# ON AGING, BEHAVIOR AND THE ROLE OF PA28αβ IN PROTEIN HOMEOSTASIS

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*On Aging, Behavior and the role of PA28aβ in Protein Homeostasis*

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Till Einar, som kämpade för ett samhälle med allas
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*hu*Man*s* will become better when ~~you~~*they* make ~~him~~*themselves*
*try to* see what *they*~~he~~ ~~is~~*are* like

- Anton Chekhov (*and less famous person*)

# ABSTRACT

As life expectancy increases, understanding challenges related to the processes of aging are more relevant than ever. Common age-related diseases progress as consequences of accumulative protein damage and protein aggregates. PA28αβ has previously demonstrated protective effects against proteinopathy and is involved in removal of protein damage early in mammalian embryonic development. In this thesis project, female and male mice overexpressing PA28αβ have been followed and analyzed throughout their lifespan to investigate the molecular function of PA28αβ and what physiological and behavioral effects its overexpression induces.

Herein, the finding of a chaperone-like function of PA28αβ is demonstrated by enhanced aggregation prevention in hippocampal extracts from mice overexpressing PA28αβ. This function correlates to enhanced cognitive capacities represented as improved learning and memory in young adults and as exploratory activity in aging mice, the latter a strong behavioral marker of aging. Thus, we have found a previously unprecedented role of PA28αβ in neuronal protein homeostasis, which improves cognitive behavior in mice, but with altered behavioral outcomes in young and old mice.

The neuronal role of PA28αβ and its cognitive effects combined with PA28αβ's molecular mechanism of preventing protein aggregation, highlight a therapeutical potential of PA28αβ in combating proteinopathies, especially neurogenerative diseases.

## KEYWORDS

Aggregation prevention, Aging, Animal ethics, Cataract, Exploratory behavior, F2 hybrid mice, Healthy aging, Learning and memory, PA28αβ, Proteasome capacity, Sex comparisons, Water-based behavioral tests

# SAMMANFATTNING

Åldrande är den biologiska process av fysiologiska förändringar som ökar risken att dö med stigande ålder. Medellivslängden har ökat dramatiskt i världen de senaste 50 åren och åldersrelaterade sjukdomar och komplikationer är nu mycket vanligt förekommande i befolkningen. Att förstå hur och varför vi åldras är en nyckel för att på bästa sätt kunna bota eller lindra dessa sjukdomar.

Åldrande sker på alla nivåer i kroppen, från molekyl-nivå till organ-nivå och beteende-nivå. Inne i celler kopplas åldrande till att proteinskador ökar vilket gör att proteiner inte kan fungera som de ska i cellens olika processer, bl a för att de kan klumpa ihop sig på ett skadligt sätt. Det tidiga embryot har lika höga nivåer av ålders-relaterade proteinskador som den vuxna individen, men några dagar efter befruktningen, i samband med att cellerna specialiserar sig för att bilda en ny individ, försvinner de plötsligt. PA28αβ behövs för denna embryonala process av skade-utrensning. PA28αβ har också bevisats minska mängden skadliga proteinklumpar i sjukdom som uppstår på grund av skadade proteiner. I detta arbete har PA28αβ studerats för att undersöka potentiella skyddande effekter mot åldrande och åldersrelaterad proteinsjukdom.

För att kunna utforska funktionen av PA28αβ under en hel livslängd har genmodifierade möss med uppreglerat uttryck av PA28αβ analyserats. Eftersom åldrande påverkar kroppen på många olika sätt sträcker sig studien från analyser på molekylär nivå till beteende-tester.

Denna avhandling visar att PA28αβ har en ny, tidigare oupptäckt, roll i hjärnans minnescenter hippocampus, där PA28αβ kan minska mängden proteinklumpar genom att förhindra att de uppstår, en s k chaperon-funktion (förklädes-funktion). Därmed är den välkända rollen av PA28αβ som proteinskade-nedbrytare inte den enda funktionen PA28αβ har, utan PA28αβ kan också samspela med andra proteiner på ett sådant sätt att de inte bildar skadliga proteinklumpar. Denna molekylära funktion korrelerar med förbättrad inlärnings- och minnes-förmåga hos unga vuxna möss. De möss som har mer PA28αβ upprätthåller också ett utforskande beteende som generellt försämras markant med åldrande.

Vi tror att PA28αβ:s två skilda molekylära funktioner, som proteinskade-nedbrytare och som chaperon, kan regleras och ger varierande nytta i olika situationer. Att PA28αβ har en roll i hjärnans funktion har inte heller varit känt sedan tidigare. Dessa upptäckter gör PA28αβ intressant att studera från ett terapeutiskt perspektiv mot sjukdomar som innefattar proteiner som klumpar ihop sig och påverkar kognitiva funktioner såsom bl a Alzheimers och Parkinsons sjukdom.

# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Adelöf, J., Andersson, M., Porritt, M., Petersen, A., Zetterberg, M., Wiseman, J., Hernebring, M.

*PA28aβ overexpression enhances learning and memory of female mice without inducing 20S proteasome activity.*

BMC Neuroscience 2018; 19: 70–85

II. Adelöf, J., Ross, J.M., Lazic, S.E., Zetterberg, M., Wiseman, J., Hernebring, M.

*Conclusions from a behavioral aging study on male and female F2 hybrid mice on age-related behavior, buoyancy in water-based tests, and an ethical method to assess lifespan.*

Aging (Albany, NY) 2019; 11: 7150-7168.

III. Hernebring, M., Adelöf, J., Wiseman, J., Petersen, A., Zetterberg, M.

*$H_2O_2$-induced cataract as a model of age-related cataract: lessons learned from overexpressing the proteasome activator PA28aβ in mouse eye lens.*

Manuscript

IV. Adelöf, J., Wiseman, J., Zetterberg, M., Hernebring, M.

*PA28a overexpressing female mice maintain exploratory behavior and capacity to prevent protein aggregation in hippocampus as they age.*

Manuscript

# CONTENT

# ABBREVATIONS

| | |
|---|---|
| Ad lib | Ad libitum; free-feeding |
| AGE | Advanced glycation end-product |
| ALE | Advanced lipoxidation end-product |
| AMC | 7-amino-4-methylcoumarin |
| AMPK | AMP-activated protein kinase |
| ATP | Adenosine triphosphate |
| CML | Nε-carboxymethyllysine |
| ES cells | Embryonic stem cells |
| $H_2O_2$ | Hydrogen peroxide |
| HSPs | Heat shock proteins |
| MEFs | Mouse embryonic fibroblasts |
| MCO | Metal-catalyzed oxidation |
| MHC-I | Major histocompatibility complex class I |
| Mw | Molecular weight |
| PA28αOE | PA28α overexpressing |
| PN | Proteostasis network |
| ROS | Reactive oxygen species |
| TOR | Target of Rapamycin |
| UPS | Ubiquitin proteasome system |
| WT | Wildtype |

# PRELUDE

For many, aging has predominately negative associations. But to age is a privilege. Aging is a biological process which happens to those who are alive long enough and is distinct from life expectancy which reflects the multifactorial probability to reach a certain age. Before aging is further discussed, it must be acknowledged that by far the best intervention to increase life expectancy is and has always been welfare, including education in how to live well.

We all have a relationship to aging; we have seen it, experienced it and probably imagined it all the way to the end of life as we know it. Philosophical thoughts on life and death are inevitable, but also fundamental with regards to purpose and ethics of research. Measurements of aging involves both lifespan and healthspan, where healthy aging includes delaying the progression of age-related diseases and importantly, upholding quality of life. Aging, or growing old, can also be regarded as a journey towards something new for those who, like Jim Morrison, believe in "a long, prolonged, derangement of the senses in order to obtain the unknown". Immortality and the dilemmas that come with it, are beyond the scope of this thesis and left for a late hour discussion.

# PURPOSE AND DELIMITATIONS

This thesis project was initiated on the basis of the emerging protective effects of proteasome activator PA28αβ in protein homeostasis. Findings from the PhD project of my supervisor Malin Hernebring includes the involvement of PA28αβ in the clearance of protein damage in mouse embryonic stem cells (Hernebring et al. 2013). Damage accumulates in cells as a consequence of living and is transferred from germ cells to the early embryo. However, during mouse embryonic stem cell differentiation, it seems that the damage is cleared in a rejuvenating process (Hernebring et al. 2006). In understanding how cells from old individuals can form young individuals, rejuvenation processes need to be further investigated. The dependency of PA28αβ in damage clearance in embryonic stem cells, as well as its demonstrated protective effects in several disease models, makes PA28αβ interesting from an aging perspective, since one of the hallmarks of aging comprises accumulation of protein damage. Thus, with the rationale that if PA28αβ can decrease the levels of protein damage, could an overexpression of PA28αβ serve as an ongoing resistance against damage accumulation and in such a way decelerate the process of aging?

The purpose of this thesis was to study the role of PA28αβ in aging and disease. Since the effect of aging is physiologically widespread and with the aspiration to assess both lifespan and healthspan, this work stretches from molecular to behavioral level, with PA28α overexpressing hybrid mice as research model. Besides behavioral studies to assess cognitive effects, the major focuses have been to investigate the function of PA28αβ overexpression during aging in i) the heart, to follow up on previous studies, ii) hippocampus, to address behavioral findings and iii) the lens, to study if PA28αβ could have a protective effect in age-related proteinopathy using cataract as model disease.

## RESEARCH QUESTIONS

- Does PA28αβ overexpression influence maximum lifespan, median lifespan or healthspan of the PA28α overexpressing mouse model?
- Does PA28αβ overexpression have an effect on the proteinopathy cataract in the PA28α overexpressing mouse model?
- What is the molecular mechanism of PA28αβ?

## DELIMITATIONS

- In this work, mice have been used as a model organism and translatability of findings to other organisms is unknown.
- The study does not include a knock-out or a knock-down model as a proof of concept to the additive biological functions of the knock-in model.
- At gene level, only the α-subunit of the PA28αβ complex is inserted and is overexpressed, as confirmed by mRNA levels. At protein level, however, both the α-subunit and the β-subunits are upregulated (as shown in Paper I and III). The overexpression of both subunits, in addition to their affinity when folded, makes the assembly of PA28αβ heterodimer complexes likely, although we cannot exclude that the subunits function alone or in other formations.
- The only proteinopathy investigated in this work is cataract and this work does not cover PA28αβ's effects on any other proteinopathy or disease.

# AGING

As a general definition, aging is functional changes accompanied by decreasing fitness and increasing mortality as time elapse. Aging cells lose the ability to repair themselves and remain functional although paradoxically they have the capacity to live forever written in their genetic code, as demonstrated by cancer cells and the cellular reprogramming that happens when germ cells fuse. Thus, before how we age is addressed, it is of importance to reflect upon why we age at all.

## EVOLUTION OF AGING

Theories of the evolution of aging arose early and Alfred Wallace, co-discoverer of natural selection wrote

> *"when one or more individuals have provided a sufficient number of successors, they themselves as consumers of nourishment in a constantly increasing degree, are an injury to those successors. Natural selection therefore weeds them out, and in many cases favors such races that almost immediately die after they have their successors"* (Wallace 1858).

In 1952, Medawar rephrased what Wallace had observed 200 years earlier and stated that natural selection favors traits that are advantageous early in life and concluded that the force of natural selection decreases with age (Medawar 1952). This includes selection of genes that are beneficial early in life and, for example, increase fecundity even if they are proven harmful at old age, as well as genes increasing longevity in species whose offspring require parental aid for survival (Williams 2001, Bourke 2007).

### TO DIE FOR YOUR CHILDREN

The well-known correlation between reproduction and aging can be explained by two different theories, the antagonist pleiotrophy theory which is on population level and the disposable soma theory on individual level. The antagonist pleiotropy theory states that genes promoting early reproduction have the cost of decreased longevity and vice versa that genes resulting in old age have negative effects on development and fecundity. Genes promoting longevity and reproduction late in life

can be selected for by alterations in extrinsic mortality such as reduced environmental pressure. Over generations, these types of selection pressure can also impact the rate of aging (Austad 1988, Williams 2001).

The disposable soma theory, on the other hand, claims that the soma – the body – is a carrier of DNA and that limited amount of resources is either invested in reproduction or in maintenance and longevity (Kirkwood 1977). In accordance, Drosophila females increase their lifespan if kept isolated from males or having their ovaries removed (Maynard Smith 1958). The observed life extension due to restricted caloric intake also indicates a shift in resources towards increased cellular maintenance at the cost of for example lowered reproduction capacity and reduced body size (Sohal et al. 1996).

In both theories above, aging is considered to be unprogrammed. That aging could be programmed is feasible on group level since the fitness of populations is proposedly greater with frequent death and growth cycles. But, natural selection which pushes evolution occurs on an individual level and programmed aging would require an organismal altruism immensely difficult to scientifically prove. Semelparity (dying after reproduction), displayed by for example salmon is not linked to aging nor a sign of programmed aging but rather a reproduction strategy, maximized to lethality, for species with low probability of reproducing more than once (Vijg et al. 2016).

## SENESCENCE AND REJUVENATION

Aging is affected by intertwining processes; senescence, maintenance and rejuvenation. Senescence is gradual deterioration leading to functional decay and is referred to when aging is generally discussed. However, the process of aging is also affected by counteracting effects, maintenance and rejuvenation, which includes mechanisms of regeneration, protection and repair. Simplified, the sum of senescence, maintenance and rejuvenation equals aging. Mechanisms and interventions to slow down the rate of aging and prolong healthspan can therefore aim to decelerate senescence, increase maintenance, enhance rejuvenation or a combination of these three.

The genetic code sets a basic prerequisite of aging rate which is affected by environmental and social conditions as well as lifestyle. For example, factors known to accelerate aging include malnutrition, pollution, smoking, inaccessibility to health care and excessive caloric intake. Maintenance and rejuvenating factors include ex-

ercise, sleeping and healthy diet. As aging does not start at old age, initiation of longevity interventions and healthy aging are most advantageously introduced early in life to stimulate processes to maintain youth-like properties of cells, tissues and organs.

Interventions of aging can be measured by the maximum and/or median lifespan and healthspan. Maximum lifespan extensions involve timewise lengthening of life, median lifespan focuses on shifting a majority to live longer and extending healthspan is prolonging the time before the risk of getting diseases becomes high.

## FOUNTAINS OF YOUTH AND FROZEN DEAD MILLIONAIRES

Scientific research aiming to understand and extend lifespan and healthspan are advancing rapidly, but one-pill solutions, fountains of youth or reviving what is already dead is still science fiction. Specific cellular pathways have been identified to correlate with aging and targeting these by different means such as dietary interventions has proven effective in animal studies. Reducing ad libitum (free-feeding) food intake, long-term by 30-40% without malnutrition, is defined as dietary restriction and caloric restriction for specifically reducing calories. Dietary restriction is well documented to extend both maximum and median lifespan for both invertebrates and vertebrates. Research spanning from budding yeast to mice has unraveled evolutionary conserved nutrient signaling pathways such as insulin/insulin-growth-like factor signaling, amino acid sensing (TOR/AMPK) pathway and histone deacetylation by sirtuins (e.g. Mair et al. 2008, Kapahi et al. 2017). Administration of pharmaceutical drugs for example resveratrol, rapamycin and metformin are known to target these pathways, and retard aging and age-related diseases in animal models (as reviewed in Mouchiroud et al. 2010). Studies on rhesus monkeys confirm the lifespan effects in nonhuman primates and give translatable insights to how caloric restriction would affect human longevity (Anderson et al. 2009, Fontana et al. 2010). As overeating is currently a major cause of health problems, introducing long-term food deprivation as a preventative intervention against age-related disease is most likely not appreciated or achievable. Fortunately, several interventions found to stimulate the same mechanisms as dietary restriction have been confirmed in research models and successfully applied to humans. For example intermittent fasting, fasting mimicking diet and low protein intake, where food sources as well as reduced caloric intake are regulated for periods of time (Brandhorst et al. 2015, Mattison et al. 2017). In addition to dietary interventions, promising rejuvenating strategies include transfer of blood factors as demonstrated by heterochronic parabiosis studies where old mice, systemically connected with young mice, reverse their

aging profile (Conboy et al. 2005). Elimination of senescent cells by senolytic drugs or genetic ablation has also been proven to enhance rejuvenation in research models (Baar et al. 2017).

As aging affects the whole organismal body, anti-aging interventions are most successful when they affect a wide range of biological functions. All processes of aging are interconnected on different physiological levels but to widen the understanding of what happens on a molecular level it is necessary to study cellular processes of aging separately.

## HALLMARKS OF AGING

As aging is known to affect cells in a myriad of ways, research to understand and target these processes is often divided and referred to as hallmarks of aging. Dividing aging into different processes is of course a simplification and understanding how they are connected is as important as understanding them separately. With aging, DNA loses integrity and stability which is reflected by **genomic instability**, **telomere attrition** and **epigenetic alterations**. These processes, together with **cellular senescence** and **stem cell exhaustion** are linked to impairment of cell cycles and the two latter also result in declining tissue regeneration. **Altered intercellular communication** and **deregulated nutrient sensing** involves age-related cellular changes in response to signals such as inflammation or insulin. In addition, and as will be further discussed in the following chapter, **mitochondrial dysfunction** and **loss of proteostasis** are also key components in the aging process (Lopez-Otin et al. 2013, Kennedy et al. 2014).

# PROTEIN HOMEOSTASIS

Protein homeostasis – proteostasis – is the maintenance of proteome integrity within cells. Loss of proteostasis is characterized by accumulating aggregates of non-native proteins and is correlated to aging. The underlying causes leading to formation of aggregates are impairments of protein quality control systems and an increase in damaging factors such as reactive molecules.

## GENERATION OF AND RESPONSES TO PROTEIN DAMAGE

Cells pay a high price for being aerobic. Although oxygen is linked to the efficient energy production of respiration, it also requires major precaution strategies in resisting the challenges associated with having oxygen molecules intracellularly. This chapter begins with the threats to protein homeostasis and continues with how cells counteract them.

### MITOCHONDRIA AND ROS PRODUCTION

As cells and mitochondria age, the respiratory chain loses efficacy, leading to increased electron leakage and reduced ATP production. Electrons leaking from the electron transport chain and reacting with oxygen is the major source of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide ($H_2O_2$) (Brand 2016, Zhao et al. 2019). In cells, these highly reactive molecules can oxidize DNA, lipids and proteins and create detrimental havoc (Sohal et al. 1996, Finkel et al. 2000, Halliwell 2007).

Multiple diseases and physiological functions are associated with ROS. High levels of ROS are linked to cancer, cardiovascular and neurodegenerative diseases and low ROS levels, although less prevalent, are related to specific autoimmune diseases e.g. chronic granulomatous disease (CGD) with patients suffering from immunodeficiency, impaired thyroid and cognitive function (Brieger et al. 2012). Although ROS is generally associated with causing harm, they also have intracellular signaling function which is highly important for cell function. More specifically, local and transi-

ent increases of ROS has a positive effect on cell growth, development and differentiation (Knoefler et al. 2014). High concentrations of ROS however, results in oxidative damage and is referred to as oxidative stress and is known to correlate with aging (Halliwell 2007, Rigoulet et al. 2010).

## OXIDATIVE STRESS AND PROTEIN MODIFICATIONS

ROS can react with proteins in many ways, both reversibly and irreversibly. Reversible modifications are often involved in redox-regulated signaling pathways and may act as a buffering antioxidant system whilst irreversible modifications may interfere with structure and function of proteins (Dahl et al. 2015). Oxidation can result in polypeptide backbone cleavage, cross-linking of amino acids or modifications on amino acids side chains such as advanced glycation end-products (AGEs). Nε-carboxymethyllysine (CML) is a well characterized AGE formed on proteins by several different pathways of glucose oxidation (glycoxidation). In addition, CML can also be a product generated from oxidation of lipids (lipid peroxidation) which is properly termed advanced lipoxidation end-products (ALE; Fu et al. 1996). AGEs on proteins are partly a result of oxidative stress but they may also induce oxidative stress themselves. AGEs are known to increase with age, especially in long lived proteins such as crystallins or collagens. In addition to aging, accumulation of AGEs is associated with a high risk of developing diabetes, inflammation, neurodegeneration and cataract (as reviewed in Baynes 2001, Semba et al. 2010). Other products of irreversible oxidation are carbonyl derivatives which are induced by a metal-catalyzed oxidation (MCO) reaction, forming a highly reactive carbonyl group composed of a carbon double bonded to oxygen on several amino acids (Stadtman 2006). Proteins with highly reactive carbonyl groups (ketones or aldehydes) often have an impaired dysfunctional structure and are found in protein aggregate formations if they escape degradation (Stadtman et al. 2003, Nyström 2005). Carbonylation of proteins increases with age and has been found to play a role in many pathogeneses such as Alzheimer's disease, Parkinson's disease, diabetes, chronic lung disease and renal failure, cancer and cataract (Levine 2002, Dalle-Donne et al. 2003, Nyström 2005, Stadtman 2006).

As a primary defense mechanism against ROS, the cell produces and relies upon antioxidants. Antioxidants, a widely used term, has been defined as any substance that can prevent or delay oxidation of other organic molecules (Halliwell et al. 1995). Antioxidants are however not enough to protect the cell from oxidative stress which calls for the requirement of a complex multicomponent system.

