SASP Modulates Neuronal Activity in A Human *in-vitro* Brain Model

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Abstract

Ageing and many neurodegenerative diseases are accompanied by subtle but chronic inflammation. Senescent cells are known as a hallmark of ageing with strong connotations to ageing and disease. A key feature of senescent cells is the evasion of apoptosis, hence leading to their accumulation with progressive age. Many senescent cells will adopt a state, known as the secretory-associated senescence phenotype (SASP), which is defined by the release of pro-inflammatory molecules in their surrounding environment. Therefore, the accumulation of senescent cells with age might be a major contributor to this age-related increase in inflammation. Historically, senescence was associated with mitotic cells, but more recent evidence changed this long standing belief, as it has been shown that postmitotic cells can also adopt a senescent-like phenotype. Because longevity is a defining characteristic of neurons, the accumulation of senescent neurons, and subsequent acquisition of the SASP could be an important driver of neuroinflammation, which might contribute to a myriad of neurodegenerative diseases. Here, we examine the role of SASP on inflammation, neuronal activity, and neurodegeneration in the context of the human brain by using a human neural *in-vitro* model.

Contents

1 Introduction

Ageing is one of the most significant factors for disease onset. Kirkwood and Austad describe it as the progressive loss of function accompanied by decreasing fertility, and increasing mortality with advancing age [1]. Adding the fact, that in developed countries ageing is considered the most severe risk factor for disease onset, ageing poses a major thread to modern and future society [2]. While the individual and global life expectancy steadily increases, the cases of medical conditions are likely to follow the same trend. If unsolved, this would lead to a large proportion of society to suffer from chronic or frequent health issues, which is not only a huge burden for the suffering individuals, but also a major strain on national and global economics. It is therefore of great interest to increase the human health-span alongside life-span to avoid the excruciating effects of age-related afflictions. Although the specific mechanisms, which cause and drive ageing are still largely shrouded in mystery, Lopez-Otin *et al.* published a widely accepted framework of ageing by identifying nine major hallmarks of ageing, which together deliver a comprehensible synopsis of the ageing process and its phenotype [3].

1.1 About the Role of Senescent Cells for Ageing and Inflammation

One of these aforementioned hallmarks of ageing is cellular senescence, which first and foremost is characterized as the induction of permanent cell cycle arrest, but also by changes in metabolism, inflammation, a flat and enlarged morphology, appearance of senescence-associated heterochromatic foci (SAHF), epigenetic changes, DNA segments with chromatin alterations, and evasion of apoptosis [4, 5, 6].

Hayflick first observed that somatic cells undergo a limited number of divisions before they enter a terminal static phase defined by a permanent arrest of cell division. This so-called replicative senescence was first solely attributed to the continuous attrition of telomeres with every cell cycle, but could since be observed as a response to stress stimuli in general. This latter definition of senescence is known as cellular senescence [7].

Which purpose cellular senescence naturally fulfills has divided the fields of cancer and ageing research. The former attributed cellular senescence a protective role, capable to prevent critically damaged cells to undergo cancer transformation. Meanwhile, ageing research described cellular senescence as a sign of attrition evoked by the accumulation of cellular damage in ageing cells [8]. Although we still have to learn a lot about cellular senescence, it is likely that the truth lies somewhere in between. Regardless of its primary purpose, the concept of senescence has historically been inseparably tied to cell division, and research was therefore limited to the scope of mitotic cells for a long time.

This long-standing belief slowly crumbles as more and more emerging evidence supports the presence of a senescence-like phenotype in terminally differentiated or postmitotic cells, which is also known as *Amitosenescence*. Many of

the features, which define senescence in mitotic cells, can also be observed in postmitotic cell types, like neurons and cardiomyoctes [9, 10].

Ageing is accompanied by a subtle, but constant increase of low-grade inflammation - often labelled as *Inflammaging* [11]. This chronic inflammatory response in the brain is suspected to be involved in the development of neurodegenerative diseases like Alzheimer's disease (AD) [12]. The exact underlying mechanisms have yet to be discovered but are characterized by elevated levels of reactive oxygen (ROS) and nitrogen species, defective autophagy, cellular senescence, and DNA damage response (DDR) activation [13, 12, 14]. As senescent cells accumulate with progressing age they can cause inflammation, which makes them a primary suspect for *Inflammaging*.

