. Goedert, A. Aigner, and G. U. Höglinger. "Tau Silencing by Sirna in the P301s Mouse Model of Tauopathy." *Curr Gene Ther* 14, no. 5 (2014): 343-51. https://dx.doi.org/10.2174/1566523214051409261606O2.

Xu, J., F. W. Tse, and A. Tse. "Atp Triggers Intracellular Ca2+ Release in Type Ii Cells of the Rat Carotid Body." *J Physiol* 549, no. Pt 3 (Jun 15 2003): 739-47. https://dx.doi.org/10.1113/jphysiol.2003.039735.

Yamboliev, I. A., L. M. Smyth, L. Durnin, Y. Dai, and V. N. Mutafova-Yambolieva. "Storage and Secretion of Beta-Nad, Atp and Dopamine in Ngf-Differentiated Rat Pheochromocytoma Pc12 Cells." *Eur J Neurosci* 30, no. 5 (Sep 2009): 756-68. https://dx.doi.org/10.1111/j.1460-9568.2009.06869.x.

Yang, W., L. Chen, Y. Ding, X. Zhuang, and U. J. Kang. "Paraquat Induces Dopaminergic Dysfunction and Proteasome Impairment in Dj-1-Deficient Mice." *Hum Mol Genet* 16, no. 23 (Dec 1 2007): 2900-10. https://dx.doi.org/10.1093/hmg/ddm249.

Yip, L., T. Woehrle, R. Corriden, M. Hirsh, Y. Chen, Y. Inoue, V. Ferrari, P. A. Insel, and W. G. Junger. "Autocrine Regulation of T-Cell Activation by Atp Release and P2x7 Receptors." *Faseb j* 23, no. 6 (Jun 2009): 1685-93. https://dx.doi.org/10.1096/fj.08-126458.

Yoshiyama, Y., M. Higuchi, B. Zhang, S. M. Huang, N. Iwata, T. C. Saido, J. Maeda, T. Suhara, J. Q. Trojanowski, and V. M. Lee. "Synapse Loss and Microglial Activation Precede Tangles in a P301s Tauopathy Mouse Model." *Neuron* 53, no. 3 (Feb 1 2007): 337-51. https://dx.doi.org/10.1016/j.neuron.2007.01.010.

Yu, Y., S. Ugawa, T. Ueda, Y. Ishida, K. Inoue, A. Kyaw Nyunt, A. Umemura, M. Mase, K. Yamada, and S. Shimada. "Cellular Localization of P2x7 Receptor Mrna in the Rat Brain." *Brain Res* 1194 (Feb 15 2008): 45-55. https://dx.doi.org/10.1016/j.brainres.2007.11.064.

Zebisch, M., M. Krauss, P. Schäfer, P. Lauble, and N. Sträter. "Crystallographic Snapshots Along the Reaction Pathway of Nucleoside Triphosphate Diphosphohydrolases." *Structure* 21, no. 8 (Aug 6 2013): 1460-75. https://dx.doi.org/10.1016/j.str.2013.05.016.

Zebisch, M., M. Krauss, P. Schäfer, and N. Sträter. "Crystallographic Evidence for a Domain Motion in Rat Nucleoside Triphosphate Diphosphohydrolase (Ntpdase) 1." *J Mol Biol* 415, no. 2 (Jan 13 2012): 288-306. https://dx.doi.org/10.1016/j.jmb.2011.10.050.

Zetterberg, H. "Cerebrospinal Fluid Biomarkers for Alzheimer's Disease: Current Limitations and Recent Developments." *Curr Opin Psychiatry* 28, no. 5 (Sep 2015): 402-9. https://dx.doi.org/10.1097/yco.0000000000000179.

Zetterberg, H. "Review: Tau in Biofluids - Relation to Pathology, Imaging and Clinical Features." *Neuropathol Appl Neurobiol* 43, no. 3 (Apr 2017): 194-99. https://dx.doi.org/10.1111/nan.12378.

Zetterberg, H. "Blood-Based Biomarkers for Alzheimer's Disease-an Update." *J Neurosci Methods* 319 (May 1 2019): 2-6. https://dx.doi.org/10.1016/j.jneumeth.2018.10.025.

Zetterberg, H., D. Wilson, U. Andreasson, L. Minthon, K. Blennow, J. Randall, and O. Hansson. "Plasma Tau Levels in Alzheimer's Disease." *Alzheimers Res Ther* 5, no. 2 (2013): 9. https://dx.doi.org/10.1186/alzrt163.

Zhang, C., R. A. Rissman, and J. Feng. "Characterization of Atp Alternations in an Alzheimer's Disease Transgenic Mouse Model." *J Alzheimers Dis* 44, no. 2 (2015): 375-8. https://dx.doi.org/10.3233/jad-141890.

Zheng, L. M., A. Zychlinsky, C. C. Liu, D. M. Ojcius, and J. D. Young. "Extracellular Atp as a Trigger for Apoptosis or Programmed Cell Death." *J Cell Biol* 112, no. 2 (Jan 1991): 279-88. https://dx.doi.org/10.1083/jcb.112.2.279.

Zimmermann, H. "5'-Nucleotidase: Molecular Structure and Functional Aspects." *Biochem J* 285 (Pt 2), no. Pt 2 (Jul 15 1992): 345-65. https://dx.doi.org/10.1042/bj2850345.

Zimmermann, H. "Ectonucleotidases in the Nervous System." *Novartis Found Symp* 276 (2006): 113-28; discussion 28-30, 233-7, 75-81.

Zinngrebe, J., A. Montinaro, N. Peltzer, and H. Walczak. "Ubiquitin in the Immune System." *EMBO Rep* 15, no. 1 (Jan 2014): 28-45. https://dx.doi.org/10.1002/embr.201338025.

Zubenko, G. S., W. N. Zubenko, S. McPherson, E. Spoor, D. B. Marin, M. R. Farlow, G. E. Smith, Y. E. Geda, J. L. Cummings, R. C. Petersen, and T. Sunderland. "A Collaborative Study of the Emergence and Clinical Features of the Major Depressive Syndrome of Alzheimer's Disease." *Am J Psychiatry* 160, no. 5 (May 2003): 857-66. https://dx.doi.org/10.1176/appi.ajp.160.5.857.