## PROTEOSTASIS NETWORK

The three-dimensional structure of a protein is determined through the properties of the amino-acid building blocks. Hydrophobic effects drives the process that results in the formation with the lowest energy – the native form of a protein (Kellis Jr et al. 1989). Maintaining the integrity of proteins during normal and challenged states, such as environmental stress and metabolic alterations, is key in proteostasis and coordinated by the proteostasis network (PN). The PN consists of multidimensional components which controls protein quality from formation to localization, function and degradation. Under normal conditions the robust PN systems strive to rapidly and dynamically avert any imbalance in proteostasis. Upon stress-induced cellular changes, the adaptive PN systems may alter the point of proteostastic balance to ensure proteome functionality and solubility (Morimoto et al. 2014). If stress becomes chronic, prolongation of this altered proteostasis eventually becomes proteotoxic (Powers et al. 2009, Hipp et al. 2014). Components of the PN include molecular chaperones and co-chaperones, protein clearance mechanisms such as the ubiquitin-proteasome system (UPS) and the autophagy system (Young et al. 2004, Arndt et al. 2007). With age, PN activity declines resulting in lower capacity to buffer against cellular challenges and reduced protein homeostasis. Loss of proteome fidelity contributes to the progression of aging and pathogenesis of age-related, neurodegenerative diseases (Vilchez et al. 2014, Labbadia et al. 2015).

## CHAPERONES

In native form, proteins contain hydrophobic regions buried in the core and since these hydrophobic regions are adhesive, they are prone to form aggregates if exposed. In the highly crowded cell, proteins require help to fold properly, acquire and maintain their active state. Molecular chaperones assist in all steps of protein processing, folding and trafficking, sequestering and disaggregation. Some chaperones are constitutively expressed and others expressed under stress conditions such as heat and oxidative stress (Hartl 1996). Although many chaperones go under the name heat shock proteins (Hsps), they are also induced by conditions other than heat. Small Hsps do not require ATP and sequester proteins to avoid aggregation (Haslbeck et al. 2015). Hsp70s are the most central chaperones in the cell and consist of an ATP binding domain that locks substrates to a binding domain which enables folding, refolding, degradation and sequestering of proteins. Hsp90 is primarily involved in de novo protein folding and the family of Hsp40 act as co-chaperones which bind proteins and recruit Hsp70. Chaperones can bind to many different co-chaperones and form various complexes, resulting in a plethora of protective functions and assembly with other cellular components. For example, chap-

erones are involved in all steps needed for protein degradation, substrate recognition, delivery and attachment to the proteolytic complex, the proteasome (Hartl et al. 2011).

## THE PROTEASOME

Dysfunctional, excessive, damaged or unfolded proteins can be substrates for targeted degradation by attachment of ubiquitin (Arndt et al. 2007). The signal for degradation is held by polymer chains of ubiquitin (poly-ubiquitination) formed by lysine 48(K48)-linking by ubiquitin ligases. Once a substrate is poly-ubiquitinated, it is targeted for degradation by the catalytic proteasome (Pickart et al. 2004). Alternatives to ubiquitin are direct proteasome signals (DPSs) such as amino acid sequences, post-translational modifications and protein charge which can mediate protein degradation signalling (Kudriaeva et al. 2019).

Proteasomes are complexes comprising of the proteasomal core (also referred to as Core Particle and Multicatalytic protease) together with proteasome activators (also referred to as regulatory particles) as demonstrated in figure 1. The constitutive proteasome complex is the 26S which consists of the core 20S and the ATP-dependent proteasome activator 19S (PA700) (Pickering et al. 2012). In eukaryotes, 20S is a 700 kDa barrel structure formed by two outer α-rings on the ends of the barrel and two center β-rings which make up the proteolytic core (Murata et al. 2009). There are three β-subunits that have hydrolytic properties; β1 with caspase-

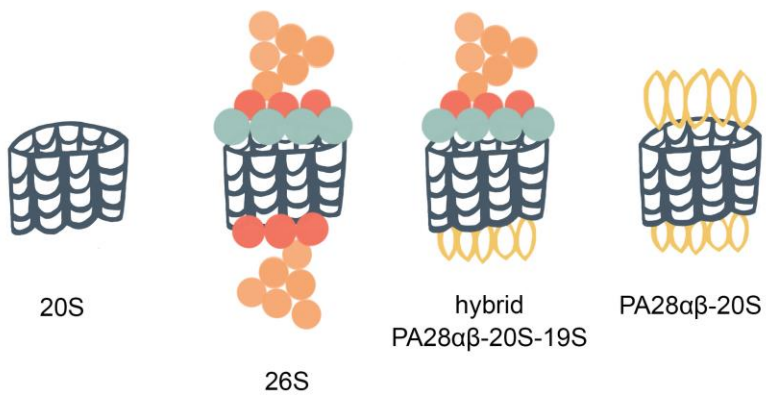



***FIGURE 1. Schematic illustration of proteasome complexes.*** *The 20S proteasome core, the proteasome complex 26S; composed by 20S and two 19S. The hybrid PA28αβ-20S-19S and the PA28αβ-20S with two PA28αβ on both sides of the 20S.*

like activity cleaves proteins after acidic residues (Glu and Asp), β2 with trypsin-like activity cleaves after basic residues (Lys and Arg) and β5 with chymotrypsin-like activity which cleaves after hydrophobic amino acids (Kisselev et al. 1999, Kisselev et al. 2003, Heinemeyer et al. 2004). A gate can be formed by N-terminus protrusions of α-subunits which gives 20S the capacity to ATP-independently degrade nonubiquitinated substrates. The opened conformation of the catalytic core is initiated by proteasome activators. Proteasome activator 19S is composed of two subcomplexes; lid and base. The ATP-dependent base complex composed of 10 subunits is attached close to the gate region on either both or one side of the 20S (Glickman et al. 1998). The lid sub-complex is made up of 9 non-ATPases. One of these (Rpn11) is located in proximity to the pore entry and has deubiquitinating activity which implements the degradation of ubiquitinated substrates (Verma et al. 2002). In vitro analysis of purified 19S, demonstrates that the base has been found to both prevent protein aggregation independently of ATP and to ATP-dependently refold proteins which signifies chaperone-like functions of 19S, physiological relevance of this mechanism is however unknown (Braun et al. 1999).

## THE IMMUNOPROTEASOME

Pro-inflammatory cytokines stimulate the upregulation of an alternative proteolytic core particle called the immunoproteasome (20Si). Upon discovery in the 1990's, the immunoproteasomes were found predominately expressed in antigen presenting cells. Their substitute proteolytic β-subunits LMP2 (β1i), MECL-1 (β2i) and LMP7 (β5i) are incorporated in de novo synthesized core particles which also enables the occasional formation of 20S intermediates composed of both 20S and 20Si β-subunits (Griffin et al. 1998, Guillaume et al. 2010). The proteolytic subunits of the immunoproteasome are known to cleave peptides for major histocompatibility complex class I (MHC-I) antigen presentation (Ferrington et al. 2012). However, recent studies demonstrate that in addition to inflammation, the immunoproteasome is upregulated by oxidative stress and may play a role in the degradation of oxidatively damaged proteins. These findings suggest highly important alternative functions of the immunoproteasome (Pickering et al. 2010, Jung et al. 2013, Petersen et al. 2016).

## PROTEASOME ACTIVATORS

Combinations of core structures (20S and 20Si) and various proteasome activators enable formation of several subtypes of proteasomes with variations of catalytic

properties and substrate preferences under different cellular conditions (Murata et al. 2009). Alternative and ATP-independent proteasome activators are PA200, PA28γ and PA28αβ (Chen et al. 2007, Schmidt et al. 2014). PA200 is known to target transcription factors regulating the ribosomal protein gene Sfp1 and to maintain homeostasis in the mitochondria (Dange et al. 2011). PA28γ is a homoheptamer found in the nucleus which assists in the degradation of small proteins such as p53, p21, SRC-3 (Ma et al. 1992, Li et al. 2007). Studies in knock-out mice demonstrates that reduction of PA28γ expression decreases body size, alters the cell cycle and causes male infertility due to impaired spermatogenesis (Murata et al. 1999, Huang et al. 2016). The third ATP-independent proteasome activator – PA28αβ – will be further discussed in the following sections.

## MEASURING PROTEASOME ACTIVITY

Proteasome capacity can be analyzed by measuring levels of fluorogenic peptides such as AMC (7-amino-4-methylcoumarin) cleaved by the proteolytic sites incorporated into the 20S core. Depending on the substrate, one of three enzymatic peptidases catalyzes the cleavage; β1 for caspase-like activity, β2 for trypsin-like activity and β5 for chymotrypsin-like activity. For example, Suc-LLVY (succinyl-Leu-Leu-Val-Tyr) is a succinyl bound peptide which can be covalently linked to AMC. When Suc-LLVY-AMC is cleaved by β5, the chymotrypsin-like activity of the proteasome in cell lysate can be measured by the levels of AMC. Proteasome independent degradation can be analyzed by adding inhibitors such as epoxomicin which blocks proteolytic sites of the 20S. To perform the assay, cells are extracted in lysis buffers. This however, changes the conditions in which the proteasome is embedded. Composition of the buffer is important for extracting the different complexes since purified proteasome complexes (20S with regulator) have been found to differ in their stability and concentrations of salt and detergent affect the proteasome assembly (Rivett et al. 2002). In the literature, some protocols use a standard salt concentration to assay all proteasome activity (Basaiawmoit 2010, Bonet-Costa et al. 2019). In this work however, different concentrations of salt (NaCl), ATP and detergents have been used in lysis and assay buffers to separate the complexes and individually assess 26S, PA28αβ-20S and 20S activity (Paper I and IV). Depending on lysis buffer(s), comparison between different studies on proteasome activity can be difficult. However, as demonstrated in figure 3 (from Paper IV), we found that β5 peptidase activity in 26S but not PA28αβ-20S increased with age in heart highlighting importance of measuring the activities separately.

# PA28αβ IN PROTEOSTASIS

Proteasome activator PA28αβ (also referred to as 11S and REG) is a regulatory particle to the 20S and 20Si core proteasomes. PA28αβ is composed of 4 α and 3 β-subunits of 28 kDa in a heteroheptamer which can either assemble into a PA28αβ-20S-PA28αβ (homo-PA28αβ-20S) proteasome, a hybrid PA28αβ-20S-19S complex or a less stable and active heptamer of PA28α solely to 20S (Johnston et al. 1997, Tanahashi et al. 2000, Huber et al. 2017). Formations of the complexes are mediated by PA28αβ C-terminus docking into α-subunits binding pockets of the core particle. Opening of the core particle gate is enabled by internal activation loops of PA28α and PA28β which access α-subunits of the 20S and induces conformational alterations (Zhang et al. 1998, Whitby et al. 2000, Förster et al. 2005). In high-throughput screens, sites for post-translational modification (specifically: phosphorylation, methylation, ubiquitination, succinylation and lysine acetylation) have been found for both the α-subunit and the β-subunit, but the functions of these sites are unknown (as found at Phosphosite under PSME1 and, PSME2 (24/3/2020)). The hybrid complex may enhance 26S-like proteasomal functions, as it reportedly could increase the proteolytic activity for specific substrates and also yielded distinct peptide products (Tanahashi et al. 2000). In contrast, the PA28αβ-20S complex has not been found to degrade ubiquitinated substrates (Cascio et al. 2002). In vitro, charge mediated DPS was shown to be most efficiently degraded independently of ATP by proteasome complexes composed of PA28α or PA28γ together with 20S (Kudriaeva et al. 2019).

PA28αβ can be induced by IFN-γ signaling upon intensified immune response together with 20Si and the βi-subunits have been shown to generate peptides for MHC-I (Major histocompatibility complex class I) more efficiently than the 20S (Sijts et al. 2002, Schroder et al. 2004, Shanley et al. 2020). Antigens presented on MHC-I originates from cytosolic peptides and are important for the immune system to recognize virus infected and tumor cells (Janeway 1992, Hewitt 2003). No additive effect in antigen presentation has been found upon cooperation between the 20Si and PA28αβ, but studies show that the complex generated smaller and more hydrophobic epitopes which resulted in more heterogeneous array of antigens (Strehl et al. 2005, de Graaf et al. 2011, Raule et al. 2014). Nonetheless, it has been elucidated that PA28αβ is associated to the endoplasmic reticulum (ER) membrane indicating a peptide delivery chaperone function with PA28αβ physically linking the proteasome and cleaving peptides to the ER for MHC-1 loading (Yamano et al. 2002).

## PA28αβ IS UPREGULATED BY OXIDATIVE STRESS

PA28αβ has been found present in a variety of tissues and organs including immune privileged sites which never evoke an immune response upon infection which indicates additional functions unrelated to antigen processing (Noda et al. 2000, Kapphahn et al. 2007). PA28αβ was also found upregulated by oxidative stress though Nrf2 signal transduction pathway. This upregulation of PA28αβ was linked to reduced oxidative damage and inhibition of protein aggregation, demonstrating a protective role of PA28αβ during oxidative stress (Pickering et al. 2012). In addition, during embryonic stem cell differentiation, increased expression of PA28αβ and 20Si together with enhanced proteasomal activity, coincided with clearance and reduction of damaged protein levels (Hernebring et al. 2006). Conversely, inhibition of PA28α expression by miRNA increased protein damage highlighting the importance of PA28αβ in rejuvenating the early embryo (Hernebring et al. 2013).

## PA28αβ IN PROTEINOPATHIES

Proteasome dysfunction is observed in proteinopathies. In experimental model of cardiac myopathy, cultured neonatal rat cardiomyocytes overexpressing PA28αβ enhanced the proteasomal degradation of misfolded proteins (Li et al. 2011a). In this study, degradation efficiency was measured by fusing GFP to degron CL1 (GFPdgn). Degron is a misfolded substrate which requires unfolding by chaperones followed by proteasomal proteolytic activity for degradation (Bence et al. 2001). Since the degron measurement of degradation efficiency is dependent on both chaperone function and the proteasome, it is a proteasome unspecific method to assess proteolytic activity. The enhanced degradation efficiency by PA28αβ overexpression could therefore be induced by improved unfolding of degron, enhanced proteasome activity or a combination of both.

Furthermore, PA28αβ have been overexpressed in cultured neonatal rat cardiomyocytes treated with $H_2O_2$ to induce oxidative stress. In these conditions, PA28αβ overexpression reduced the accumulation of endogenously damaged proteins caused by the oxidative stress. (Li et al. 2011a). In accordance with these results, knocking down PA28α in cultured mice cardiomyocytes resulted in the accumulation of protein aggregates (Li et al. 2011b). Together, these findings demonstrate that PA28αβ protects against oxidative damage in cultured cells. The protective effects of PA28αβ have also been investigated in a cardiac proteinopathy mouse model with ischemia/reperfusion injury. In this in vivo model, PA28α overexpression limited the infarct size and the reperfusion injury, and prolonged the lifespan of the mice. In accordance with findings in cultured cells, heart extracts from mice

overexpressing PA28α were found to have reduced protein aggregate formations (Li et al. 2011b).

## Additional functions of PA28αβ

Once an aggregate is formed it can be reduced by degradation or disaggregation. But, in addition to degradation, different mechanisms can inhibit damaged proteins to aggregate, such as maintenance of protein stuctures or hindering proteins to aggregate. Chaperones are essential for protein degradation as they can destabilize protein structures to make them more accessible for proteasomal degradation. In addition, chaperones can refold proteins back to their innate structures and also prevent aggregates from forming by preventing disturbing interactions that could lead to aggregation. Previous studies highlight that overexpression of PA28αβ inhibited aggregate formation. But since the degron approach for measuring degradation is dependent on chaperones, it is unclear if PA28αβ actually enhances degradation directly or if it enhances the possibility of proteins to be degraded. In line with this, PA28αβ has been found to be essential in Hsp90 mediated refolding of denatured luciferase, together with Hsc70 and Hsp40 (Minami et al. 2000). This finding demonstrates the possibility of a chaperone-like function of PA28αβ and an alternative mechanism as to how overexpression of PA28α protects against oxidative stress and proteinopathies.

## Effect of PA28αβ in heart and hippocampus

In this work, the effects of PA28αβ overexpression have been analyzed in heart and hippocampus from young/ mature adult, middle-age and old female and male mice. The heart was selected because of previous work demonstrating PA28αβ protective effects against cardiomyopathy, oxidatively induced damage and protein aggregation in the heart/ cardiomyocytes (Li et al. 2011a, Li et al. 2011b). The hippocampus was selected for its importance in relation to behaviors enabling the linkage of molecular function to healthy aging through biochemical and phenotypic assay. The PA28αOE mice have the constitutive promotor CAG driving the expression of a murine PA28α gene inserted into the *Rosa26* locus (Paper I and on page 54 in Methodology). Overexpression was stable with age and no sex differences in PA28α levels were detected (Figure 2, from Paper IV)). The ratio of PA28α protein levels in PA28αOE compared to WT mice was 7-fold higher in heart and 5-fold higher in hippocampus. Since the levels are relative, differences in overexpression between

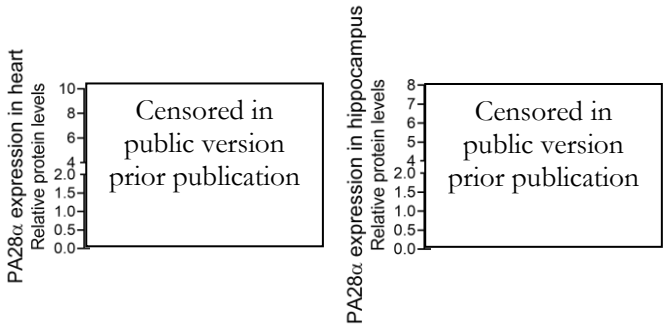



***FIGURE 2.*** *Expression of PA28α in heart and hippocampus of PA28αOE and WT mice. In hippocampus levels of PA28α in females increased over time in PA28αOE and WT. WT females and males differed in expression with age. Relative protein levels were obtained by western blotting of tissue extracts from female and male C57BL/6N × BALB/c F2 hybrid mice, except 4 months hippocampal extracts which are from C57BL/6N mice. (Paper IV; Modified Fig. 2a).*

tissues could either originate from inequivalent innate levels or overexpression, or protein stability in cells. Regarding innate PA28α levels in the hippocampus of female and male WT mice, the expression demonstrated opposite directions with age for the sexes and there was an increase in PA28α levels from 7 to 22 months of age in female mice, but not male mice. Interestingly, a similar trend of age-related increase of PA28α expression was found in PA28αOE females. As PA28αβ is closely linked to proteasomal degradation and known as an alternative activator to the 20S core, peptidase activity for the 20S, 26S and PA28αβ-20S complexes were measured separately to assess the effect of overexpressing PA28α on proteasomal activity (Figure 3, from Paper IV). To verify that the method measured PA28αβ-20S activity, cell lysate from IFN-γ treated MEF was used as positive control (Paper I, Supp. Fig. 11). Surprisingly, the PA28αβ-20S activity in both heart and hippocampus was lower in PA28αOE as compared with WT mice. Also interestingly, PA28αβ-20S capacity increased with age in heart but not hippocampus, an increase which did not correlate with the levels of PA28α expression. The proteolytic capacity of 26S and 20S proteasomes were the same in extracts from PA28αOE and WT mice and did not significantly decline with age.

PA28αβ has previously been found to be strongly associated with degradation of protein damage. Therefore, we analyzed the levels of two different irreversible damage induced modifications; carbonylated proteins (Figure 4a, from Paper IV) and CML, an advanced glycation end-product (Figure 4b, from Paper IV). PA28α overexpression did not reduce the levels of protein damage in heart or hippocampus as no differences were observed between PA28αOE and WT mice. Protein

damage is known to increase with age and the levels of both protein carbonyls and CML accordingly increased from 7 to 22 months of age in the heart. However, the protein damage load of hippocampal extracts did unexpectedly not increase upon aging.