One key mechanism for how senescent cells can cause chronic inflammation is by adapting a loosely defined phenotype, known as the senescence-associated secretory phenotype (SASP). The term SASP summarizes a plethora of similar phenotypes, defined by the aberrant secretion of cytokines, growth factors, and other factors in senescent cells [15]. Acquisition of the SASP and the subsequent secretion of pro-inflammatory molecules causes inflammation in the surroundings of senescent cells; hence creating a pro-inflammatory micro-environment, which negatively affects or damages nearby cells, and aids the development of disease.

This inflammatory micro-environment was quickly associated to be at least partially responsible for the correlation between age and cancer development [16]. Furthermore, many other ageing-related diseases also correlate with SASP acquisition, including kidney failure, osteoporosis, and several neurodegenerative diseases [17, 14, 18]. For example, the release of inflammatory cytokines and chemokines can be observed at the onset of atherosclerosis by senescent cells, where they drive pathology [19].

The phenomenon of SASP was first described by Campisi *et al.*, when they showed that senescent cells would induce tumorigenesis in nearby pre-malignant cells by aberrant secretion of molecules that cause inflammation and oncogenesis [20, 21]. Other early evidence for the SASP-Inflammation hypothesis was observed in senescent fibroblasts, where the expression of matrix-degrading proteases and inflammatory chemokines is strongly up-regulated, thus resembling the phenotype of activated fibroblasts during wound healing [22].

Although the predominantly negative connotations of SASP, one also has to put its beneficial effects into perspective. Arguably its best known beneficial effect is in promoting wound healing [23]. In contradiction to the publications linking SASP and senescence to tumorigenesis, other sources report that SASP mediates cell cycle arrest and therefore effectively functions as a tumor suppressor via secretion of IL-8, IL-6, PAI-1, and IGFBP7 [24, 25, 26].

The profile of secreted factors is known as the secretome. It is strongly heterogeneous and depends on the cell type, time, and further cellular context. Analysis of soluble SASP factors between fibroblasts and epithelial cells revealed that between both cell types only about 9-23% factors are shared [21, 27, 28].

The soluble secreted factors can roughly be categorized as follows: Soluble signaling factors, secreted proteases, and secreted insoluble proteins/extracellular matrix (ECM) components [29, 30, 31].

More evidence for the hypothesis that SASP contributes to Inflammaging comes from the expression of many pro-inflammatory genes, which are usually up-regulated with advanced age. Accordingly, the expression of IL-6, GRO1, PAI1, and TGF-beta all increase with age, while IGF1 decreases. These factors, together with IL-8 and IGFBP7, further support the senescence-induced growth arrest [32, 25].

IL-6 is a pleiotropic pro-inflammatory cytokine, which appears to be a core component for the inflammatory nature of SASP and oncogene-induced senescence. If knocked out it strongly attenuates the inflammatory response, as well as oncogene-induced cellular senescence [33, 34]. Secretion of IL-6 might be directly controlled by ATM and CHK2 in response to persistent DNA damage[30]. Because IL-6 is consistently expressed during SASP, it might considerably contribute to the inflammatory stress response of neighbouring cells harboring the IL-6 receptors gp80 and gp130 signaling complex during SASP exposure [31].

Like during neuronal cell death, acquisition of the SASP can also be traced back to the DDR pathway and aberrant activation of cell cycle factors. For example, SASP activation in response to DNA damage can be observed in patients undergoing chemotherapy or radiation therapy [35, 36, 37].

Persistent DNA damage forms lesions and double strand breaks, which lead to the secretion of cytokines like IL-6. The release of cytokines is regulated downstream of the DDR pathway, and relies on the recruitment of ATM, NBS1, and CHK2. Thus, malignant phenotypes evoked by IL-6 can be attenuated by upstream inhibition of ATM. Because mostly cycling cells express IL-6, it further emphasises the connection between DDR, cell cycle, inflammation, and cell death. However, the secretion of IL-6 can also occur independently of p53 and RB, which suggests that some SASP inducing pathways may be bypassed [30].