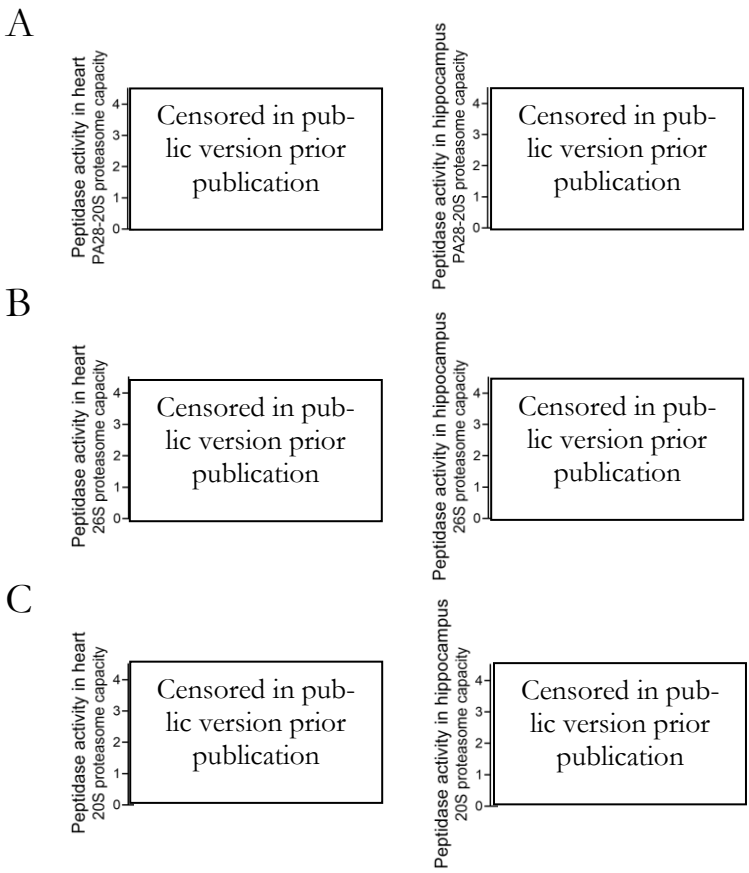



***FIGURE 3.*** *Proteasome activity in heart and hippocampus of PA28αOE and WT mice. No sex effects were obtained and therefore the female and male data was pooled (Paper IV; Supp. Fig. 2). Activity in heart were analysed from 7-, 15- and 22-month-old animals, and in the hippocampus from 15- and 22-month-old animals A) Peptidase activity in PA28αβ-20S decreased in PA28αOE mice in both heart and hippocampus. In heart, PA28αOE expression increased for both PA28αOE and WT mice with age. There were no differences between PA28αOE and WT animals in peptidase activity in B) 26S or C) 20S. (Paper IV; Fig. 2b,c,d).*

Aggregate formation can be avoided by proteasomal degradation of damaged proteins or by inhibiting proteins from aggregating through for example chaperone functions like unfolding/refolding or sequestering. Under conditions of oxidative stress, PA28αβ overexpression has been shown to protect against protein damage in models of disease. Results from the proteasome activities in this study however, indicate that the protective effects of PA28αβ may be independent of proteolytic capacity since overexpression did not enhance proteasome activity. Therefore, it was of interest to investigate if PA28α overexpressing mice had an alternative function, similar to chaperone function, which could impact protein aggregation and proteostasis. Aggregation prevention was assayed by measuring aggregation of luciferase in hippocampal extracts. The assay could not be performed in heart extracts however, likely due to coagulation factors present in protein extracts counteracting their ability to prevent luciferase aggregation. As shown in Figure 5 (from Paper IV), aggregation prevention was enhanced and maintained with age in PA28αOE females but not in males when compared to WT littermates.

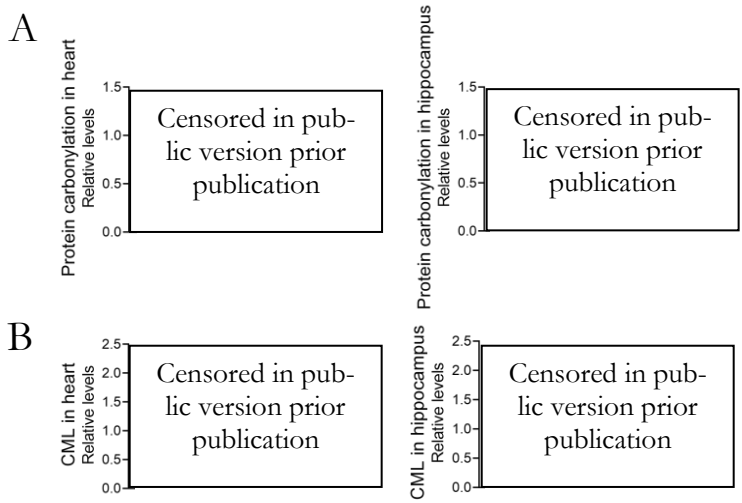



***FIGURE 4.*** *Protein damage in heart and hippocampus of PA28αOE and WT mice. The sexes were pooled because no differences between females and males were found (Paper IV; Supp. Fig. 3). The levels of A) carbonylated proteins and B) CML was the same in extracts of heart and hippocampus from PA28αOE and WT mice (Paper IV; Fig. 3). In heart, both protein carbonylation and CML increased with age, in the hippocampus however, no age-related effect was detected. PA28αOE mice had, in the heart, higher levels of protein carbonylation at 7 months and lower levels of CML at 15 months, as compared to WT mice. All extracts were taken from hybrid mice, except 4 months hippocampal extracts which were from C57BL/6N mice. (Paper VI; Fig. 3).*

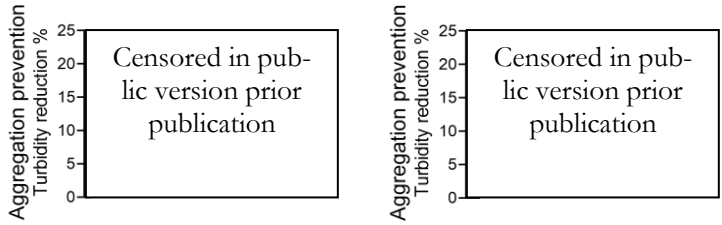



***FIGURE 5.*** *Aggregation prevention in female and male PA28αOE and WT mice analyzed by turbidity reduction of luciferase. The percentage of non-aggregated luciferase at 42°C was higher in PA28α overexpressing females as compared to WT females, PA28α overexpressing and WT males. Hippocampal extracts from males 4 months were on C57BL/6N background, all other were from C57BL/6N × BALB/c F2 hybrid mice. (Paper IV, Fig. 1 and Supp. Fig. 1).*

The clearance of protein damage in embryonic stem cells was found dependent on PA28αβ's proteasome activity and linked to the rejuvenation process of young offspring. In this study however, overexpressing PA28α did not reduce protein damage load with age or affect lifespan as shown in Figure 6 (from Paper IV).

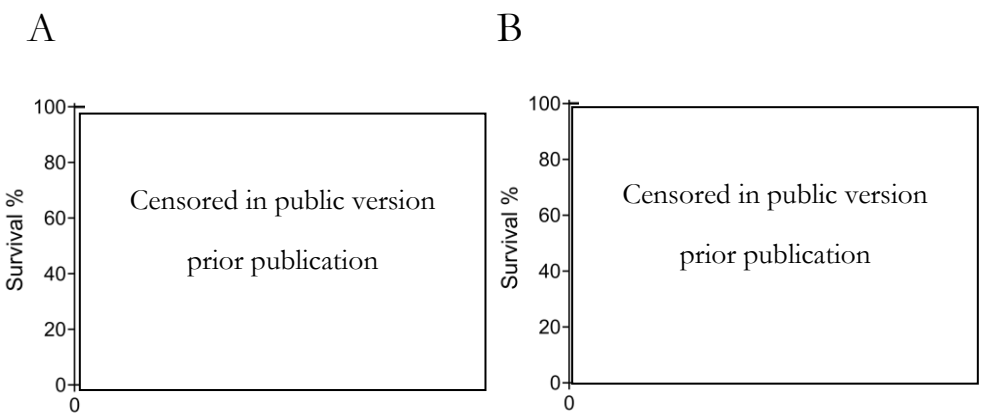



***FIGURE 6.*** *Lifespan of A) female and B) male, PA28αOE and WT littermates. PA28α overexpression did not affect the lifespan of mice. The lifespan is displayed by two survival curves representing minimum lifespan and maximum lifespan which are dependent on categorization of euthanized animals as described in Paper II and on p. 60. (Paper IV, Fig. 4).*

## PA28α OVEREXPRESSION RESULTS IN ENHANCED CHAPERONE-LIKE FUNCTION IN FEMALE BUT NOT MALE HIPPOCAMPUS

Our results demonstrated a sex difference in chaperone-like function of PA28αOE mice. This may be addressed by different conclusions i) the mechanism of PA28α may only be visible in females, but the finding may be translatable to other organisms in a sex-independent manner or ii) there may be a sex-dependent reason for why the effect is only observed in female mice and this may or may not be translatable to other species.

## SPECULATIONS REGARDING THE SEX DIFFERENCE OF PA28α OVEREXPRESSION IN HIPPOCAMPUS

Sex identity for 98-99% of the human population refers to genetic, gonadal and genital endowment (3G) being aligned into females or males (Joel 2012). The 3G-sex model highlights that sex differences are almost completely dimorphic and by this concludes that no organ, except reproductive, exists in binary form. Primarily originating from the genitals, systemically dispersed sex hormones drive secondary sex characteristics as well as impacting organs and tissues in various ways. Since the brain is not dimorphic, nor has any part of the brain been found to be completely dimorph, there is actually no "female" or a "male brain", but rather a unique mosaic of regions affected by female and male sex hormones. This is emphasized by the considerable distribution overlap, allomorphism, that has been found for all sex differences in the brain (Cosgrove et al. 2007, Joel 2012).

Sex differences originating from neurogenesis have been heavily studied and found to play important roles for sex characteristics and behavior in adulthood. A high level of rigidity is required for functions that occur in early development to exist throughout infancy to puberty, adulthood and aging. In addition, many neuronal and behavioral sex differences have been demonstrated to continuously change as sex differences from genes to behavior can be persistent or transient and context dependent or independent (Rippon et al. 2014, Joel et al. 2017). For example, females have greater density of dendritic spines in the hippocampus in comparison to males, but under stressful conditions the opposite is found (Shors et al. 2001). The chaperone-like function in PA28αOE females was maintained from young to old age, suggesting persistency and certain context-independency. However, as stressful and hormonal conditions can impact brain regions, including the hippocampus, of females and males differently, cellular or structural environments found in only female mice may induce or allow for a PA28αβ chaperone-like function.

Estrogen is the main female sex hormone and has been found to impact both lifespan and healthspan. Estradiol has enhanced hippocampal function and memory formation (Frick et al. 2018) and administration of 17-α estradiol extended the lifespan in mice although only seen in males (Garratt et al. 2018). We hypothesized that the PA28α chaperone-like function might be linked to estradiol signaling, and if so, this would be detected by comparing PA28αOE females to WT females. Therefore, in Paper I, levels of β-estradiol in serum and hippocampal S105-phosphorylated estrogen receptor were analyzed, but there were no differences between samples from 7-month-old PA28αOE and WT females (Paper I, Additional file 9). These results indicate that the molecularly improved function related to PA28α overexpression cannot be explained by increased levels of β-estradiol or phosphorylated estrogen receptor. If estradiol however, intracellularly triggers a pathway connecting to PA28αβ remains unknown. These are however only speculations and further investigations are required to address the sex differences of PA28αβ in hippocampus.

## THE PROTEINOPATHY CATARACT AND PA28αβ

Cataract is a proteinopathy which causes visual impairments and affects one out of two individuals at old age (Kahn 1977). Cataract is not reversible and if left untreated it can cause blindness. The only therapeutically successful approach available today is surgery and in Sweden, cataract surgery is the most common of all surgical operations (Behndig 2019). Because of limited access to cataract surgery worldwide, cataract is the leading global cause of blindness (35% in 2015; Bourne 2017). Cataract is easily identified by clouded grey lenses formed by light scattering aggregates of crystallin proteins (Beebe et al. 2010). Crystallins can lose their stability and polymerize into high molecular weight (Mw) protein aggregates as a consequence of oxidative stress. The formation of cataract can be biochemically characterized through the oxidatively damaged proteins and how their aggregation decreases protein solubility (Truscott 2005, Moreau et al. 2012). In Paper III, we use two approaches to induce and assess cataract to investigate if PA28αβ overexpression can protect against the oxidative stress or protein aggregation causing this proteinopathy. The two approaches to induce cataract are further examined on page 61 in Methodology.

### IN VIVO AND EX VIVO INDUCTION OF CATARACT IN OCULAR LENSES OF PA28α OVEREXPRESSING MICE

In the ocular lens of PA28αOE mice, both subunits; PA28α and PA28β were found upregulated at the protein level, enabling possible formation of PA28αβ complex (Paper III; Fig. 1). Cataract was induced by age in vivo and at 7, 15 and 22 months of age, ocular lenses from PA28αOE and WT littermates were analyzed for degree of cataract followed by storage in -80°C. In parallel, lenses from 3-4 months old mice were dissected and cultured for ex vivo hydrogen peroxide ($H_2O_2$) induction of cataract. Exposure to $H_2O_2$ introduced opacity within 7 days and cataract like progression of ocular lenses from both PA28αOE and WT were assessed. There was however no difference between PA28αOE and WT ocular lenses in either onset or degree of cataract for in vivo or ex vivo induced cataract (Figure 7, from Paper III).

However, an aggregation prevention effect of PA28α overexpression might have been counteracted by the large amount of crystallins in the lens. In the heart, R120G mutation of αB-crystallins causes desmin-related myopathy. Mouse models of this specific proteinopathy accumulate protein aggregates and have a reduced lifespan. When PA28α was overexpressed in this model, the deteriorating effects of the αB-crystallin mutation was counteracted resulting in inhibition of protein aggregation and prolongation of lifespan (Li et al. 2011b). These results indicate that PA28αβ compensates for the effect of R120G mutation of αB-crystallin and suggest similar functions. In accordance with this, in vitro α-crystallins have been found to have anti-aggregation properties through sequestration of non-native proteins and collaborating with ATP-dependent chaperones (Haslbeck et al. 2016). Therefore, a chaperone-like function of PA28αβ may be difficult to confirm as crystallins are highly abundant in the lens (90% of all proteins; Haslbeck et al. 2016), and this could explain that no effect of PA28αβ overexpression was found in the cataract models.

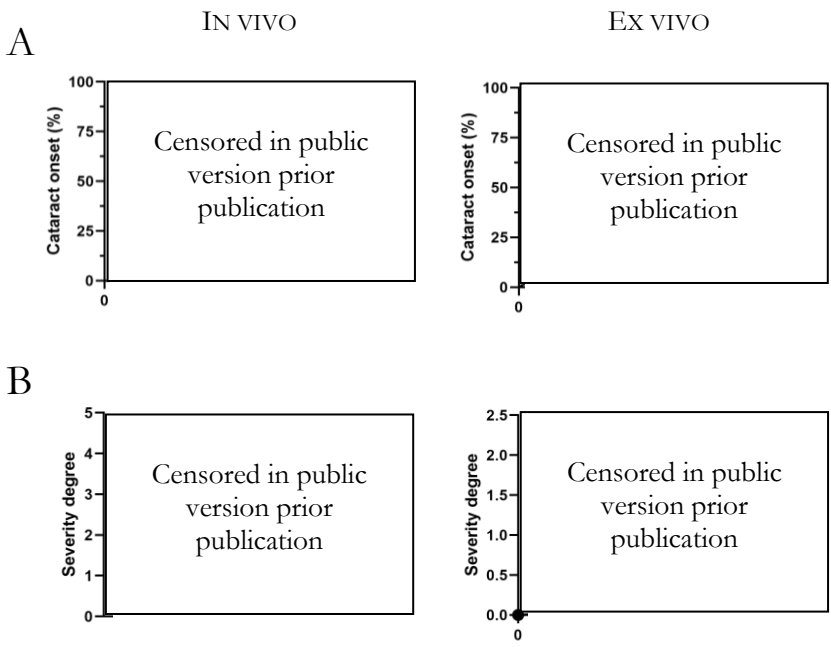



***FIGURE 7.*** *PA28α overexpression did not protect ocular lenses against either in vivo or ex vivo induced cataract. A) No difference in onset of cataract between ocular lenses from PA28αOE and WT mice was detected. Cataract onset in vivo was analyzed by percentage of cataract lenses at 7, 15 and 22 months of age. Ex vivo onset of cataract was visually assessed by opacification and after 8 days of $H_2O_2$ exposure, all lenses were opaque. B) Severity degree of cataract, both in vivo and ex vivo, did not differ between ocular lenses from PA28αOE and WT lenses. Lenses from both sexes were used. (Paper III, Fig. 2 and Table 1 for severity assessments).*

# BEHAVIOR

Behavioral analyses are commonly used to assess phenotypes of animal models used in biomedical research. The assessments control for adverse behaviors in disease models and for drug development but are also important in understanding physiological processes regarding development and aging.

## AGE-RELATED BEHAVIOR

As life expectancy of the world population increases and deaths by communicable diseases continues to decline, advancing age is the most correlated risk factor of mortality. In high income countries with the highest life expectancy in the world, the effect of medical advancements continues to result in improved prevention and therapies for the most common diseases such as cardiovascular diseases, cancer and respiratory diseases (WHO 2017). This introduces other age-related diseases as primary causes of death. In Sweden, where the median age of death for the population is 77 and 82 years for men and women respectively, there has been a reduction in cardiovascular disease related deaths in the last 15 years whilst mental and behavioral disorders and diseases of the nervous system increases, predominately in women (The Swedish National Board of Health and Welfare. 2017). This clear trend highlights emerging challenges, and the importance of understanding cognitive decline and behavioral alterations associated with aging of the mammalian brain.

Behavioral analyses can be used to analyze and decipher behavioral markers of aging, healthy aging and the effects of anti-aging interventions. Analyses to assess behaviors or phenotypes requires robustness, specificity and awareness of confounding factors. Conclusions regarding any behavior are ideally confirmed using several analyses, but problematically several behavioral analyses require naivety of mice which could be incompatible with extensive physiologic and phenotypic test batteries. Controlling for confounding effects in behavioral analyses of aging can be problematic. For example, to assess cognitive function of caloric restricted non-mammalian primates, tests with motivational cues (often food rewards) are generally used (Anderson et al. 2009). This is problematic because it is difficult to control if fasted animals differ in motivation to acquire food rewards as compared to ad lib

fed animals. In addition, there are sex differences in for example visual navigation which can strongly affect comparisons of female and male memory assessments (further addressed on page 58 in Methodology).

## BEHAVIORS OF AGING MICE

For rodents, there are many tests for assessing various behaviors such as memory capacities, anxiety, depression and exploration. For example, in memory analyses rodents demonstrate similar patterns of remembering capacities to certain stimuli as humans (predominately episodic memory; Foster et al. 2012, Shoji et al. 2019, Febo et al. 2020). Assessments of learning and memory in rodents generally include test batteries of spatial tests, such as a variety of mazes (Morris water, Radial, and Barnes maze) and habituation, and non-spatial tests, i.e. object recognition, passive avoidance, active avoidance, cued and contextual fear-conditioning (Crawley 1999). Learning and memory has by combinations of different tests been found to decline with age in mice (Benice et al. 2006, Foster et al. 2012, Shoji et al. 2019) but with varying results depending on for example sex (Jonasson 2005, Benice et al. 2006, Koss et al. 2017). Other behaviors such as anxiety and depression have been assessed in mice but with various results with regard to aging (Fahlstrom et al. 2009, Fahlstrom et al. 2012, Shoji et al. 2019). In paper II, mice on a hybrid background did not demonstrate decreased learning and memory with advancing age. Exploratory behavior, however, was confirmed to decline with age in both females and males as found previously in inbred mice (Benice et al. 2006, Fahlstrom et al. 2009, Fahlstrom et al. 2012, Shoji et al. 2019, Szentes et al. 2019), rats (Glenn et al. 2008, Katharesan et al. 2016) and dogs (Siwak et al. 2001).

Exploratory behavior is assessed by recoding activity during the initial phase, generally 3-5 minutes, of a mouses' experience in a novel environment, for example an open field arena (Paper II, Fahlstrom et al. 2009, Fahlstrom et al. 2012, Shoji et al. 2019). Latency to transition from dark to light arenas can also be used to assess exploration and increases with age, signifying decreased explorative behavior (Bourin et al. 2003, Fahlstrom et al. 2012, Shoji et al. 2019). Tendencies to explore, together with motor skills are fundamental in most behavioral tests. A decrease in exploration may also result in passivity in other tests for example to assess memory and anxiety. For example, in the elevated plus maze, several studies demonstrate that the explorative momentum of the test (e.g. numbers of entries) declines with age but the time spent in exposed area (e.g. time spent in open areas) remains relatively unaltered with advancing age (Fahlstrom et al. 2009, Fahlstrom et al. 2012, Shoji et al. 2019). Therefore, tests that contain explorative components but aim to

analyze other behaviors make it difficult to draw any conclusions on age-related differences regarding the non-exploratory behavior. Taken together, exploratory behavior which declines sex-independently in a continuous manner makes a robust behavioral marker of aging and its age effect may explain findings of age-related decline in other behaviors.

## BEHAVIORAL EFFECTS OF PA28α OVEREXPRESSION

In addition to biochemical assessments of PA28αOE females and males, all mice were behaviorally assayed in a phenotypic test battery at 7, 15 and 22 months of age.

### BEHAVIORAL EFFECTS IN MATURE ADULT PA28αOE FEMALES

At the first timepoint of phenotypic assessment, PA28αOE females exhibited cognitive enhanced performance in several behavioral analyses (Paper I). Mature adult PA28αOE males did not demonstrate any of the behaviors found in PA28αOE females (Paper I, Supp. Fig. 8).

PA28αOE mature adult females habituated faster into a familiar environment compared to WT female littermates (see page 59 in Methodology, for a comprehensive explanation of the habituation assessment). On the second day of activity box analysis, habituation is indicated by reduced activity as compared to the first day. PA28αOE females demonstrated decreased locomotion and rearing and increased corner time, signifying habituation (Figure 8, from Paper I). In addition, the same females were found to exhibit improved learning and memory in the well-known passive avoidance test Shuttle-box (Bammer 1982). In this test, mice are placed in a lit compartment and are given the choice to enter a dark compartment which they innately prefer. Once they pass through a centrally placed mechanical sliding door, the door closes, and they receive a mild electric shock from the metal grid floor. The following day, the mice are once again placed in the lit compartment and given the same choice to enter the dark one. Mice are considered to remember what happens upon entry to the dark compartment if they stay a prolonged time in the lit area or never enter the dark compartment. Markedly showing remembrance, no PA28αOE females re-entered the dark compartment in contrast to the majority of WT females (Figure 9a, from Paper I).

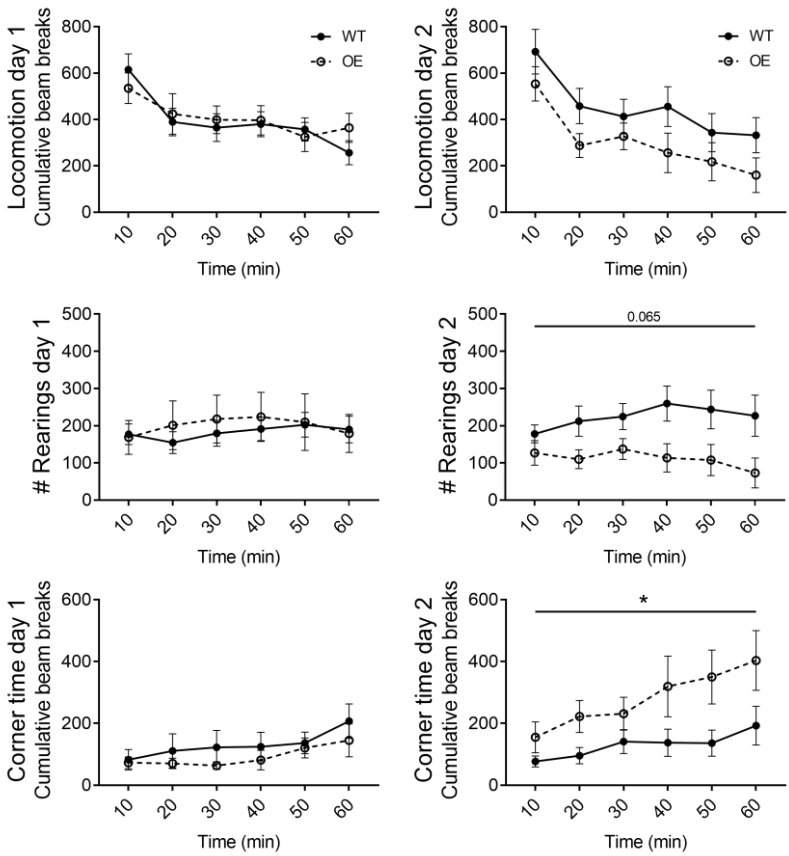



***FIGURE 8.*** *Differences in habituation assessed in the open field test for 7 months old female mice. Several parameters of activity from the Activity Box – open field test demonstrated that there is no difference in general activity or anxiety-like behavior shown on day 1. But on day 2, PA28αOE mice displayed faster intrasessional (slope) and intersessional (start point) habituation as compared to WT as shown with a faster increase in corner time and decreased locomotion and rearings, signifying enhanced intersessional habituation i.e. learning and memory (Rearings: $p_{WT\text{-}OE}$=0.065 F(1, 14) = 3992, and corner time: $p_{WT\text{-}OE}$=0.034 F(1, 14) = 5498; two-way ANOVA repeated measurements, followed by Sidak test). Values are mean ± SEM. $n_{WT}$=10, $n_{OE}$=6. (Paper I; Fig. 3).*

Additionally, PA28αOE females were more active in the forced swim test as demonstrated by swimming activity and distance travelled (Figure 9b, from paper I). The forced swim test is a commonly used to assess depressive-like behavior for anti-depressive drug discovery (David et al. 2003). In the forced swim test, mice are swimming in cylinders and their activity in water is recorded. Immobility in this test is considered a measurement of depressive-like behavior. However, that PA28αOE

females were more active does not imply that the WT mice demonstrate depressive-like behavior. Since there was no difference in general locomotion between PA28αOE and WT mice, swimming activity due to locomotor differences does most likely not explain the results in the forced swim test (Figure 8, day 1, from Paper I). The swimming behavior in the forced swim test therefore indicates that PA28αOE mice demonstrated improved coping mechanisms to a stressful event.

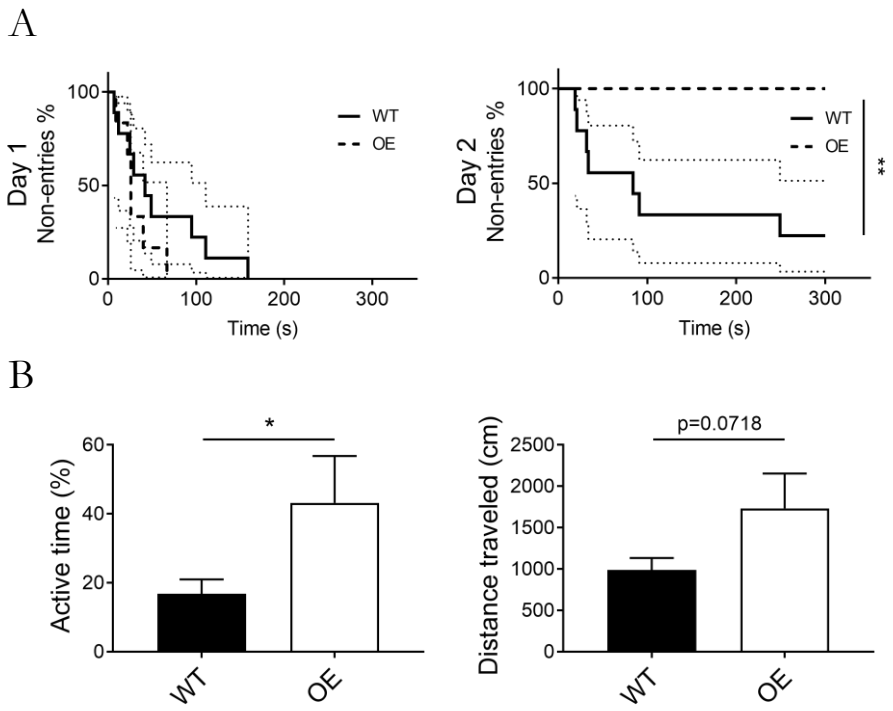



***FIGURE 9.*** *PA28αOE females exhibited improved learning and memory assessed by passive avoidance and increased swimming activity in the forced swim test. A) In the shuttle-box, 6/6 PA28αOE compared to 2/10 WT females did not re-enter on the second day of the test protocol (p= 0.0056; Mantel-Cox survival test). B) PA28αOE females showed higher percentage of active swim time and longer distance travelled in the forced swim test of depressive-like behavior (p= 0.0476; Student's t test). Values are mean ± SEM.* $n_{WT}=10$, $n_{OE}=6$. *(Paper I; Fig. 3 and 2).*

## AGE-RELATED EFFECTS IN PA28αOE MICE

To address age-related behavioral changes and differences between PA28αOE mice and their wildtype littermates, cohorts of naïve mice were analyzed in the same behavioral tests described previously. Considering WT animals as a control of aging and lifespan, 7 months old WT mice represented mature adults and the mice at 15 and 23 months of age represented middle-age and old age, respectively. No mice had died at 7 months, however at 15 months survival was 90% and at 22 months 71% and 74% for males and females respectively (Paper II). It was planned that the order of tests in the test battery remained similar throughout the timepoints, especially those requiring naivety to be performed early. However, this was not technically or practically feasible for the open field measurements of 15 months old females which seemed to have affected the analysis and therefore this data has been excluded. None of the other behavioral analyses generated results that indicated an influence by the order of the testing.

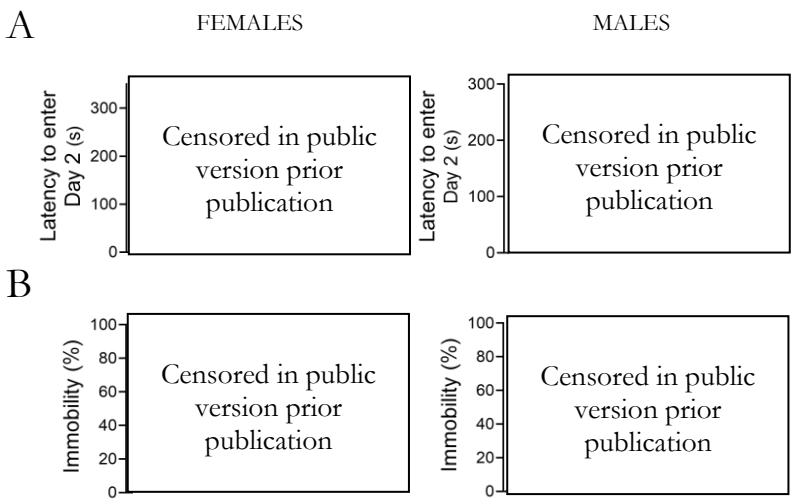



***FIGURE 10.*** *Behavioral tests of PA28αOE mice as they age; learning and memory assessed by Shuttle-box passive avoidance test, and depressive-like behavior analyzed with immobility in the forced swim test. A) At 7 months PA28αOE females displayed enhanced learning and memory capacity, but the improved performance as compared to WT females was reduced with age (see Fig. 8a for latency to enter on day 1). Neither PA28αOE and WT male mice demonstrated age-related trends in learning and memory. B) PA28αOE females were more active in the forced swim test at 7 months and 15 months but not 22 months of age. No clear trends of age-related depressive like behavior was observed in PA28αOE or WT males. (Paper IV; Fig. 5 (females) and Supp. Fig. 4 (males)).*

No clear age-related difference was found in either learning and memory as analyzed with the shuttle-box passive avoidance test (Figure 10a, from Paper IV), depressive-like behavior as analyzed with forced-swim test (Figure 10b, from Paper IV) or habituation, analyzed in an open field test, for either WT or PA28αOE mice (Figure 11, from Paper IV). The difference in improved cognitive function between 7-month-old PA28αOE females and WT females in these tests was either reduced with age or could not be detected using the same tests as aging progressed.

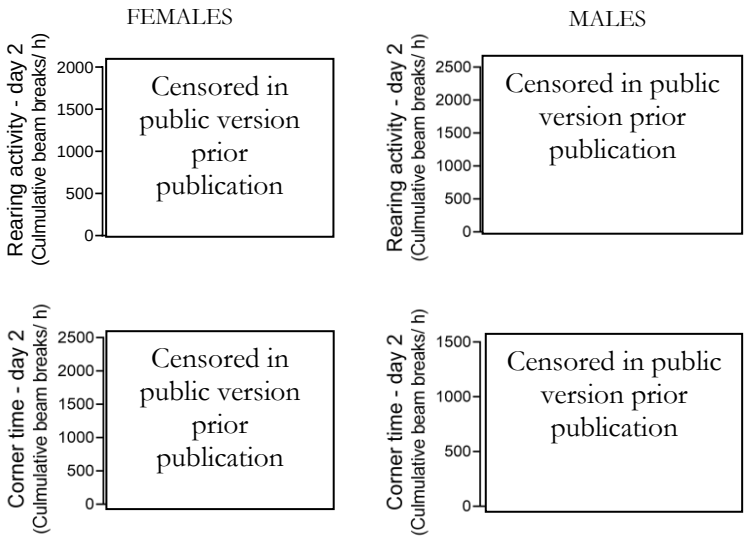



***FIGURE 11.*** *Habituation analyzed by activity in Activity box open field test on the day following naïve testing. In this acquainted environment, PA28αOE females demonstrated decreased rearing activity at 7, but not 22 months old and increased corner time. No habituation was found for females at 22 months or PA28αOE and WT males at 7, 15 or 22 months of age. (Paper IV; Fig. 5 (females) and Supp. Fig. 4 (males)).*

Interestingly, PA28αOE mice, particularly the females, exhibited maintained exploratory behavior with age whilst, as expected, their WT littermates demonstrated continuously declining exploration from young to old age (Figure 12, from Paper IV). Impressively, 22 months old PA28αOE females were as horizontally active as 7 months old females. Male PA28αOE mice maintained their horizontal exploration until 15 months of age but at 22 months, their activity had declined to levels seen in WT males' (Figure 12a, from Paper IV). Exploratory rearing activity was similarly

maintained in female PA28αOE mice but no differences between PA28αOE and WT males was found at either 15 and 22 months of age. No clear differences in activity during one-hour assessments were found between PA28αOE and WT females and males of similar age (Figure 13, from Paper IV). This signifies that it is exploratory behavior alone and not general locomotor function that is maintained in PA28αOE mice. That female, and possibly male, PA28αOE mice demonstrated maintained exploratory behavior with age indicates that overexpressing PA28α positively influences a fundamental age-related behavior. Maintaining youth-like behavior can signify preservation of cognitive capacities and prolonged healthspan.

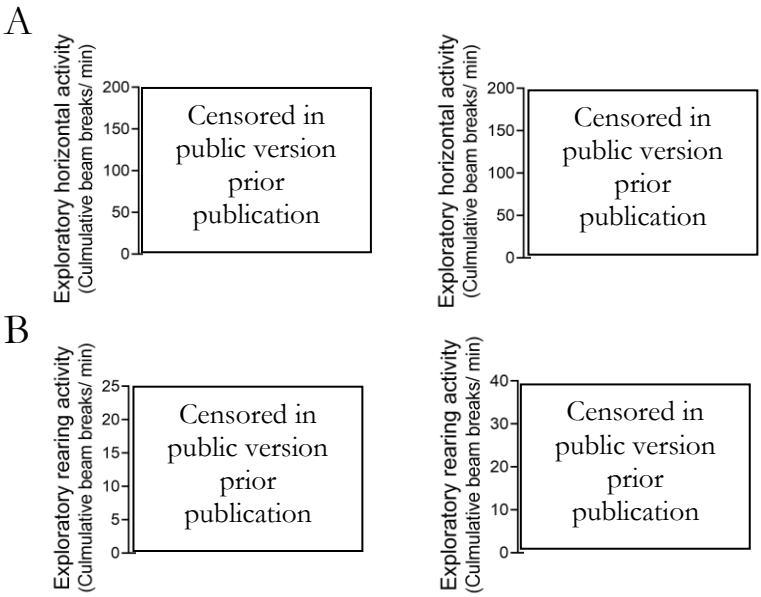
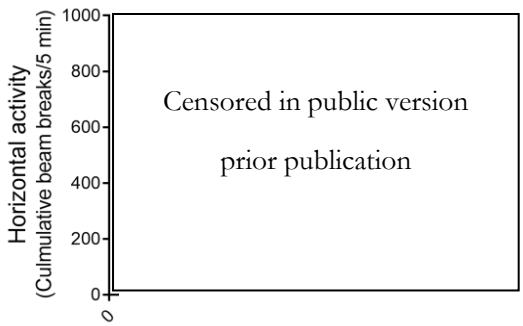



***FIGURE 12.*** *Exploratory behavior of PA28αOE and WT mice. The first 5 minutes in the activity box open field test measures exploratory behavior. This behavior decreased as expected in WT while it was maintained in 22-month-old PA28αOE females. A) Females: Horizontal activity decreased in WT but not for PA28αOE resulting in a difference in activity at 22 months. Males: WT males demonstrated decreased horizontal activity with age. At 15 months, PA28αOE males were as active as at 7 months which is significantly higher than WT males. B) Females: Rearing activity declined for wildtype from 7 to 22 months whilst PA28αOE females maintained their rearing activity, resulting in a difference in activity at 22 months. Males: Rearing activity declined with age, however not significantly in PA28αOE, most likely due to large variation. (Paper IV; Fig 6 (females) and Supp. Fig. 5 (males)).*

A




B

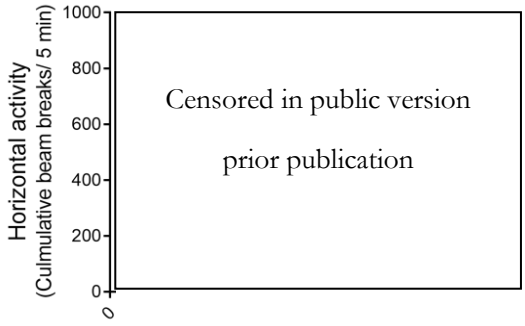



***FIGURE 13.*** *Activity in the activity box open field test in 5-minute bins over an hour. A) Female horizontal activity was similar for all mice between minutes 10 and 60. B) Male horizontal activity was the same for all mice from 10 to 60 minutes. (Paper IV).*

# RESEARCH MODELS

Animal research enables the study of particular biological questions under regulated and simplified conditions with the aim to create knowledge translatable or beneficial to humans. Using simplified model systems is a way to increase the understanding of whole organisms, since complex systems are immensely difficult to study. To do this, it is highly important with awareness of benefits and limitations of animal models for research purposes.

## OF MICE AND MEN

The mouse, *Mus musculus,* is the most commonly used mammalian model organism and mouse research has contributed to much of what we know in life sciences today. Despite the obvious differences of appearance between humans and mice, we are similar enough for fundamental biological principles to be studied in mice models and applied in humans and other organisms. Through genome sequencing it has been demonstrated that the mouse genome is 14% smaller than the human genome but both contain approximately 30 000 coding genes. Out of the coding genes, 80% of mouse genes have a single identifiable human ortholog, and only 1% of mouse genes have no detected homology in the human genome and therefore it is often claimed that mice share 99% of the human genome (Chinwalla et al. 2002). It is the similar, albeit not identically composed, expression of homologous proteins that supports translatability from mice to humans.

The mouse genome is amenable to genetic engineering evidenced by thousands of published transgenic mouse models which enables biological questions to be addressed in living mammalian systems. Accordingly, transgenic mice have been demonstrated as successful models of human diseases for example neurodegenerative diseases (Aguzzi et al. 1994, Brusa 1999), hereditary cancer syndromes (Ward et al. 2004) and in lysosomal storage diseases (Suzuki et al. 2004). However, mouse models have also failed to mimic human pathophysiology, particularly metabolic (Elsea et al. 2002) and inflammatory diseases (Seok et al. 2013). Their small size is well-suited for laboratory research and allows for economical animal husbandry in controlled environments. Large litters and relatively short lifespan also allow for mice to be models of gerontological research and age-related diseases.

## INBRED VS. HYBRID

Traditionally, various inbred mice are used for research in biomedical disciplines, but advantages in robustness make hybrid mice an emerging option. Inbreeding results in homogeneity at all genetic loci i.e. homozygosity. Thus, inbred mice have controlled genetic backgrounds, stable characteristics and can have organs and tissues easily transplanted within the strain (Lipman 1997). Favorably, any difference between transgenic inbred mice and wild-type members of the same strain can be designated the genetic modification. Unfavorably though, having two identical alleles at all loci creates genetic weakness and sensitizes mice to diseases and pathogenic conditions. Inbred mice are prone to develop strain-specific diseases such as organ and tissue specific tumors, skin and eye diseases, and for example, the commonly used C57BL/6 background typically develops ulcerative dermatitis with advancing age (C57BL/6NNia: Andrews et al. 1994, C57BL/6NNia: Turturro et al. 2002, C57BL/6Crl: Hampton et al. 2012). Disease, especially uniformly diseased animals, complicates research studies in several ways as it may require treatment or affect the animals prior to the rise of symptoms which in turn may lead to difficulties in interpreting results. Most research is advantageously performed using young mice before any maladies are displayed, but with the caveat that these studies use recently sexually mature but not fully-grown adult mice. Strain-specific diseases make inbred mice particularly suboptimal for aging research considering that studies aimed at investigating aging effects may instead report disease-related processes. Regarding phenotypic assays and behavioral analyses, inbred strains have strain-specific idiosyncrasies and differ in e.g. coordination, learning capacity, and anxiety-like behavior (Crawley et al. 1997, Võikar et al. 2004) which limits any finding to the strain used. Behavioral studies, like aging studies, may therefore be limited to pick up strain-specific phenotypes which reduces translatability of behaviors to other mouse strains, species or organisms. In addition, a meta-analysis comparing phenotypic studies demonstrated that outbred and inbred mice show similar variability in the same experiments and proposes that genetic variability acts as a stabilizing force of phenotypic research (Tuttle et al. 2018).

Using homogenic cohorts facilitates controlling for what is studied is "actually" studied but never reflects the variation found in "natural settings". Any finding in these genetically uniform mice thus reflects biology in one individual and may be, but not necessarily, a finding which is similarly displayed in other individuals. One way to try to address this dilemma is by using hybrid mice. Hybrid mice are generated by crossing different inbred strains, preferably twice to form a F2 generation. F2 hybrids are genetically similar but never uniformly homozygous and are considered advantageous for aging and behavioral studies (Miller et al. 1999, Miller et al. 2000, Sumien et al. 2006). Increased genetic robustness results in a population of

hybrid mice less likely to uniformly develop the same diseases and in addition, genetic variability better reflects other heterogenetic populations which improves extrapolation to other mammals (Miller et al. 1999, Rivera et al. 2008, Tuttle et al. 2018). A finding in a group of individuals is more likely biologically significant than a finding in a single individual. With the rationale that if a process or trait is biologically integral, it will be distinguishable even with the increased variation found in more genetically diverse models. On the other hand, a finding in an inbred strain – one individual – is only guaranteed to exist in that strain and additional studies are needed to verify translatability to other strains.