Overall, it remains unclear which exact mechanisms mediate the induction of the SASP. Because the SASP is not precisely defined, multiple pathways are likely to be involved in the manifestation of its symptoms. Similar to the heterogeneous composition of the secretome the signalling cascades upstream of SASP also seem to depend on a variety of factors.

Downstream of the discussed DDR pathway, NF-κB appears to be a major regulator for the expression of pro-inflammatory factors in SASP. NF-κB itself is considered to be a master regulator of the innate immune response and is also increasingly expressed with higher age [38]. In general, activation of NF-κB promotes the expression of inflammatory genes [39]. In turn, the inflammatory cytokines activate the NF-κB pathway in senescent cells and prevent them from undergoing apoptosis, which in turn could cause their transformation to senescent cells, leading to even more senescent cells. NF-κB often interacts with C/EBP to regulate the transcription of many SASP genes [38].

Another stress induced factor, which also happens to be induced by inflammatory cytokines, is Mammalian p38 mitogen-activated protein kinase (p38MAPK) [40]. p38MAPK can induce cell cycle arrest through p53 and RB, and might activate NF-κB, and ultimately cause induction of SASP [41, 38]. p38MAPK itself gets activated in response to various extracellular stimuli, including inflammatory cytokines and growth factors as secreted during SASP, but also from UV light, heat, or osmotic shock [42, 43, 44, 45]. Downstream, the activation of p38 promotes the expression of pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α [46]. This could be a reason why p38 is involved in AD development [47].

1.1.1 Senescence and the Cell Cycle

One major and the name-giving feature of postmitotic cells is their inability to proliferate. Not too long ago it was widely believed that mature neurons and other postmitotic cells permanently lose their ability to proliferate after they are fully differentiated. This clearly distinguishes them from quiescent cells, which enter a state of reversible cell cycle arrest, capable to reenter cell cycle if needed [8]. However, the belief about whether the fate of postmitotic cells is indeed terminal is being challenged by the hypothesis that neurons' chronic arrest in the G0 phase relies on a persistent and controlled inhibition of cell cycle progression. Failure in this system can lead to activation of otherwise suppressed cell cycle factors, which are suspected to cause a myriad of problems, and might render the cell vulnerable to neurodegeneration [48, 49]. Activation of cell cycle factors might be a response to major stress events, which disrupt core components of the cell cycle's inhibitory complex and thus awake the neurons' cell cycle machine from its somber sleep in the G0 phase. Although some hypotheses attribute this to a postmitotic cells' desire to reenter the cell cycle it probably only affects some parts of the cell cycle program [50, 51, 52, 49].

Following persistent stress stimulation, the DDR pathway gets activated, just like it happens in mitotic cells. In mitotic cells, the DDR response pathway is a potent inhibitor of cell cycle progression. This prevents damaged cells to undergo cell division until the DNA damage is repaired, which is crucial to avoid cancer transformation [53, 54]. Usually, the DDR-pathway is activated when damage persists, and in response will recruit Phosphatidylinositol-3 kinase-related kinases Ataxia telangiectasia mutated (ATM) and ATM and RAD3-related (ATR) kinases. The latter stabilizes p53, leading to the expression of p21, a potent cell cycle inhibitor, which works by inhibiting CDK1/2 and Cyclin A/E. Cyclins and CDKIs are required to trigger the transition of the cell cycle from G1 to S-phase, which depends on the dissociation of E2F from RB. Before E2F can be released, RB needs to be phosphorylated by Cyclin D and CDK 4/6 [9, 55, 56, 57].

The DDR pathway is also responsible for the induction of apoptosis. This is important, because the evasion of apoptosis is a key feature of senescent cells, leading to the accumulation of senescent cells with advancing age. It is estimated that senescent cells constitute around 20% of cells in very old primates [58]. However, the exact mechanisms about whether a cell is killed in response to persistent damage or transforms into a senescent cell are still unclear. Some sources claim that cell death in postmitotic cells is induced when a cell attempts to reenter the cell cycle. Although this hypothesis does not necessarily have to hold true, many genes and proteins classically associated with the cell cycle machinery are involved in senescent and apoptotic pathways. However, induction of senescence in mitotic as well as postmitotic cells is very heterogeneous and can be the result of different stress stimuli and pathways.