## ISSUES OF TRANSLATABILITY

Despite highly interesting discoveries and breakthroughs using animal models, little research progress beyond utilizing one strain, one species or, even more disastrously, one study. According to the scientific community there are major issues of reproducibility in many scientific disciplines, including biology and medical science (Baker 2016). Published studies often fail in being transparent and only 2-12% include replication efforts (Wallach et al. 2018) and empirical studies aimed to investigate these issues suggests that as much as 75-89% are not reproducible (Prinz et al. 2011, Begley et al. 2012). Irreproducibility is a consequence of the existing publication pressure that leads to biased analyzing and scientific misconduct (Tijdink et al. 2014, Begley et al. 2015, Grimes et al. 2018). Since repeating the same study in the same organism has proven difficult, it is not surprising perhaps that extrapolating findings from one species to another – translating science – is a major perplexity in research.

In the context of animal research to support the development of pharmaceutical treatments for human diseases, translatability can be measured by success in clinical trials. As clinical trials put patients at risk it is of ethical importance to ensure translatability from preclinical to clinical studies. But, measuring translatability from animal research to humans is however difficult because publication tradition selects for successful studies and not those with negative results (Leenaars et al. 2019). Another measurement of translatability in preclinical studies is how many therapies are approved for use in the clinic. Taken into consideration all preclinically developed treatments, approximately 10% are approved for patients (FDA 2004, Shih et al. 2017). A large review which investigated clinical trials from 1998-2008 and was followed up in 2015, demonstrated that clinical trial failure is due to inadequate drug or treatment efficacy (57%), safety reasons (17%), and commercial reasons (22%) (Hwang et al. 2016). Efficacy and safety issues are often referred to as lack of

internal validity and/or external validity. Internal validity in animal research signifies poor study setup, in particular, biases which may be resolved by proper blinding and randomization. External validation aims at biological parameters separating the preclinical cohorts from the targeted patient group, for example that young mice are used as models of old patients (van der Worp et al. 2010, Pound et al. 2018). Mouse models enter studies after weaning once they become fertile which occurs around 3 months of age. The developmental stage in which they are initially used for investigation is, by human standard, the equivalent to teenagers and young adults. In Paper II (Fig. 2), we highlight that when mice are around 7 months old, they have reached a body weight which will be maintained throughout their lifespan, an indication that they are fully grown adult mice. In addition, as human diseases generally take time to progress and despite shared symptoms between disease models and targeted patients, the pathogenesis may be insufficiently similar for findings to be translatable.

As previously discussed with inbred strains, there are issues of robustness with homogenetic models and therefore research may fail to translate to heterogenetic population. Not only do the general research models consist of one set of genes but also, they are of the same sex. There are, however, predictable and unpredictable biological differences between females and males.

A neuroscience publication survey in 2009 found that nearly six times more males than females were used in research (Beery et al. 2011). The male norm has predominately been explained with the rationale of female hormonal fluctuations increasing variability of data (Wald et al. 2010). Controversies with this reasoning include that several studies demonstrate that sex differences are independent of the estrous cycle and neglect male hormone levels, since testosterone levels amongst males can differ five-fold depending on dominance hierarchy (Machida et al. 1981, Palanza et al. 2001, Meziane et al. 2007). Both testosterone and estrogen are known to be strong neuromodulators and therefore, hormones are no scientific justification for using only males (McEwen et al. 2015). In addition, meta-analyses in rodents demonstrate that there is no difference in variation caused by female hormonal fluctuation between female and male data (Prendergast et al. 2014, Becker et al. 2016). Preclinical studies may therefore be poorly translated to a patient cohort consisting of women and men because the finding may be sex dependent. Introducing age, sex and heterogeneity in the process of target validation, and not when moving into humans, would add knowledge and specificity about drugs and interventions that most likely would improve the success rate and safety of clinical trials. Nonetheless, mouse models are highly important in the discovery of new medicines and although 10% of successful translatability from preclinic to clinic seems like a

small number, it is life changing for many patients. Addressing issues of translatability will improve the research to generate even more successful therapies to humans.

## YOU CAN TAKE THE HUMAN (RESEARCHER) OUT OF SOCIETY, BUT YOU CAN NEVER TAKE SOCIETY OUT OF THE HUMAN (RESEARCHER)

Behavioral analyses with model organisms are performed with the aim of understanding the biological processes behind behavior. Important to note is that any activity or trait needs to be confirmed using several different tests and parameters before any behavioral conclusion can be drawn. The use of behavioral assessments also informs drug development in validating targets and screening. However, it is problematic to determine what and how behaviors are shared between species. Although there are examples of behaviors such as learning that are translatable from mice to humans (Soliman et al. 2010, Casey et al. 2011), it is with major caution that any behavior in mice should be directly translated to any other organism.

The idea of using a model to study behavioral sex differences is interesting since it makes it easier to isolate biological effects and attempt to control for cofounders. Whether "sex" and "gender" are separable in humans is an ongoing dispute, but it is generally concluded that only sex is found in mice. Gender is commonly defined as socially constructed sex characteristics and includes norms, roles and relationships (WHO 2019) and humans are the only species known to have this social perception and implication to their own sex (Eliot et al. 2016). As females and males differ in behaviors and diseases linked to behavior, it is of interest to try to study and understand the biological mechanism behind these differences. But, as sex and gender biases are well-integrated in humans, it is important to ensure objective research methodology and avoid assigning sex differences which matches gender expectations, even to model organisms.

# METHODOLOGY

## GENERATION OF THE PA28αOE MOUSE MODEL

To investigate the role of PA28αβ in aging and disease, we created a hybrid mouse model overexpressing PA28α. The transgenic mouse model was generated through a knock-in strategy of the coding region of murine PA28α. A targeting vector carrying the PA28α gene was electroporated into C57BL/6N mouse embryonic stem (ES) cells. The vector was inserted in the genome by homologous recombination at the *Rosa26* locus, a genomic target locus considered not to impair any exons (endogenous gene coding parts of DNA). Since the endogenous Rosa26 promotor is known to change its capacity with age, the vector insertion included the CAG promotor to constituently and ubiquitously drive PA28α expression. Cre-Lox recombination was used to excise the positive selection markers from the targeting vector (Figure 14, from Paper I). Targeted insertion into ES cells was confirmed by PCR screening and Targeted Locus Amplification.

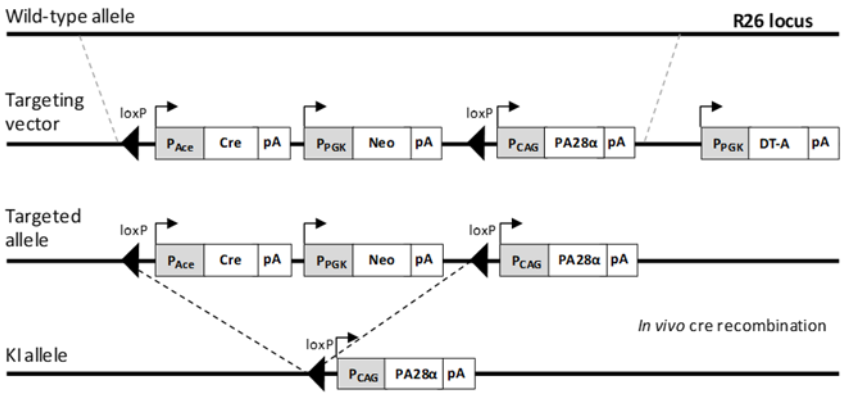



***FIGURE 14**. The design of the targeting construct used for generation of PA28αOE mice. (Paper I, Fig. 1a).*

The C57BL/6N ES cell clones were expanded and injected into embryos from donor females at blastocyst stage and injected into pseudoimpregnated females. All females were white-coated. Chimeric offspring, which are patchwork of normal and genetically modified cells were generated and males were later bred with C57BL/6JOlaHsd females to generate offspring carrying the inserted transgene in

all cells, identified by black coat color. To control for proper gene insertion, all founder mice were genotyped on both sides of the homology arms for integration of PA28α in the *Rosa26* locus and also confirmed by Targeted Locus Amplification. All mice were bred in accordance with ethical certificate permit number 91-2013 approved by the Animal Ethics Committee in Gothenburg, Sweden.

In the founder C57BL/6N it was confirmed that overexpressing mRNA encoding the α-subunit (Figure 15a, from Paper I) results in overexpression of the PA28α protein detected by western blot (Figure 15c, from Paper I). At the mRNA level there was no difference in PA28β expression (Figure 15b, from Paper I) but overexpression of the α-subunit induces an upregulation of the β-subunit at the protein level (Figure 15c, from Paper I), this is in accordance with previous studies demonstrating that overexpression of PA28α stabilizes PA28β and increases the complex (Li et al. 2011a). Nonetheless, and as pointed out in delimitations on page 18, it cannot be excluded that PA28α-subunit alone may have a monomeric function.

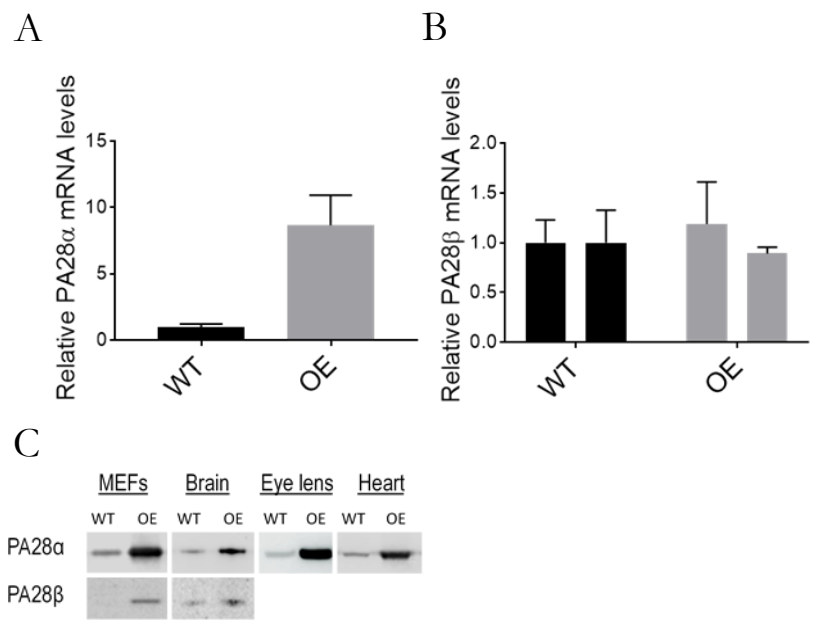



***FIGURE 15**. Expression of the PA28α and PA28β A) PA28α mRNA levels are upregulated almost tenfold in the PA28αOE mice. B) mRNA expression of PA28β is the same for WT and PA28αOE mice. The bars represent two different splicing variants of the PA28β transcript for each genotype. C) Western blot analysis of PA28α and PA28β confirms that PA28α are overexpressed in MEF cells, and brain (front cortex and striatum), eye lens and heart of PA28αOE (OE) as compared to wildtype (WT) in the founder C57BL/6 male mice. Expression of PA28β was non-detectable in eye lens and heart. (Paper I; Fig. 1).*

The PA28αOE mouse line was established on a pure C57BL/6N genetic background. To create a robust aging study the founder C57BL/6N mouse line was crossed with wildtype BALB/c generating F1 C57BL/6NxBALB/c mice. Siblings from these F1 animals were bred to generate a F2 C57BL/6NxBALB/c with mixed genetic background.

There has been no sign of decreased fertility, body size or changes in physical appearance for the PA28αOE mice compared to WT mice. F2 hybrid mice PA28αOE and WT littermates were analyzed for physiological and behavioral profiling and organs were harvested at three timepoints. The mice are referred to as 7, 15 and 22 months of age at the timepoints. But, since the in vivo phenotyping took approximately 8 weeks to complete, the animals were exactly, 6.6-7.8±0.2, 14.5-15.6±0.1 and 21.8-22.5±0.2 months old at time of testing (age at test period start - age at test period end ±SD of age variance in the cohort). Experimental mouse studies were performed in accordance with ethical permit no. 164-2015, approved by the Animal Ethics Committee in Gothenburg, Sweden. All studies were carried out following EU Directive 2010/63/EU for animal experiments.

## BODY COMPOSITION IMPACTS WATER-BASED BEHAVIORAL ASSESSMENTS

The importance of studying both females and males as well as acknowledging sex differences has previously been raised in this thesis. In Paper II, we detected a sex difference in the forced swim test which implied that females demonstrated more depressive-like behavior than males (Figure 16, from Paper II). But, after correlating physiological measurements to the results, we concluded that the results in the forced swim test were to a large extent dependent on sex differences in fat mass. Thus, for the first time we showed that body composition affects performance and results in water-based tests.

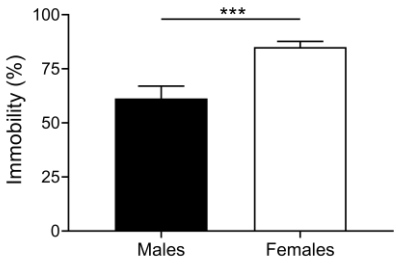



***FIGURE 16**. Females are more immobile in the forced swim test (p=0.0007; Mann-Whitney). Data are from 7 and 15 months old F2 hybrid mice. Values are mean ± SEM. $n_{Males}$=17, $n_{Females}$=21. (Paper II; Supp. Fig. 2a).*

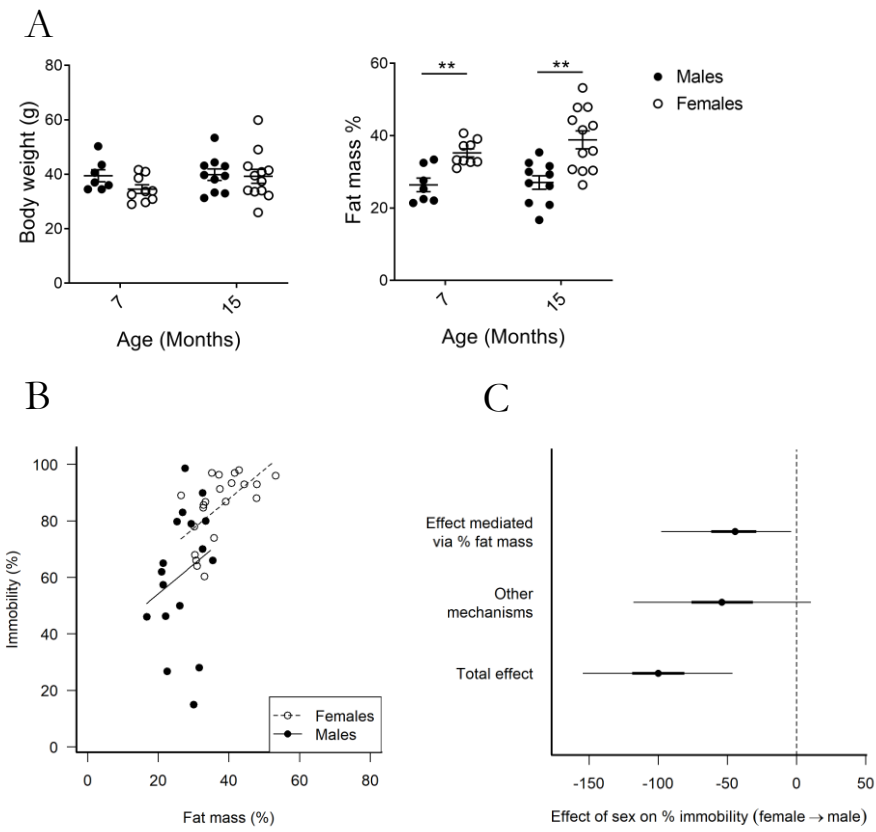



***FIGURE 17.*** *Immobility in forced swim test correlate with fat mass. A) Although no sex difference in body weight, females had more fat mass as compared to males ($p_7$=0.0023, $p_{15}$=0.0012). B) Immobility (%) plotted against fat mass (%) for each animal demonstrate a correlation of the two variables. C) Fat mass was found to account for 46% of the sex difference effect in immobility (Bayesian p=0.986; Bayesian mediation analysis). Values are mean ±50% (thick lines) and 95% confidence interval (thin lines); $n_{M7}$=7, $n_{M15}$=10, $n_{F7}$=9, $n_{F15}$=12. (Paper II; A is extracted from Fig. 2a and 2c; B and C, Supp. Fig. 2b and 2c, respectively).*

The forced swim test is commonly used test to assess depressive-like behavior and anti-depressant screening in rodents. Mice are placed in a water tank (25 cm in diameter and 60 cm high), all fur-coated mice float in water but the novel sensation of water invokes swimming movements. Mice are recorded for 6 minutes and 20 seconds out of which the last 4 minutes are used, during this time activity in the water changes. Initially, the mice show high activity and try to find ways out of the

water but as time elapses this behavior can change, and immobility is considered a measurement of depressive-like behavior. (Porsolt et al. 1978, Bourin et al. 1998, David et al. 2003, Fernández-Guasti et al. 2017). In this test, the sensation of water and buoyancy are key factors for the outcome of the test.

Commonly in phenotypic and behavioral tests, animals are controlled for body weight since it is known to correlate with results. Nonetheless, in a high-throughput phenotype mice data study, 57% of the continuous data sets of traits (e.g. lean mass) was found to differ due to sex effect, when including body weight as a covariate (Karp et al. 2017). Our physiological analysis of females and males showed no difference in body weight but with full body scan we could detect differences in body composition, more specifically, females had higher percentage of body fat (Figure 17a, from Paper II). Interestingly, body fat percentage correlated to decreased activity in water (Figure 17b, from Paper II) and with Bayesian mediation analysis (Figure 17c, from Paper II) we could confirm that the forced swim test analysis was directly affected by sex differences in fat. Because of the effect of fat mass in this water-based test we could not draw conclusions regarding any sex differences in depressive-like behavior, nonetheless, the results highlight the importance of controlling for physiological parameters in behavioral tests.

According to the current dogma, female rodents have poorer learning and memory capacities as compared to males. This is supported by research where females performing less well in the commonly used spatial, cued and water-based cognition tests (Mishima et al. 1986, Berger-Sweeney et al. 1995, LaBuda et al. 2002, Frick et al. 2003, Benice et al. 2006). As concluded, water-based tests are dependent on body composition for swimming activity and no studies have so far taken this factor into consideration when comparing females and males. In addition, spatial reference tests are also unsuitable for sex comparisons of cognitive function since females and males have been found to use different reference cues to navigate, and thus these tests often favor one sex over the other (Kanit et al. 1998, Sandstrom et al. 1998, Roof et al. 1999, Kant et al. 2000). We found no sex difference in our behavioral aging study when we investigated learning and memory in mice with the shuttle-box passive avoidance test (Paper II, Fig. 4). In accordance with this, but not as frequently discussed, females and males have been found to perform equally well in non-spatial and non-water-based learning and memory tests, for example object memory consolidation (Benice et al. 2006, Fahlstrom et al. 2012), active avoidance tests (Mishima et al. 1986, Frick et al. 1999, Sumien et al. 2006) nor habituation (our unpublished data).

## HABITUATION IS A SIMPLE FORM OF MEMORY

In Paper I, we presented a novel protocol to assess learning and memory in mice by using the open field test to analyze habituation. Open field tests have been used for many years to study general activity, exploratory behavior and anxiety in rodents (Denenberg 1969, Crawley 1999). The simple procedure of recording the activity of rodents through breaks of infrared beams in a box have been given additional dimensions, such as size, lighting, exposure, repetitions and time spent in box, to expand the parameters of the test. The time of recording varies depending on protocol used, often only the first 3-5 minutes are recorded and considered as general activity. In other study protocols, the first 3-5 minutes are considered as exploratory activity since the innate mouse behavior in a novel environment is to explore. With extended recording time, approximately one hour, and as the mouse explores a novel environment, it adapts and changes its behavior (Prut et al. 2003, Lau et al. 2008, Fahlstrom et al. 2012). The process of adaptation makes it therefore important to take time into consideration when comparing open field tests. In our studies, we regarded the initial 5 minutes as exploratory behavior and the extended one-hour recordings as general activity in adapted environment. In addition, our protocol includes repetition of the study session on the following day, to record the activity in a now familiar environment. In the first session, the environment transitions from being novel to habituated and the mice adapt to the box, this is referred to as intrasessional (within session) habituation. In the second and following session, as the environment is already familiar, the mice are then intersessionally (between sessions) habituated (Fraley et al. 1981, Leussis et al. 2006). Since habituation is one of the simplest forms of learning and memory (Bolivar 2009), intrasessional and intersessional habituation in an open field test, reflects how well mice remember what they already have explored. Repeated measurements in the open field test to assess habituation have been performed previously (Platel et al. 1984), but to our knowledge not for an hour of recording. As demonstrated by our repeated open field data (Figure 8, from Paper I, on page 43 in Behavioral effects of PA28αβ overexpression), habituation between animals with different learning and memory capacity was evident at the second session when recording for an hour, but not the initial 5-10 minutes. Thus, our protocol extended intra- and intersessional analyses that broadens the sensitivity and robustness of habituation assessments with open field testing.

## How to ethically perform a lifespan analysis and still enable comparisons to other aging studies

Traditionally, animals in lifespan studies are kept alive until they die from intrinsic causes and they are only euthanized because survival is estimated to less than a week. This makes aging studies especially challenging to combine with good animal ethics and welfare. It is however crucial to determine lifespan to enable investigation on longevity effects by genetic modifications, interventions and with drugs.

In our lifespan study, we euthanized mice upon signs of pain or severe disease strictly following our study ethical certificate (permit number 164-2015). To enable comparison with other aging studies which had no or very low percentage of euthanized animals, we mathematically generated two life curves resulting in a survival span as presented in paper II, here visualized in figure 18.

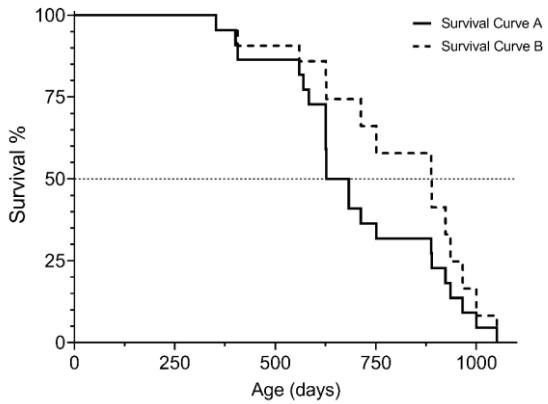



***Figure 18. Example survival curves to visualize lifespan.*** *In survival curve A, euthanized mice are considered to have reached their maximum lifespan, in survival curve B, time of death is considered unknown for euthanized animals. Within the span created by the two survival curves lies the probable lifespan for the cohort (Paper II; wildtype females, extracted from Fig. 1).*

The method involves statistical categorization of animal fate. In lifespan analyses labeling euthanized animals as dead by natural cause generates an underestimation of natural lifespan. On the other hand, labeling euthanized animals as "censored" at the time of euthanization (i.e. treated as healthy but with unknown time of death), natural lifespan is overestimated. These differing categorizations of euthanized animals generate two distinct survival curves, one minimum curve (Survival Curve A) and one maximum curve (Survival Curve B). Albeit time of death of intrinsic causes

(i.e. death by disease) remains unknown for the euthanized animals, it is likely that their state would limit their life expectancy in comparison to healthy mice and thus the most probable time point of actual death lies within the span created by two survival curves.

Hence, this novel method gives a qualitative estimation and visualization of the natural lifespan without the expense of animal suffering and allows for comparisons at different timepoints as well as in the end of studies. For example, this method enabled us to compare the "maximum lifespan" of F2 hybrid female and male WT mice with inbred strains in addition to a lifespan "frame" to determine when mice were considered mature adults, middle-aged and old.

## $H_2O_2$-INDUCED CATARACT AS A MODEL OF AGE-INDUCED CATARACT

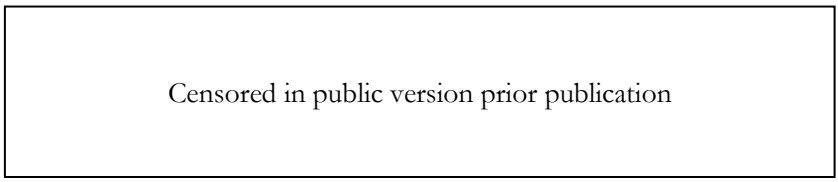
To assess if PA28α overexpression had an effect on cataract, ocular lenses from mice in the aging study were analyzed and compared to hydrogen peroxide ($H_2O_2$) exposed ocular lenses dissected from 3-4 months old mice, in paper III. Age-related opacification is referred to as in vivo cataract and the $H_2O_2$ exposed lenses is an alternative ex vivo model of cataract. Although no protective effects against cataract in PA28αOE mice were found, comparisons of the two procedures of inducing cataract gave valuable insights for modelling and investigating cataract.

Oxidative stress has been found to cause cataract and is a connecting component of the risk factors (aging, UV-radiation, heavy metal exposure, metabolic stress and eye injury/ inflammation). Not surprisingly, administration of $H_2O_2$, causing oxidative stress in the lens, is commonly used to induce cataract in ex vivo models of the disease. Enhanced ROS disturbs crystalline stability and solubility which results in crystalline aggregation clouding the lens and in loss of transparency (Truscott 2005, Moreau et al. 2012).

The occurrence of cataract increased with aging and at termination of 7, 15 and 22 months of age, ocular lenses were dissected and stored in -80°C. In addition, ocular lenses from 3-4 months old mice were collected and maintained under standard culture conditions and exposed to 100μM $H_2O_2$ every day, following a 7-day protocol (Petersen et al. 2004). Cataract progression was documented daily and the $H_2O_2$ exposure introduced swelling of the lens, measured by lens diameter, and an opaque ring formation with hat-like appearance as demonstrated in figure 19 (from Paper III). The swelling of the lens is considered most likely to be a consequence of failed osmotic balance.

**IN VIVO INDUCED CATARACT**

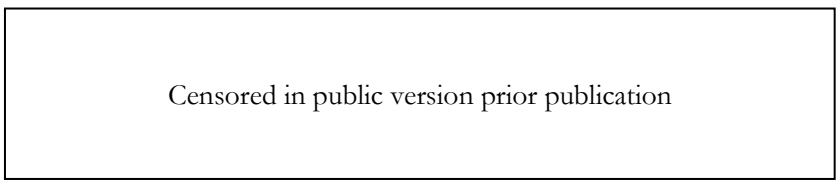
CLEAR DISTINCT OPACITY OPAQUE




**EX VIVO INDUCED CATARACT**

UNTREATED CONTROLS

DAY 1 DAY 8




$H_2O_2$ TREATED

DAY 1 DAY 8

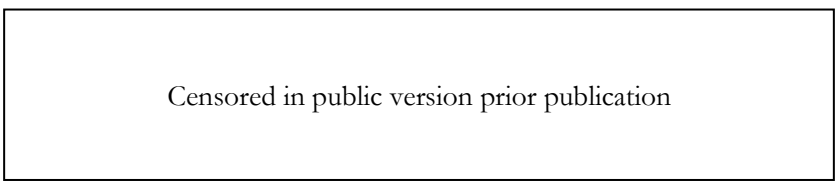



***FIGURE 19.*** *In vivo and ex vivo induced cataract. Ocular lenses with age-related cataract displayed complete opacification. Ocular mouse lenses exposed to 100 uM $H_2O_2$ for 8 consecutive days displayed opacification, swelling and a hat-like formation (Paper III; Fig. 4 and 5).*

Ocular lenses with varying degrees of cataract/opacification, from both in and ex vivo induced cataracts, were homogenized and analyzed for solubility and oxidative damage through levels of carbonylated proteins. Protein aggregation which opacifies the lens also negatively impacts solubility. As demonstrated in figure 20a, there was a pronounced effect on solubility in the age-induced cataract lenses which was not seen in the $H_2O_2$-exposed lenses. Crystallins, which make up 90% of all proteins in the lens (Haslbeck et al. 2016), were not found to differ in carbonylation either by age or $H_2O_2$-exposure as observed by the dominant low molecular weight (Mw) band in figure 21 (from Paper III). Carbonylated proteins of high Mw (>35kDa) however increased both in vivo and ex vivo, although most pronounced in $H_2O_2$-induced cataract (Figure 20b, from Paper III).

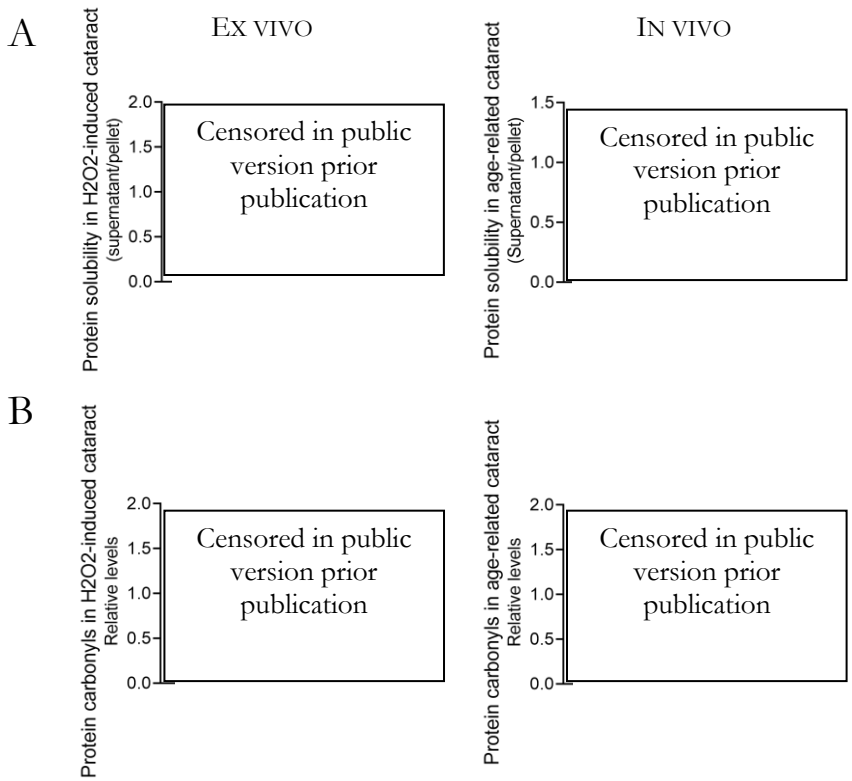



***FIGURE 20.*** *Protein solubility and carbonylated proteins in ex vivo and in vivo induced cataract lenses. A) Protein solubility did not markedly decrease in ex vivo induced cataract by $H_2O_2$ exposure of ocular lenses. In age-induced in vivo cataract however, there was a strong decrease as analyzed with ocular lenses from 15- and 23-month-old female and male mice. B) High molecular weight protein carbonyl levels increased in both ex vivo and in vivo induced cataract. (Paper III; Fig. 4 and 3).*

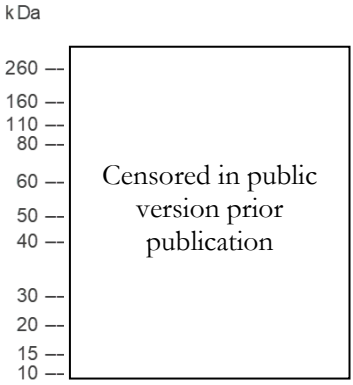



***FIGURE 21**. Western blots of representative protein carbonyl levels. 1) clear ex vivo lens, 2) H2O2-treated semi-opaque ex vivo lens, 3) clear in vivo aged lens and 4) opaque in vivo aged lens. (Paper III, Fig. 3a).*

Although high Mw carbonylated proteins increased in both cataract models, protein aggregation analyzed by protein solubility could not be detected in $H_2O_2$-induced cataract and the swelling and hat-like opacification indicate poor translatability of cataract markers. Interventions aimed to alleviate cataractogenesis by targeting these markers should cautiously and preferably not be assessed ex vivo by $H_2O_2$ exposure.

# SUMMARY OF FINDINGS

## PAPER I

- PA28αOE F2 hybrid mice overexpressed PA28α in murine embryonic fibroblasts (MEFs), brain, ocular lens and heart. PA28β overexpression could be detected in MEFs and brain.
- 7-month-old females overexpressing PA28α exhibited improved learning and memory capacity and anti-depressive-like behavior.
- PA28αOE did not increase proteasome degradation as demonstrated by i) PA28αβ-20S proteolytic capacity was not higher in hippocampal and mouse embryonic fibroblast extracts from PA28αOE females than that of WT and ii) a slight increase in K48-linked polyubiquitinated proteins in hippocampal extracts from PA28αOE and WT females.
- There were no differences in serum levels of β-estradiol or S105-phosphorylated estrogen receptor in hippocampus comparing 7-month-old PA28αOE and WT females.
- Hippocampal extracts from PA28αOE females prevented aggregation formation more efficiently than hippocampal extract from WT littermates, indicating that a chaperone-like function may explain the enhanced cognitive function.

## PAPER II

- No age-related effect on learning and memory or depressive-like behavior was found for either female or male hybrid mice.
- Exploratory behavior declined continuously with age in both female and male mice of the robust F2 hybrid background.
- Body composition was for the first time demonstrated to affect water-based behavioral tests.
- An alternative method to assess lifespan in aging studies was presented. Categorization of euthanized animals created minimum and maximum survival curves forming a survival span which allowed for comparison of studies with protocols following different animal ethics.

## PAPER III

- PA28α and PA28β were overexpressed in ocular lenses from PA28αOE mice.
- PA28αβ overexpression in the lens did not protect against age-related or $H_2O_2$-induced cataract.
- $H_2O_2$-induced cataract is problematic as a model of age-related cataract.

## PAPER IV

- PA28α overexpression prevented protein aggregation in hippocampal extracts of mature adult, middle-aged and old female mice, indicating a chaperone-like function maintained with age.
- Innate levels of PA28α did not correlate to proteolytic activity of the PA28αβ-20S proteasome in heart extracts from WT mice.
- PA28α overexpression decreased PA28αβ-20S proteasome capacity in extracts of heart and hippocampus from PA28αOE compared to WT mice.
- Innate PA28αβ-20S proteasome capacity in heart increased with age.
- Overexpression of PA28α had no effect on the accumulation of damaged proteins in aging mice.
- Overexpressing PA28α did not affect the lifespan of F2 hybrid mice.
- The improved cognitive function demonstrated by enhanced learning and memory and anti-depressive-like behavior of mature adult female PA28αOE mice could not be detected with the same tests at old age.
- Female mice overexpressing PA28α exhibited youth-like exploratory behavior at old age. Male mice overexpressing PA28α showed indications of maintained exploratory behavior up until middle-age.

# TO CONCLUDE

The effects of aging discussed in this thesis ranges from single molecules such as ROS to advanced behavior. This comprehensive scope of biological functions was important in addressing the molecular function of PA28αβ and the effect its overexpression embodies in mice. Although the existence of PA28αβ has been known for more than twenty years, its cellular role is quite intricate to grasp. This work adds fundamental knowledge about mechanisms of PA28αβ in a mammalian system. In addition, following mice overexpressing PA28αβ throughout their full lifespan allowed for research on molecular and behavioral markers of aging, and age-related cataract with similar pathogenesis as in humans.

## CHAPERONE-LIKE FUNCTION; A NOVEL ROLE OF PA28αβ IN HIPPOCAMPUS

Herein, data has been presented suggesting a chaperone-like function of PA28αβ, and possibly a dual role of PA28αβ as a proteasome activator and a chaperone. Assessment of the chaperone-like function could unfortunately not be analyzed in the heart, but the aggregation prevention of PA28αβ overexpression in disease models of the heart found in previous studies could be a consequence of a chaperone-like role of PA28αβ. Since PA28αβ overexpression has previously been found to counteract myocardiopathy induced by mutation of a crystallin, which hold protein aggregation properties, it is likely that the chaperone-like function of PA28αβ can compensate for the loss of aggregation prevention which may cause this proteinopathy. That we found no effect of PA28αβ overexpression in cataract may be that this particular protective function may already be saturated by the abundance of crystallins.

A decrease of PA28αβ-20S proteolytic capacity in PA28αOE extracts was detected in conditions optimized for that specific proteasome complex formation. This challenges the previous findings that PA28αβ overexpression enhances the degradation of oxidatively damaged proteins. In addition, the proteolytic capacity of PA28αβ-20S was found to be independent of PA28αβ protein levels and the PA28αβ-20S proteasome activity seems to be regulated by cellular factors other than abundance of the activator. During early differentiation of embryonic stem cells, removal of

damaged proteins is dependent on an upregulation of PA28αβ and PA28αβ-20S proteasome activity. This process may be a result of this unknown regulation in combination with the rise of PA28αβ levels which allows for the formation of the complexes. In this work, upregulating the levels of PA28αβ did not increase proteasome activity but enhanced aggregation prevention. Thus, PA28αβ harbors two types of molecular functions: i) an activator of 20S proteasome and ii) a chaperone. These two functions of PA28αβ-20S could be regulated by post-translational modifications (e.g. phosphorylation), since post-translational sites with unknown function have been found for both PA28α and PA28β.

### CHAPERONE-LIKE FUNCTION OF PA28αβ IN THE BRAIN IMPROVES COGNITIVE FUNCTION AND EXTENDS HEALTHSPAN?

PA28αβ chaperone-like function correlates to enhanced cognitive capacity as demonstrated by behavioral analyses in mature adult hippocampi. The PA28αβ-induced enhanced behavioral performance as assessed by learning and memory and in the forced swim test, cannot be detected in old mice. In hippocampal extracts from 4-month-old mice, there was no differences in levels of carbonylated proteins or CML between PA28αOE and WT. Thus, a cognitive effect induced by PA28αβ overexpression is present at adult age and it is independent of protein damage/aging markers in hippocampus. This implies that in young mice, the mechanism behind is not by reducing damage or compensating for any function lost by age, but rather an improvement on cognitive function induced by PA28αβ overexpression.

As the brain ages, several changes are introduced which affect cognition and behavior. Thus, any cognitive effect can vary in how it is presented and may be observable in different behavioral tests depending on age. The maintained youth-like behavior in explorative activity at old age correlates to enhanced hippocampal protein aggregation prevention, as does improved learning and memory for mature adults. This suggests that overexpression of PA28αβ correlates with enhanced cognitive capacities, with altered behavioral outcomes as age progresses.

It is highly unclear why the chaperone-like function of PA28αβ is only improved in female mice and not in males. We have been able to exclude the apparent target; conventional estradiol signaling, since there were no differences in levels of serum estrogen serum and/or hippocampal phosphorylated estrogen receptor β in PA28αOE females compared to WT mice.

If the function of PA28αβ is regulated by post-translational modifications, sex differences in signaling pathways that can activate the chaperone-like function, could explain why only PA28αOE female mice demonstrate enhanced aggregation prevention.

## THE POTENTIAL OF PA28αβ

The finding of a mechanism which protects against protein aggregation and protein dysfunction is of therapeutical interest for many, if not all proteinopathies. The three most common neurodegenerative diseases in humans; Alzheimer's, Parkinson's and Huntington's disease, are all characterized by misfolding of proteins, nondegraded proteins and excessive proteins resulting in accumulation of protein aggregates. The combination of the chaperone-like function and the cognitive effects in PA28αOE females makes PA28αβ an interesting therapeutic candidate in neurodegenerative medicine research. The behavioral effects of PA28αβ were demonstrated in mature adult mice as enhanced memory and during aging as maintained exploratory behavior. These results suggest that the chaperone-like function exert direct positive effects and possibly also protective effects in hippocampus upon aging. With this reasoning, PA28αβ overexpression could in neurodegenerative diseases either i) enhance the overall cognitive capacity and partly counterbalance deteriorating symptoms or ii) act protectively against the mechanisms of pathogenesis and alleviate disease progression.

## IN THE ECLIPSE OF EXCITING FINDINGS

In parallel to a scientific context which favors the publishing of novelty and differences, it is important to acknowledge and constantly scrutinize the fundaments that research is laid upon such that no incorrect and detrimental conclusions are drawn. In both Paper II and III, commonly used methods and research models were challenged with the aim to improve the quality of science and translatability. Although negative results or insights into methodology are not as thrilling or funding attractive, they are highly important components for robust, ethical and safe research. I also encourage the use of both sexes in animal research, since important physiological and molecular mechanisms may be affected by sex as demonstrated by this very thesis.

# ACKNOWLEDGEMENTS

As an Industrial PhD I have had the privilege to have four excellent supervisors and teams at both Sahlgrenska Academy and AstraZeneca, Gothenburg. I would like to thank my head-supervisor Madeleine for experienced guidance and support along the way. I will never seize to be fascinated by your ability to keep your focus on so many things at the same time, your endless motivation and never fading smile. Thank you, Anne for making me feel so welcomed in the beginning, your scientific experience and kindness made me feel comfortable and never hesitant to ask stupid questions. My industry supervisor John, thanks for your confidence in me, for being supportive and giving me autonomy.

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Thank you, Gothenburg Women Scientists for being such a supportive network and for shedding light on inequality in academia.

During this PhD it has become clear to me that my work stretches far into my private life and vice versa. I have had so many dreams about mice and colorful gels, and some of the best ideas have popped into my head during a run in the forest, in my studio or whilst sinking in a swimming pool (due to my body composition…). But also, during these years it has become extremely evident for me that when something happens in the private part of life it cannot be simply left behind when you come in to work. During this PhD, I have learnt how to live one day at a time. My mother recovered from her breast cancer, but my brother-in-law Tobias lost the fight against his brain tumor. It has been disconcerting to study life and aging, and truly realize how much is taken for granted. I am grateful for the support, understanding and extra time given to me by my supervisors and financers.

I would of course not be who I am or where I am if it was not for my sprawling family that has kept me on my toes, always. Conversely, I acknowledge that my fiery temperament and creative vices must have been challenging for you (it is so great that I have left all that behind). Mamma, you have taught me how to fight for what is important and given me the courage to explore what I do not understand and beyond. Pappa, your wholehearted enthusiasm and endless energy is something I would like to have inherited even more of. I am grateful to my sisters for all the love and tough love; it has shaped me. Anna, thank you for demonstrating that hard work pays off and believing that I can do the same. Maria, it still amazes me that you became my best friend after 19 years of being my enemy. Seeing you suffer without being able to do anything is the hardest thing my heart and reason ever have experienced. Lars, maybe in a few years when you have learnt how to read, you will pick up this book and find it written that I have loved you always.