A large body of evidence suggests that cell cycle inhibitors, such as p16, p21, and p53 take on a protective role by mitigating cell death, but promote the induction of senescence. Accordingly, many cell cycle inhibitors are often found to be up-regulated in senescent cells, including postmitotic ones [59, 60, 61, 62]. This would make sense in a neural context; because our brains require neurons to be exceptionally long-lived, and are very limited in their regenerative capacities it makes sense for neurons to prioritize cell survival over the acquisition of a senescent-like state.

While RB phosphorylation and release of E2F leads to proliferation in mitotic cells, the same events have been observed to cause degeneration of neurons [63, 64]. On that note increased levels of the cell cycle promotor Cyclin D1–associated kinase was detected in apoptotic cortical neurons, while over-expression of the cyclin-dependent kinase inhibitors $p16^{Ink4a}$, $p21^{waf/cip1}$, $p27^{kip1}$, as well as a double knockout of CDK4/6 could rescue neurons from DDR mediated apoptosis [65]. Furthermore, the artificial inhibition of cell cycle transition proteins such as CDK4 and CDK6 could also interfere with the damage response [64, 66, 65]. Interestingly, CDK4/6 and RB are part of the same pathway and when activated by Cyclin D, CDK4/6 can directly phosphorylate RB to promote cell cycle progression [67]. Mutation of RB phosphorylation sites, including the CDK 4/6 site, which inhibits E2F release from RB, also prevents cell death [66]. Accordingly, cerebellar expression of the SV40 T antigen, capable of binding RB and p53, led to the degeneration of Purkinje cells and resulted in an ataxia-like phenotype in mice [63]. Direct targeting of the transcription factor and RB target E2F by mutating DP1, a protein required for E2F activation, also rescues neurons from cell death, which further emphasizes the role of proteins involved in cell cycle progression on cell death [66].

The same components of the cell cycle, including Cyclins (A,B,D,E), CDKs, and CDKIs have also been reported to be elevated in AD patients, thus establishing a link between cell cycle mediated neuron death and neurodegeneration [49, 68, 52, 69]. However, at least one publication observed a correlation of brain atrophy and NFT burden with increasing levels of CDKN2a, the gene encoding for $p16^{INK4a}$ and $p14^{ARF}$, in a murine AD model [70].

1.2 About the Role of $p16^{INK4a}$ in Senescence

$p16^{INK4a}$ and $p14^{ARF}$ are both encoded by the CDKN2A gene, and highly relevant for senescence induction as its transcription is activated in cells undergoing senescence, and up-regulated during ageing [59, 60, 61]. p16 itself is a potent inhibitor of the cell cycle, and loss of p16 is strongly associated with cancer transformation [71, 72, 73].

Canonically p16 acts through the RB pathway by preventing the interaction between CDK4/6 and Cyclin D, which is required for RB phosphorylation and subsequent E2F release; thereby leading to a cell cycle arrest in the G1 phase [74]. Additionally, various non-canonical pathways downstream of p16 have been described. These affect 1) Nucleotide metabolism via mTORC1; 2) NF-κB via p65; 3) Oxidative stress response; 4) Mitochondrial biogenesis; 5) AP-1 activation; 6) Protein translation via eEF1A2; and 7) Expression of tumor suppressive miRNA via CDK4-SP1 [74].

Stress stimuli are major drivers of p16 expression. Hence, p16 expression levels naturally increase with advanced age in response to DDR, but independent of telomere attrition [75]. Another well-known driver of p16 expression is oncogenic stress; here the expression of oncogenic Ras positively correlates with an increase in p16 and p21 levels. Ras proteins themselves are capable of inducing senescence by altering the intracellular levels of ROS [76]. Oxidative stress from ROS might induce a $p16^{INK4a}$ and $p14^{Arf}$ dependent decline in hematopoietic stem cell function, suggesting that $p16^{INK4a}$ expression participates in cell-autonomous ageing *in-vivo* [77, 78, 59].

Besides p16, the ectopic expression of oncogenic Ras also leads to an increase in p53, which together with p16 can induce cell cycle arrest. On the other hand, expression of oncogenic Ras in cells lacking either p16 or p53 does not induce cell cycle arrest [79]. In fish cells, which are considered to be resistant to senescence, ectopic expression of p16 in the cell line EPC showed a similar phenotype as senescent mammalian cells regarding morphology, SA-β-Gal expression, and an increase in SASP related mRNA levels of pro-inflammatory factors [80].