Bosse, thank you for being the first to discuss biology with me. I am grateful for the calmness you bring to Mamma when everything feels as if it's blowing in the wind and times are a-changing. Thank you, Karin and Klara for always being empowering and that you, together with Pappa, remind me to enjoy good things in life.

Tack Mormor, Farmor och Farfar, Eva-Lena, Johan och Ingela för stöttning och visat intresse.

My dear shoe-box, friends, wolfpack, cosmic twins, sham twin, artistic souls, dance partners, sailor mates, travel buddies, et bon vivants, I surely hope that none of you skipped reading the section on rejuvenation because I am truly looking forward to growing old with you.

Yours sincerely,

Julia Helena Herner Adelöf

# REFERENCES


Aguzzi, A., S. Brandner, U. Sure, D. Rüedi and S. Isenmann (1994). "Transgenic and Knock-out Mice: Models of Neurological Disease." Brain Pathology **4**(1): 3-20.

Anderson, R. M., D. Shanmuganayagam and R. Weindruch (2009). "Caloric restriction and aging: studies in mice and monkeys." Toxicologic pathology **37**(1): 47-51.

Andrews, A. G., R. C. Dysko, S. C. Spilman, R. G. Kunkel, D. W. Brammer and K. J. Johnson (1994). "Immune Complex Vasculitis with Secondary Ulcerative Dermatitis in Aged C57BL/6NNia Mice." Veterinary Pathology **31**(3): 293-300.

Arndt, V., C. Rogon and J. Höhfeld (2007). "To be, or not to be — molecular chaperones in protein degradation." Cellular and Molecular Life Sciences **64**(19): 2525.

Austad, S. N. (1988). "The adaptable opossum." Sci Am (Feb): 98-104.

Baar, M. P., et al. (2017). "Targeted Apoptosis of Senescent Cells Restores Tissue Homeostasis in Response to Chemotoxicity and Aging." Cell **169**(1): 132-147.e116.

Baker, M. (2016). "1,500 scientists lift the lid on reproducibility." Nature **533**(7604): 452–454.

Bammer, G. (1982). "Pharmacological investigations of neurotransmitter involvement in passive avoidance responding: A review and some new results." Neuroscience & Biobehavioral Reviews **6**(3): 247-296.

Basaiawmoit, R. V., Rattan, S.I.S. (2010). Protein Misfolding and Cellular Stress in Disease and Aging. Methods in Molecular Biology (Methods and Protocols). Totowa, NJ, Humana Press.

Baynes, J. W. (2001). "The role of AGEs in aging: causation or correlation." Experimental Gerontology **36**(9): 1527-1537.

Becker, J. B., B. J. Prendergast and J. W. Liang (2016). "Female rats are not more variable than male rats: a meta-analysis of neuroscience studies." Biology of sex differences **7**: 34-34.

Beebe, D. C., N. M. Holekamp and Y. B. Shui (2010). "Oxidative damage and the prevention of age-related cataracts." Ophthalmic Res **44**(3): 155-165.

Beery, A. K. and I. Zucker (2011). "Sex Bias in Neuroscience and Biomedical Research." Neuroscience and biobehavioral reviews **35**(3): 565-572.

Begley, C. G. and L. M. Ellis (2012). "Raise standards for preclinical cancer research." Nature **483**(7391): 531-533.

Begley, G. C. and J. P. A. Ioannidis (2015). "Reproducibility in Science." Circulation Research **116**(1): 116-126.

Behndig, A. L., M. Serring, I. Montan, P. Kugelberg, M. Nilsson, I and Zetterberg, M (2019). "Svensk Kataraktkirurgi, Patientanpassad Årsrapport 2018 baserad på data från Nationella Kataraktregistret.".

Bence, N. F., R. M. Sampat and R. R. Kopito (2001). "Impairment of the Ubiquitin-Proteasome System by Protein Aggregation." Science **292**(5521): 1552-1555.

Benice, T. S., A. Rizk, S. Kohama, T. Pfankuch and J. Raber (2006). "Sex-differences in age-related cognitive decline in C57BL/6J mice associated with increased brain microtubule-associated protein 2 and synaptophysin immunoreactivity." Neuroscience **137**(2): 413-423.

Berger-Sweeney, J., A. Arnold, D. Gabeau and J. Mills (1995). "Sex differences in learning and memory in mice: Effects of sequence of testing and cholinergic blockade." Behavioral Neuroscience **109**(5): 859-873.

Bolivar, V. J. (2009). "Intrasession and intersession habituation in mice: from inbred strain variability to linkage analysis." Neurobiology of learning and memory **92**(2): 206-214.

Bonet-Costa, V., P. Y. Sun and K. J. A. Davies (2019). "Measuring redox effects on the activities of intracellular proteases such as the 20S Proteasome and the Immuno-Proteasome with fluorogenic peptides." Free Radic Biol Med **143**: 16-24.

Bourin, M., M. C. Colombel, J. P. Redrobe, J. Nizard, m. Hascoët and G. B. Baker (1998). "Evaluation of efficacies of different classes of antidepressants in the forced swimming test in mice at different ages." Progress in Neuro-Psychopharmacology and Biological Psychiatry **22**(2): 343-351.

Bourin, M. and M. Hascoët (2003). "The mouse light/dark box test." European Journal of Pharmacology **463**(1): 55-65.

Bourke, A. F. G. (2007). "Kin Selection and the Evolutionary Theory of Aging." Annual Review of Ecology, Evolution, and Systematics **38**(1): 103-128.

Bourne, R. R. A., SR Flaxman, T Braithwaite, MV Cicinelli, A Das, JB Jonas, J Keeffe, JH Kempen, J Leasher, H Limburg, K Naidoo, K Pesudovs, S Resnikoff, A Silvester, GA Stevens, N Tahhan, TY Wong, HR Taylor & G Vision Loss Expert (2017). "Magnitude, temporal trends, and projections of the global prevalence of blindness and distance and near vision impairment: a systematic review and meta-analysis." Lancet Glob Health **5**: e888-e897.

Brand, M. (2016). "Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling." Free Radical Biology and Medicine **100**.

Brandhorst, S., et al. (2015). "A Periodic Diet that Mimics Fasting Promotes Multi-System Regeneration, Enhanced Cognitive Performance, and Healthspan." Cell Metabolism **22**(1): 86-99.

Braun, B. C., M. Glickman, R. Kraft, B. Dahlmann, P.-M. Kloetzel, D. Finley and M. Schmidt (1999). "The base of the proteasome regulatory particle exhibits chaperone-like activity." Nature Cell Biology **1**(4): 221-226.

Brieger, K., S. Schiavone, F. J. Miller, Jr. and K. H. Krause (2012). "Reactive oxygen species: from health to disease." Swiss Med Wkly **142**: w13659.

Brusa, R. (1999). "Genetically modified mice in neuropharmacology." Pharmacological Research **39**(6): 405-419.
Cascio, P., M. Call, B. M. Petre, T. Walz and A. L. Goldberg (2002). "Properties of the hybrid form of the 26S proteasome containing both 19S and PA28 complexes." The EMBO Journal **21**(11): 2636-2645.
Casey, B. J., E. J. Ruberry, V. Libby, C. E. Glatt, T. Hare, F. Soliman, S. Duhoux, H. Frielingsdorf and N. Tottenham (2011). "Transitional and translational studies of risk for anxiety." Depression and anxiety **28**(1): 18-28.
Chen, X., L. F. Barton, Y. Chi, B. E. Clurman and J. M. Roberts (2007). "Ubiquitin-independent degradation of cell cycle inhibitors by the REGγ proteasome." Mol Cell **26**(6).
Chinwalla, A. T., et al. (2002). "Initial sequencing and comparative analysis of the mouse genome." Nature **420**(6915): 520-562.
Conboy, I. M., M. J. Conboy, A. J. Wagers, E. R. Girma, I. L. Weissman and T. A. Rando (2005). "Rejuvenation of aged progenitor cells by exposure to a young systemic environment." Nature **433**(7027): 760-764.
Cosgrove, K. P., C. M. Mazure and J. K. Staley (2007). "Evolving knowledge of sex differences in brain structure, function, and chemistry." Biological psychiatry **62**(8): 847-855.
Crawley, J. (1999). "Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests1Published on the World Wide Web on 2 December 1998.1." Brain Research **835**(1): 18-26.
Crawley, J., et al. (1997). "Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies." Psychopharmacology **132**: 107-124.
Dahl, J.-U., M. J. Gray and U. Jakob (2015). "Protein Quality Control under Oxidative Stress Conditions." Journal of Molecular Biology **427**(7): 1549-1563.
Dalle-Donne, I., D. Giustarini, R. Colombo, R. Rossi and A. Milzani (2003). "Protein carbonylation in human diseases." Trends in Molecular Medicine **9**(4): 169-176.
Dange, T., D. Smith, T. Noy, P. C. Rommel, L. Jurzitza, R. J. B. Cordero, A. Legendre, D. Finley, A. L. Goldberg and M. Schmidt (2011). "Blm10 Protein Promotes Proteasomal Substrate Turnover by an Active Gating Mechanism." Journal of Biological Chemistry **286**(50): 42830-42839.
David, D. J. P., C. E. Renard, P. Jolliet, M. Hascoët and M. Bourin (2003). "Antidepressant-like effects in various mice strains in the forced swimming test." Psychopharmacology **166**(4): 373-382.
de Graaf, N., M. J. van Helden, K. Textoris-Taube, T. Chiba, D. J. Topham, P. M. Kloetzel, D. M. Zaiss and A. J. Sijts (2011). "PA28 and the proteasome immunosubunits play a central and independent role in the production of MHC class I-binding peptides in vivo." Eur J Immunol **41**(4): 926-935.
Denenberg, V. H. (1969). "Open-field behavior in the rat: what does it mean?*." Annals of the New York Academy of Sciences **159**(3): 852-859.
Eliot, L. and S. S. Richardson (2016). "Sex in Context: Limitations of Animal Studies for Addressing Human Sex/Gender Neurobehavioral Health

Disparities." The Journal of neuroscience : the official journal of the Society for Neuroscience **36**(47): 11823-11830.
Elsea, S. H. and R. E. Lucas (2002). "The Mousetrap: What We Can Learn When the Mouse Model Does Not Mimic the Human Disease." ILAR Journal **43**(2): 66-79.
Fahlstrom, A., Q. Yu and B. Ulfhake (2009). "Behavioral changes in aging female C57BL/6 mice." Neurobiol Aging **32**(10): 1868-1880.
Fahlstrom, A., H. Zeberg and B. Ulfhake (2012). "Changes in behaviors of male C57BL/6J mice across adult life span and effects of dietary restriction." Age (Dordr) **34**(6): 1435-1452.
FDA, U. S. D. o. H. a. H. S. (2004). "Innovation or Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products.".
Febo, M., A. Rani, B. Yegla, J. Barter, A. Kumar, C. A. Wolff, K. Esser and T. C. Foster (2020). "Longitudinal Characterization and Biomarkers of Age and Sex Differences in the Decline of Spatial Memory." Front Aging Neurosci **12**: 34.
Fernández-Guasti, A., M. Olivares-Nazario, R. Reyes and L. Martínez-Mota (2017). "Sex and age differences in the antidepressant-like effect of fluoxetine in the forced swim test." Pharmacology Biochemistry and Behavior **152**: 81-89.
Ferrington, D. A. and D. S. Gregerson (2012). "Immunoproteasomes: Structure, Function, and Antigen Presentation." Progress in molecular biology and translational science **109**: 75-112.
Finkel, T. and N. J. Holbrook (2000). "Oxidants, oxidative stress and the biology of ageing." Nature **408**(6809): 239-247.
Fontana, L., L. Partridge and V. D. Longo (2010). "Extending healthy life span--from yeast to humans." Science **328**(5976): 321-326.
Foster, T. C., R. A. Defazio and J. L. Bizon (2012). "Characterizing cognitive aging of spatial and contextual memory in animal models." Frontiers in aging neuroscience **4**: 12-12.
Fraley, S. M. and A. D. Springer (1981). "Memory of simple learning in young, middle-aged, and aged C57/BL6 mice." Behavioral and Neural Biology **31**(1): 1-7.
Frick, K. M., L. A. Burlingame, J. A. Arters and J. Berger-Sweeney (1999). "Reference memory, anxiety and estrous cyclicity in C57BL/6NIA mice are affected by age and sex." Neuroscience **95**(1): 293-307.
Frick, K. M. and J. E. Gresack (2003). "Sex Differences in the Behavioral Response to Spatial and Object Novelty in Adult C57BL/6 Mice." Behavioral Neuroscience **117**(6): 1283-1291.
Frick, K. M., J. Kim and W. A. Koss (2018). "Estradiol and hippocampal memory in female and male rodents." Current Opinion in Behavioral Sciences **23**: 65-74.
Fu, M.-X., J. R. Requena, A. J. Jenkins, T. J. Lyons, J. W. Baynes and S. R. Thorpe (1996). "The Advanced Glycation End Product, N-(Carboxymethyl)lysine, Is a Product of both Lipid Peroxidation and Glycoxidation Reactions." Journal of Biological Chemistry **271**(17): 9982-9986.

Förster, A., E. I. Masters, F. G. Whitby, H. Robinson and C. P. Hill (2005). "The 1.9 Å Structure of a Proteasome-11S Activator Complex and Implications for Proteasome-PAN/PA700 Interactions." Molecular Cell **18**(5): 589-599.

Garratt, M., K. A. Lagerborg, Y. M. Tsai, A. Galecki, M. Jain and R. A. Miller (2018). "Male lifespan extension with 17-alpha estradiol is linked to a sex-specific metabolomic response modulated by gonadal hormones in mice." Aging Cell **17**(4): e12786.

Glenn, M. J., E. D. Kirby, E. M. Gibson, S. J. Wong-Goodrich, T. J. Mellott, J. K. Blusztajn and C. L. Williams (2008). "Age-related declines in exploratory behavior and markers of hippocampal plasticity are attenuated by prenatal choline supplementation in rats." Brain research **1237**: 110-123.

Glickman, M. H., D. M. Rubin, O. Coux, I. Wefes, G. Pfeifer, Z. Cjeka, W. Baumeister, V. A. Fried and D. Finley (1998). "A Subcomplex of the Proteasome Regulatory Particle Required for Ubiquitin-Conjugate Degradation and Related to the COP9-Signalosome and eIF3." Cell **94**(5): 615-623.

Griffin, T. A., D. Nandi, M. Cruz, H. J. Fehling, L. V. Kaer, J. J. Monaco and R. A. Colbert (1998). "Immunoproteasome Assembly: Cooperative Incorporation of Interferon γ (IFN-γ)–inducible Subunits." The Journal of Experimental Medicine **187**(1): 97-104.

Grimes, D. R., C. T. Bauch and J. P. A. Ioannidis (2018). "Modelling science trustworthiness under publish or perish pressure." R Soc Open Sci **5**(1): 171511.

Guillaume, B., J. Chapiro, V. Stroobant, D. Colau, B. Van Holle, G. Parvizi, M.-P. Bousquet-Dubouch, I. Théate, N. Parmentier and B. J. Van den Eynde (2010). "Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules." Proceedings of the National Academy of Sciences **107**(43): 18599-18604.

Halliwell, B. (2007). "Biochemistry of oxidative stress." Biochemical Society Transactions **35**(5): 1147-1150.

Halliwell, B. and J. M. C. Gutteridge (1995). "The definition and measurement of antioxidants in biological systems." Free Radical Biology and Medicine **18**(1): 125-126.

Hampton, A. L., G. A. Hish, M. N. Aslam, E. D. Rothman, I. L. Bergin, K. A. Patterson, M. Naik, T. Paruchuri, J. Varani and H. G. Rush (2012). "Progression of Ulcerative Dermatitis Lesions in C57BL/6Crl Mice and the Development of a Scoring System for Dermatitis Lesions." Journal of the American Association for Laboratory Animal Science : JAALAS **51**(5): 586-593.

Hartl, F. U. (1996). "Molecular chaperones in cellular protein folding." Nature **381**(6583): 571-580.

Hartl, F. U., A. Bracher and M. Hayer-Hartl (2011). "Molecular chaperones in protein folding and proteostasis." Nature **475**(7356): 324-332.

Haslbeck, M., J. Peschek, J. Buchner and S. Weinkauf (2016). "Structure and function of α-crystallins: Traversing from in vitro to in vivo." Biochimica et Biophysica Acta (BBA) - General Subjects **1860**(1, Part B): 149-166.

Haslbeck, M. and E. Vierling (2015). "A first line of stress defense: small heat shock proteins and their function in protein homeostasis." J Mol Biol **427**(7): 1537-1548.

Heinemeyer, W., P. C. Ramos and R. J. Dohmen (2004). "The ultimate nanoscale mincer: assembly, structure and active sites of the 20S proteasome core." Cellular and Molecular Life Sciences CMLS **61**(13): 1562-1578.

Hernebring, M., G. Brolén, H. Aguilaniu, H. Semb and T. Nyström (2006). "Elimination of damaged proteins during differentiation of embryonic stem cells." Proceedings of the National Academy of Sciences **103**(20): 7700-7705.

Hernebring, M., Å. Fredriksson, M. Liljevald, M. Cvijovic, K. Norrman, J. Wiseman, H. Semb and T. Nyström (2013). "Removal of damaged proteins during ES cell fate specification requires the proteasome activator PA28." **3**: 1381.

Hewitt, E. W. (2003). "The MHC class I antigen presentation pathway: strategies for viral immune evasion." Immunology **110**(2): 163-169.

Hipp, M. S., S.-H. Park and F. U. Hartl (2014). "Proteostasis impairment in protein-misfolding and -aggregation diseases." Trends in Cell Biology **24**(9): 506-514.

Huang, L., K. Haratake, H. Miyahara and T. Chiba (2016). "Proteasome activators, PA28γ and PA200, play indispensable roles in male fertility." Scientific Reports **6**: 23171.

Huber, E. M. and M. Groll (2017). "The Mammalian Proteasome Activator PA28 Forms an Asymmetric α4β3 Complex." Structure.

Hwang, T. J., D. Carpenter, J. C. Lauffenburger, B. Wang, J. M. Franklin and A. S. Kesselheim (2016). "Failure of Investigational Drugs in Late-Stage Clinical Development and Publication of Trial Results." JAMA Internal Medicine **176**(12): 1826-1833.

Janeway, J. (1992). "The T Cell Receptor as a Multicomponent Signalling Machine: CD4/CD8 Coreceptors and CD45 in T Cell Activation." Annual Review of Immunology **10**(1): 645-674.

Joel, D. (2012). "Genetic-gonadal-genitals sex (3G-sex) and the misconception of brain and gender, or, why 3G-males and 3G-females have intersex brain and intersex gender." Biology of Sex Differences **3**(1): 27.

Joel, D. and M. M. McCarthy (2017). "Incorporating Sex As a Biological Variable in Neuropsychiatric Research: Where Are We Now and Where Should We Be?" Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology **42**(2): 379-385.

Johnston, S. C., F. G. Whitby, C. Realini, M. Rechsteiner and C. P. Hill (1997). "The proteasome 11S regulator subunit REG alpha (PA28 alpha) is a heptamer." Protein Science : A Publication of the Protein Society **6**(11): 2469-2473.

Jonasson, Z. (2005). "Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioral and biological data." Neuroscience & Biobehavioral Reviews **28**(8): 811-825.

Jung, T., A. Hohn and T. Grune (2013). "The proteasome and the degradation of oxidized proteins: Part II - protein oxidation and proteasomal degradation." Redox Biol **2C**: 99-104.

Kahn, H. A., Leibowitz, H.M. Ganley, J.P. Kini, M.M. Colton, T. Nickerson, R.S & Dawber, T.R (1977). "The Framingham Eye Study. I. Outline and major prevalence findings." Am J Epidemiol **106**: 17-32.

Kanit, L., D. Taşkıran, J. J. Furedy, B. Kulali, R. McDonald and Ş. Pöğün (1998). "Nicotine interacts with sex in affecting rat choice between "look-out" and "navigational" cognitive styles in the Morris water maze place learning task." Brain Research Bulletin **46**(5): 441-445.

Kant, L., O. Yilmaz, D. Takiran, B. Kulali, J. J. Furedy, S. Demirgören and Pöün (2000). "Sexually dimorphic cognitive style, female sex hormones, and cortical nitric oxide." Physiology & Behavior **71**(3): 277-287.

Kapahi, P., M. Kaeberlein and M. Hansen (2017). "Dietary restriction and lifespan: Lessons from invertebrate models." Ageing Res Rev **39**: 3-14.

Kapphahn, R. J., E. J. Bigelow and D. A. Ferrington (2007). "Age-dependent inhibition of proteasome chymotrypsin-like activity in the retina." Experimental Eye Research **84**(4): 646-654.

Karp, N. A., et al. (2017). "Prevalence of sexual dimorphism in mammalian phenotypic traits." Nat Commun **8**: 15475.

Katharesan, V., M. D. Lewis, R. Vink and I. P. Johnson (2016). "Disparate Changes in Plasma and Brainstem Cytokine Levels in Adult and Ageing Rats Associated with Age-Related Changes in Facial Motor Neuron Number, Snout Muscle Morphology, and Exploratory Behavior." Frontiers in neurology **7**: 191-191.