Expression of $p16^{INK4a}$ induces hallmarks of senescence, such as cell enlargement, higher glucose uptake, mitochondrial activity, and drives glucose-stimulated insulin secretion during normal ageing [81]. A substantial body of evidence claims that p16 up-regulation during persistent stress stimulation is crucial for the induction and maintenance of senescence, and is often responsible for its characteristic cell cycle arrest [79, 82, 83, 84]. Hence, suppression of p16 should prevent, or at least attenuate senescence [73, 72, 74]. Age-related accumulation also interferes with the proliferation of beta cells, thus limiting their regenerative capacity [85]. Meanwhile, inhibition of p16 has been found to keep cells in a stem cell-like state [86].

Like in mitotic cells, p16 expression also positively correlates with age in postmitotic cells. Elevated levels of p16, alongside other senescent markers such as SA-β-Gal, Lamin B1 loss, and SASP could also be found in rat hippocampal and cortical neurons *in vitro* [87].

In-vivo, activation of the $p16^{INK4a}$ promotor also correlates with the increase of senescence features. In mice, cells with active p16 promotors are known to cause inflammation, and also accumulate with age [82]. In respect, suppression of p16 appears to ameliorate the SASP condition, as its knockdown lowers the levels of IL-6 and CXCL8 in oncogene-induced and DDR senescence models [88]. Deletion of p16 in *Bmi-1*$^{-/-}$ mice attenuated senescence, and the inflammatory effects of SASP as well [89].

Both $p16^{INK4a}$ and $p14^{ARF}$, are effective inhibitors of the NF-κB signalling pathway by directly interacting with its p65 subunit [38, 90]. This is interesting because NF-κB signalling usually promotes the expression of inflammatory genes, and its knockdown significantly decreases the expression levels of SASP factors up to 75% [39]. Furthermore, NF-κB-p65 seems to increase upon deletion of p16 in a Bmi-1 mouse model [89].

Due to its prominent role in ageing, cell cycle, and senescence, p16 might be an important factor in disease development. Because p16 basically functions as a cell cycle break it is not surprising that it is strongly associated with cancer. In fact, about half of all cancer cells show diminished levels of CDKN2A expression [91], and although p16 loss of function is not sufficient for cancer transformation it greatly facilitates it [84]. In the year 1999, evidence emerged that p16 co-localizes with both neurofibrillary tangles and neuritic components of plaques in humans with AD [92]. Likewise, mice with Tau-dependent neurodegenerative disease also accumulate senescent astrocytes and microglia, which are positive for $p16^{INK4a}$. Clearance of these cells prevents gliosis and the hyperphosphorylation of soluble and insoluble Tau. Thereby, it prevents the deposition of neurofibrillary tangles and consequently degeneration of cortical and hippocampal neurons; thus preserving cognitive function [93]. In addition, p16 and the metalloproteinase MMP-1 have been found in higher concentrations in astrocytes from the frontal cortex of AD patients. More evidence for the relationship between AD and p16 can be seen upon treatment with Aβ(1-42), which can trigger a senescence response *in-vitro* as indicated by elevated expression levels of p16 and SA-β-Gal. This is accompanied by a higher production of SASP related inflammatory cytokines, including IL-6 [94]. CDKN2A transcripts also correlate with brain atrophy and neurofibrillary tangles in mice. Alongside CDKN2a, several SASP genes as well as the NF-κB pathway are up-regulated in rTg(tauP301L)4510 mice; a mouse model over-expressing human Tau [70].

In humans with Huntington's disease increased levels of $p16^{INK4a}$ have been found in neural stem cells and medium spiny neurons [95].

Rat models for Amyotrophic lateral sclerosis (ALS), which harbor the ALS-mutation SOD1G93A, show nuclear localisation of p16 as well as loss of Lamin B1. Furthermore, microglia positive for p16 also adopt markers of SASP by developing a senescent-like large and flat morphology, increased SA-β-Gal activity, and are positive for p53, MMP-1, and nitrotyrosine [96].

Clearance of $p16^{INK4a}$ ameliorates ageing-related conditions in several organs, including postmitotic cardiomyocytes. Accumulation of $p16^{INK4a}$ also

correlates with a shortened life- and health-span. Corresponding ablation experiments in a murine model could extend lifespan by about 25% [83, 97].

Microglia in the human brain overall express higher levels of p16 with advanced age, but only some subpopulations of p16-expressing microglia positively correlate with age. The expression of inflammatory genes is promoted by p16 and interestingly chemokine enriched inflammatory microglia become enriched with age [98, 99].

1.3 Senolytics

Because senescent cells accumulate with age by evading cell death through activation of anti-apoptotic pathways, they might eventually acquire the SASP. This transformation can lead to a cascade, where the chronic paracrine inflammatory signalling from secreted SASP factors damages otherwise healthy proximal cells up to a point where they themselves become senescent, which consequentially extends the perimeter of the inflammatory micro-environment. Therefore, targeting of senescent cells, especially in the elderly, has been discussed as an attractive strategy to interfere with the pro-inflammatory signalling and to ameliorate neurodegeneration. Successful ablation of $p16^{Ink4a}$-positive senescent cells has already shown to delay ageing-associated disorders in mice, while late intervention could attenuate the progression of already established age-related disorders [83].

However, in contrast to transgenic modifications, chemical compounds are more accessible and easier to bring into clinical trials. Drugs designed to specifically target and kill senescent cells are called *Senolytics*, while drugs interfering with the progression and activity of senescence are termed *Senostatics*. In progeroid mice pharmacological intervention of the NF-κB activating kinase (IKK) with 8K-NBD delayed age-related symptoms [100]. NF-κB is known to regulate the apoptosis pathway [101, 102], and thus targeting it could lead to artificial induction of apoptosis, and the ablation of senescent cells. Hence, targeting the apoptotic pathway provides an interesting opportunity for the discovery of senolytic compounds. And indeed, by performing knock-down experiments on anti-apoptotic pathways many senolytic target sites could be revealed. Among the most interesting target sites were p21, and PI3KCD a member of the Pi3K pathway, well known for its role in cell cycle and apoptosis, as well as BCL-xL, which regulates mitochondrial-dependent apoptosis [103, 104, 105]. Inhibition of BCL-2 family proteins and the aforementioned BCl-xL with Navitoclax (ABT263) could successfully ablate $p16^{INk4a}$-positive senescent cells, prevent age-related bone loss [106], and reduce atherogenesis onset in mice[19] Although the use of senolytics for neurodegenerative diseases is still in its infancy, it harbors a huge potential, which has yet to be discovered. Two chemical compounds, namely Quercetin and Dasatinib, show exceptional potential to be used as senolytic drugs. Both molecules interfere with the Pi3K-Akt pathway to promote apoptosis, whereas Dasatinib inhibits AKT, and Quercetin Pi3K. Treatment with Quercetin and Dasatinib selectively removes senescent cells from a murine AD model, which had beneficial effects

on the plaque environment, reduced neuroinflammation, lessened Aβ load, and ameliorated cognitive deficits [107].

Dasatinib treatment alone could induce clearance of senescent human pre-adipocytes, while Quercetin on its own could induce cell death in senescent epithelial cells, but also in pre-adipocytes, although to a lesser degree. Treatment with a combination of both drugs showed to be more effective in inducing apoptosis, and applies to a broader spectrum of senescent cells [103]. The combination of both, as well as ABT263 treatment, could also decrease $p16^{INK4a}$ positive senescent microglia in aged mice, resulting in a reduced expression of SASP factors, and significantly improved cognitive function [108]. Furthermore, combined treatment effectively clears p16-positive cells in aged murine fat and liver [103]. Dasatinib and Quercitin treatment has also been reported to reduce NFT burden and neurodegeneration in Tau transgenic mice [70]. Finally, ABT263 also showed promising results regarding neurodegenerative diseases by successful ablation of senescent astrocytes in mouse brains [109].