Kellis Jr, J. T., K. Nyberg and A. R. Fersht (1989). "Energetics of complementary side chain packing in a protein hydrophobic core." Biochemistry **28**(11): 4914-4922.

Kennedy, B. K., et al. (2014). "Geroscience: linking aging to chronic disease." Cell **159**(4): 709-713.

Kirkwood, T. B. L. (1977). "Evolution of ageing." Nature **270**(5635): 301-304.

Kisselev, A. F., T. N. Akopian, V. Castillo and A. L. Goldberg (1999). "Proteasome Active Sites Allosterically Regulate Each Other, Suggesting a Cyclical Bite-Chew Mechanism for Protein Breakdown." Molecular Cell **4**(3): 395-402.

Kisselev, A. F., M. Garcia-Calvo, H. S. Overkleeft, E. Peterson, M. W. Pennington, H. L. Ploegh, N. A. Thornberry and A. L. Goldberg (2003). "The Caspase-like Sites of Proteasomes, Their Substrate Specificity, New Inhibitors and Substrates, and Allosteric Interactions with the Trypsin-like Sites." Journal of Biological Chemistry **278**(38): 35869-35877.

Knoefler, D., L. I. Leichert, M. Thamsen, C. M. Cremers, D. Reichmann, M. J. Gray, W. Y. Wholey and U. Jakob (2014). "About the dangers, costs and benefits of living an aerobic lifestyle." Biochem Soc Trans **42**(4): 917-921.

Koss, W. A. and K. M. Frick (2017). "Sex differences in hippocampal function." Journal of Neuroscience Research **95**(1-2): 539-562.

Kudriaeva, A., E. S. Kuzina, O. Zubenko, I. V. Smirnov and A. Belogurov, Jr. (2019). "Charge-mediated proteasome targeting." FASEB J **33**(6): 6852-6866.

Labbadia, J. and R. I. Morimoto (2015). "The Biology of Proteostasis in Aging and Disease." Annual review of biochemistry **84**: 435-464.

LaBuda, C. J., R. L. Mellgren and R. L. Hale (2002). "Sex differences in the acquisition of a radial maze task in the CD-1 mouse." Physiology & Behavior **76**(2): 213-217.

Lau, A. A., A. C. Crawley, J. J. Hopwood and K. M. Hemsley (2008). "Open field locomotor activity and anxiety-related behaviors in mucopolysaccharidosis type IIIA mice." Behav Brain Res **191**(1): 130-136.

Leenaars, C. H. C., C. Kouwenaar, F. R. Stafleu, A. Bleich, M. Ritskes-Hoitinga, R. B. M. De Vries and F. L. B. Meijboom (2019). "Animal to human translation: a systematic scoping review of reported concordance rates." Journal of translational medicine **17**(1): 223-223.

Leussis, M. P. and V. J. Bolivar (2006). "Habituation in rodents: A review of behavior, neurobiology, and genetics." Neuroscience & Biobehavioral Reviews **30**(7): 1045-1064.

Levine, R. L. (2002). "Carbonyl modified proteins in cellular regulation, aging, and disease2,3 2Guest Editor: Earl Stadtman 3This article is part of a series of reviews on "Oxidatively Modified Proteins in Aging and Disease." The full list of papers may be found on the homepage of the journal." Free Radical Biology and Medicine **32**(9): 790-796.

Li, J., K. M. Horak, H. Su, A. Sanbe, J. Robbins and X. Wang (2011b). "Enhancement of proteasomal function protects against cardiac proteinopathy and ischemia/reperfusion injury in mice." J Clin Invest **121**(9): 3689-3700.

Li, J., S. R. Powell and X. Wang (2011a). "Enhancement of proteasome function by PA28α overexpression protects against oxidative stress." FASEB J **25**(3): 883-893.

Li, X., L. Amazit, W. Long, D. M. Lonard, J. J. Monaco and B. W. O'Malley (2007). "Ubiquitin- and ATP-Independent Proteolytic Turnover of p21 by the REGγ-Proteasome Pathway." Molecular Cell **26**(6): 831-842.

Lipman, R. D. (1997). "Pathobiology of aging rodents: Inbred and hybrid models." Experimental Gerontology **32**(1): 215-228.

Lopez-Otin, C., M. A. Blasco, L. Partridge, M. Serrano and G. Kroemer (2013). "The hallmarks of aging." Cell **153**(6): 1194-1217.

Ma, C. P., C. A. Slaughter and G. N. DeMartino (1992). "Identification, purification, and characterization of a protein activator (PA28) of the 20 S proteasome (macropain)." Journal of Biological Chemistry **267**(15): 10515-10523.

Machida, T., Y. Yonezawa and T. Noumura (1981). "Age-associated changes in plasma testosterone levels in male mice and their relation to social dominance or subordinance." Hormones and Behavior **15**(3): 238-245.

Mair, W. and A. Dillin (2008). "Aging and survival: the genetics of life span extension by dietary restriction." Annu Rev Biochem **77**: 727-754.

Mattison, J. A., R. J. Colman, T. M. Beasley, D. B. Allison, J. W. Kemnitz, G. S. Roth, D. K. Ingram, R. Weindruch, R. de Cabo and R. M. Anderson (2017). "Caloric restriction improves health and survival of rhesus monkeys." Nature Communications **8**: 14063.

Maynard Smith, J. (1958). "The effects of temperature and of egg-laying on the longevity of Drosophila subobscura." J. Exp. Biol. **35**: 832-842.
McEwen, B. S., J. D. Gray and C. Nasca (2015). "Redefining neuroendocrinology: stress, sex and cognitive and emotional regulation." The Journal of endocrinology **226**(2): T67-T83.
Medawar, P. B. (1952). An unsolved problem of biology. London, Published for the College by H.K. Lewis.
Meziane, H., A. M. Ouagazzal, L. Aubert, M. Wietrzych and W. Krezel (2007). "Estrous cycle effects on behavior of C57BL/6J and BALB/cByJ female mice: implications for phenotyping strategies." Genes Brain Behav **6**(2): 192-200.
Miller, R. A., S. Austad, D. Burke, C. Chrisp, R. Dysko, A. Galeckid, A. Jackson and V. Monnier (1999). "Exotic mice as models for aging research: polemic and prospectus." Neurobiol Aging(20): 217–231.
Miller, R. A. and N. L. Nadon (2000). "Principles of Animal Use for Gerontological Research." The Journals of Gerontology: Series A **55**(3): 117-123.
Minami, Y., H. Kawasaki, M. Minami, N. Tanahashi, K. Tanaka and I. Yahara (2000). "A Critical Role for the Proteasome Activator PA28 in the Hsp90-dependent Protein Refolding." Journal of Biological Chemistry **275**(12): 9055-9061.
Mishima, N., F. Higashitani, K. Teraoka and R. Yoshioka (1986). "Sex differences in appetitive learning of mice." Physiology & Behavior **37**(2): 263-268.
Moreau, K. L. and J. A. King (2012). "Protein misfolding and aggregation in cataract disease and prospects for prevention." Trends in Molecular Medicine **18**(5): 273-282.
Morimoto, R. I. and A. M. Cuervo (2014). "Proteostasis and the Aging Proteome in Health and Disease." The Journals of Gerontology Series A: Biological Sciences and Medical Sciences **69**(Suppl 1): S33-S38.
Mouchiroud, L., L. Molin, N. Dallière and F. Solari (2010). "Life span extension by resveratrol, rapamycin, and metformin: The promise of dietary restriction mimetics for an healthy aging." BioFactors **36**(5): 377-382.
Murata, S., H. Kawahara, S. Tohma, K. Yamamoto, M. Kasahara, Y.-i. Nabeshima, K. Tanaka and T. Chiba (1999). "Growth Retardation in Mice Lacking the Proteasome Activator PA28γ." Journal of Biological Chemistry **274**(53): 38211-38215.
Murata, S., H. Yashiroda and K. Tanaka (2009). "Molecular mechanisms of proteasome assembly." Nat Rev Mol Cell Biol **10**(2): 104-115.
Noda, C., N. Tanahashi, N. Shimbara, K. B. Hendil and K. Tanaka (2000). "Tissue Distribution of Constitutive Proteasomes, Immunoproteasomes, and PA28 in Rats." Biochemical and Biophysical Research Communications **277**(2): 348-354.
Nyström, T. (2005). "Role of oxidative carbonylation in protein quality control and senescence." The EMBO Journal **24**(7): 1311-1317.
Palanza, P., L. Gioiosa and S. Parmigiani (2001). "Social stress in mice: Gender differences and effects of estrous cycle and social dominance." Physiology & Behavior **73**(3): 411-420.

Petersen, A. and M. Zetterberg (2016). "The Immunoproteasome in Human Lens Epithelial Cells During Oxidative Stress." Investigative Ophthalmology & Visual Science **57**(11): 5038-5045.

Petersen, A., M. Zetterberg, J. Sjostrand and J. O. Karlsson (2004). "A new model for assessing proteolysis in the intact mouse lens in organ culture." Ophthalmic Res **36**(1): 25-30.

Pickart, C. M. and D. Fushman (2004). "Polyubiquitin chains: polymeric protein signals." Curr Opin Chem Biol **8**(6): 610-616.

Pickering, A. M. and K. J. Davies (2012). "Degradation of damaged proteins: the main function of the 20S proteasome." Prog Mol Biol Transl Sci **109**: 227-248.

Pickering, A. M., A. L. Koop, C. Y. Teoh, G. Ermak, T. Grune and K. J. Davies (2010). "The immunoproteasome, the 20S proteasome and the PA28alphabeta proteasome regulator are oxidative-stress-adaptive proteolytic complexes." Biochem J **432**(3): 585-594.

Pickering, A. M., R. A. Linder, H. Zhang, H. J. Forman and K. J. Davies (2012). "Nrf2-dependent induction of proteasome and Pa28alphabeta regulator are required for adaptation to oxidative stress." J Biol Chem **287**(13): 10021-10031.

Platel, A., M. Jalfre, C. Pawelec, S. Roux and R. D. Porsolt (1984). "Habituation of exploratory activity in mice: Effects of combinations of piracetam and choline on memory processes." Pharmacology Biochemistry and Behavior **21**(2): 209-212.

Porsolt, R. D., A. Bertin and M. Jalfre (1978). "“Behavioural despair” in rats and mice: Strain differences and the effects of imipramine." European Journal of Pharmacology **51**(3): 291-294.

Pound, P. and M. Ritskes-Hoitinga (2018). "Is it possible to overcome issues of external validity in preclinical animal research? Why most animal models are bound to fail." Journal of Translational Medicine **16**(1): 304.

Powers, E. T., R. I. Morimoto, A. Dillin, J. W. Kelly and W. E. Balch (2009). "Biological and Chemical Approaches to Diseases of Proteostasis Deficiency." Annual Review of Biochemistry **78**(1): 959-991.

Prendergast, B. J., K. G. Onishi and I. Zucker (2014). "Female mice liberated for inclusion in neuroscience and biomedical research." Neuroscience & Biobehavioral Reviews **40**: 1-5.

Prinz, F., T. Schlange and K. Asadullah (2011). "Believe it or not: how much can we rely on published data on potential drug targets?" Nature Reviews Drug Discovery **10**(9): 712-712.

Prut, L. and C. Belzung (2003). "The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review." European Journal of Pharmacology **463**(1): 3-33.

PSME1, P. "Proteasome activator complex subunit 1." Retrieved 24/3, 2020.

PSME2, P. "Proteasome activator complex subunit 2." Retrieved 24/3, 2020.

Raule, M., F. Cerruti, N. Benaroudj, R. Migotti, J. Kikuchi, A. Bachi, A. Navon, G. Dittmar and P. Cascio (2014). "PA28αβ Reduces Size and Increases Hydrophilicity of 20S Immunoproteasome Peptide Products." Chemistry & Biology **21**(4): 470-480.

Rigoulet, M., E. D. Yoboue and A. Devin (2010). "Mitochondrial ROS Generation and Its Regulation: Mechanisms Involved in H2O2 Signaling." Antioxidants & Redox Signaling **14**(3): 459-468.

Rippon, G., R. Jordan-Young, A. Kaiser and C. Fine (2014). "Recommendations for sex/gender neuroimaging research: key principles and implications for research design, analysis, and interpretation." Frontiers in human neuroscience **8**: 650-650.

Rivera, J. and L. Tessarollo (2008). "Genetic Background and the Dilemma of Translating Mouse Studies to Humans." Immunity **28**(1): 1-4.

Rivett, A. J., S. Bose, A. J. Pemberton, P. Brooks, D. Onion, D. Shirley, F. L. L. Stratford and K. Forti (2002). "Assays of proteasome activity in relation to aging." Experimental Gerontology **37**(10): 1217-1222.

Roof, R. L. and D. G. Stein (1999). "Gender differences in Morris water maze performance depend on task parameters." Physiology & Behavior **68**(1): 81-86.

Sandstrom, N. J., J. Kaufman and S. A. Huettel (1998). "Males and females use different distal cues in a virtual environment navigation task1Published on the World Wide Web on 27 January 1998.1." Cognitive Brain Research **6**(4): 351-360.

Schmidt, M. and D. Finley (2014). "Regulation of proteasome activity in health and disease." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1843**(1): 13-25.

Schroder, K., P. J. Hertzog, T. Ravasi and D. A. Hume (2004). "Interferon-gamma: an overview of signals, mechanisms and functions." J Leukoc Biol **75**(2): 163-189.

Semba, R. D., E. J. Nicklett and L. Ferrucci (2010). "Does accumulation of advanced glycation end products contribute to the aging phenotype?" J Gerontol A Biol Sci Med Sci **65**(9): 963-975.

Seok, J., et al. (2013). "Genomic responses in mouse models poorly mimic human inflammatory diseases." Proceedings of the National Academy of Sciences of the United States of America **110**(9): 3507-3512.

Shanley, K. L., C.-L. Hu and O. A. Bizzozero (2020). "Proteasome Composition in Cytokine-Treated Neurons and Astrocytes is Determined Mainly by Subunit Displacement." Neurochemical Research.

Shih, H.-P., X. Zhang and A. M. Aronov (2017). "Drug discovery effectiveness from the standpoint of therapeutic mechanisms and indications." Nature Reviews Drug Discovery **17**: 19.

Shoji, H. and T. Miyakawa (2019). "Age-related behavioral changes from young to old age in male mice of a C57BL/6J strain maintained under a genetic stability program." Neuropsychopharmacol Rep **39**(2): 100-118.

Shors, T. J., C. Chua and J. Falduto (2001). "Sex Differences and Opposite Effects of Stress on Dendritic Spine Density in the Male Versus Female Hippocampus." The Journal of Neuroscience **21**(16): 6292-6297.

Sijts, A., Y. Sun, K. Janek, S. Kral, A. Paschen, D. Schadendorf and P.-M. Kloetzel (2002). "The role of the proteasome activator PA28 in MHC class I antigen processing." Molecular Immunology **39**(3): 165-169.

Siwak, C. T., P. D. Tapp and N. W. Milgram (2001). "Effect of age and level of cognitive function on spontaneous and exploratory behaviors in the beagle dog." Learning & memory (Cold Spring Harbor, N.Y.) **8**(6): 317-325.

Sohal, R. S. and R. Weindruch (1996). "Oxidative Stress, Caloric Restriction, and Aging." Science (New York, N.Y.) **273**(5271): 59-63.

Soliman, F., et al. (2010). "A Genetic Variant BDNF Polymorphism Alters Extinction Learning in Both Mouse and Human." Science **327**(5967): 863.

Stadtman, E. R. (2006). "Protein oxidation and aging." Free Radical Research **40**(12): 1250-1258.

Stadtman, E. R. and R. L. Levine (2003). "Free radical-mediated oxidation of free amino acids and amino acid residues in proteins." Amino Acids **25**(3): 207-218.

Strehl, B., U. Seifert, E. Krüger, S. Heink, U. Kuckelkorn and P.-M. Kloetzel (2005). "Interferon-γ, the functional plasticity of the ubiquitin–proteasome system, and MHC class I antigen processing." Immunological Reviews **207**(1): 19-30.

Sumien, N., M. N. Sims, H. J. Taylor and M. J. Forster (2006). "Profiling psychomotor and cognitive aging in four-way cross mice." Age (Dordrecht, Netherlands) **28**(3): 265-282.

Suzuki, K., E. T, T. J, J. Matsuda and M. Vanier (2004). "Are animal models useful for understanding the pathophysiology of lysosomal storage disease?" Acta paediatrica (Oslo, Norway : 1992). Supplement **92**: 54-62; discussion 45.

Szentes, N., V. Tekus, V. Mohos, E. Borbely and Z. Helyes (2019). "Exploratory and locomotor activity, learning and memory functions in somatostatin receptor subtype 4 gene-deficient mice in relation to aging and sex." Geroscience **41**(5): 631-641.

Tanahashi, N., Y. Murakami, Y. Minami, N. Shimbara, K. B. Hendil and K. Tanaka (2000). "Hybrid Proteasomes: Induction by IFN-γ and contribution to ATP-dependent proteolysis." Journal of Biological Chemistry **275**(19): 14336-14345.

The Swedish National Board of Health and Welfare. (2017). Statistics on causes of deaths 2016.

Tijdink, J. K., R. Verbeke and Y. M. Smulders (2014). "Publication Pressure and Scientific Misconduct in Medical Scientists." Journal of Empirical Research on Human Research Ethics **9**(5): 64-71.

Truscott, R. J. (2005). "Age-related nuclear cataract-oxidation is the key." Exp Eye Res **80**(5): 709-725.

Turturro, A., P. Duffy, B. Hass, R. Kodell and R. Hart (2002). "Survival Characteristics and Age-Adjusted Disease Incidences in C57BL/6 Mice Fed a Commonly Used Cereal-Based Diet Modulated by Dietary Restriction." The Journals of Gerontology: Series A **57**(11): B379-B389.

Tuttle, A. H., V. M. Philip, E. J. Chesler and J. S. Mogil (2018). "Comparing phenotypic variation between inbred and outbred mice." Nat Methods **15**(12): 994-996.

Wald, C. and C. Wu (2010). "Of Mice and Women: The Bias in Animal Models." Science **327**(5973): 1571-1572.
Wallace, A. (1858). "On the Tendency of Varieties to Depart Indefinitely From the Original Type.".
Wallach, J. D., K. W. Boyack and J. P. A. Ioannidis (2018). "Reproducible research practices, transparency, and open access data in the biomedical literature, 2015–2017." PLOS Biology **16**(11): e2006930.
van der Worp, H. B., D. W. Howells, E. S. Sena, M. J. Porritt, S. Rewell, V. O'Collins and M. R. Macleod (2010). "Can animal models of disease reliably inform human studies?" PLoS medicine **7**(3): e1000245-e1000245.
Ward, J. M. and D. E. Devor-Henneman (2004). "Mouse Models of Human Familial Cancer Syndromes." Toxicologic Pathology **32**(1_suppl): 90-98.
Verma, R., L. Aravind, R. Oania, W. H. McDonald, J. R. Yates, E. V. Koonin and R. J. Deshaies (2002). "Role of Rpn11 Metalloprotease in Deubiquitination and Degradation by the 26S Proteasome." Science **298**(5593): 611-615.
Whitby, F. G., E. I. Masters, L. Kramer, J. R. Knowlton, Y. Yao, C. C. Wang and C. P. Hill (2000). "Structural basis for the activation of 20S proteasomes by 11S regulators." Nature **408**(6808): 115-120.
WHO (2017). "World Health Statistics 2017." 29-33.
WHO, W. H. O. (2019, 2019-09-02). "Gender."
Vijg, J. and B. K. Kennedy (2016). "The Essence of Aging." Gerontology **62**(4): 381-385.
Vilchez, D., I. Saez and A. Dillin (2014). "The role of protein clearance mechanisms in organismal ageing and age-related diseases." Nat Commun **5**: 5659.
Williams, G. C. (2001). "Pleiotropy, Natural Selection, and the Evolution of Senescence." Sci. Aging Knowl. Environ. **2001**(1): cp13-.
Võikar, V., E. Vasar and H. Rauvala (2004). "Behavioral alterations induced by repeated testing in C57BL/6J and 129S2/Sv mice: implications for phenotyping screens." Genes, Brain and Behavior **3**(1): 27-38.
Yamano, T., S. Murata, N. Shimbara, N. Tanaka, T. Chiba, K. Tanaka, K. Yui and H. Udono (2002). "Two Distinct Pathways Mediated by PA28 and hsp90 in Major Histocompatibility Complex Class I Antigen Processing." The Journal of Experimental Medicine **196**(2): 185-196.
Young, J. C., V. R. Agashe, K. Siegers and F. U. Hartl (2004). "Pathways of chaperone-mediated protein folding in the cytosol." Nat Rev Mol Cell Biol **5**(10): 781-791.
Zhang, Z., A. Clawson, C. Realini, C. C. Jensen, J. R. Knowlton, C. P. Hill and M. Rechsteiner (1998). "Identification of an activation region in the proteasome activator REGα." Proceedings of the National Academy of Sciences **95**(6): 2807-2811.
Zhao, R.-Z., S. Jiang, L. Zhang and Z.-B. Yu (2019). "Mitochondrial electron transport chain, ROS generation and uncoupling (Review)." International journal of molecular medicine **44**(1): 3-15.