1.4 Directly Re-programmed Neurons as a Model for Human Ageing Research

Research of cellular senescence and senolytic treatment has yielded promising results in various animal models *in-vitro* as well as *in-vivo*; however, their effect in human postmitotic tissues is still unclear. Human neurons constitute a prime target for ageing research due to their exceptional longevity and low capacity for self-renewal, which demand effective strategies to tackle neurodegenerative diseases. Given the famous saying that *"all models are wrong, but some are useful"*, human induced neurons (iNs) directly induced from donors' skin biopsies constitute a potentially useful model system for the research of ageing in postmitotic tissues. The iN system is particularly relevant because these patient-specific neurons capture many important ageing hallmarks of their human donor, including transcriptome and epigenome [110].

This key feature differentiates iNs from other re-programmed neurons, which were differentiated from induced pluripotent stem cells (iPSCs). Yamanaka *et al.* discovered that the cell fate of differentiated somatic cells can be reversed to a more naive cell fate resembling pluripotent embryonic cells, by over-expression of Klf4, Oct3/4, c-Myc, and Sox2 [111]. During the process, iPSCs are being rejuvenated and lose their hallmarks of ageing. Neurons derived from iPSCs hence lose critical information for many ageing-related experiment set-ups. Direct conversion protocols skip this intermediate rejuvenating iPSC step and thus the cells' ageing hallmarks are preserved. Early neuronal direct conversation protocols came from Werning *et al.*; where they showed that the three factors Ascl1, Brn2/Pou3f2, and Myt1l are sufficient to convert mouse embryonic and postnatal fibroblasts into functional neurons [112]. Thereupon a plethora of new protocols to induce specific neuronal subtypes were published [113]. Many of these protocols, although not all of them, rely on the over-expression of Ascl1 and Ngn2 [113]. For example, fibroblasts can be converted to glutaminergic and GABAergic iNs by over-expression of Ascl1 and Ngn2 together with small

molecular inhibition of selected signalling pathways [114]. These protocols allow for easy and fast conversion of readily available cells such as fibroblasts from skin biopsies and thus provide a solid model for studying ageing in the otherwise very difficult accessible human nervous system. Transcriptomic as well as epigenetic methylation data shows that iNs derived from fibroblasts at different donor ages correlate with distinct ageing signatures [115, 113].

1.5 Aim of the Project

Here, we show how the inflammatory-promoting milieu generated by the SASP affects neuronal activity in a human *in-vitro* brain model. First, we induce senescence in iNs by over-expression of $p16^{INK4A}$. Using condition media from senescent-like iNs we expose our brain model to a pro-inflammatory microenvironment associated with the SASP, and quantify its effect on neuronal activity via calcium imaging by examining calcium transients with gCAMP6m [116] and spinning-disk confocal imaging. Through this project, we gain a better understanding of how neuroinflammation develops in the ageing brain. By targeting senescent cells with senolytic compounds we might be able to stop neurodegeneration in its early stages to promote health-span for many affected people.

2 Results

2.1 Ectopic Expression of $p16^{INK4a}$ Establishes Senescent-like iNs

Human fibroblasts were successfully converted into age-matched iNs by activating the transgenic tet-On Ngn2:2A:Ascl1 (UNA, Fig.1 A) construct in combination with small molecular treatment. During the three weeks of conversion the cells underwent distinctive morphological changes, and adopted a typical neuronal cell morphology. At the end of treatment, converted iNs featured a defined and small cell soma as well as distinct cell protrusions (Fig.1 D). To evaluate whether the conversion was indeed successful, some iNs were transduced with a lentiviral construct to over-express the fluorescent marker protein dsRed under the control of the neuron-specific Synapsin (Sy1) promotor. Subsequent observation of fluorescence, emitted by iNs proves that the direct conversion to mature iNs had been successful (Fig.2 B).

Fully converted iNs were further transduced with p16-ZS-green to over-express $p16^{INK4a}$, and to induce the post-mitotic senescence-like state. Successful incorporation of the p16 construct was validated by the expression of the construct's GFP-tag (Fig.2 A). To examine if over-expression of $p16^{INK4a}$ induced hallmarks of senescence in iNs, we stained iNs for SA-β-Gal, a widely used biomarker for senescence. Co-localization of $p16^{INK4a}$ and SA-β-Gal could be observed, thus validating the effectiveness of ectopic $p16^{INK4a}$ expression (Fig.2 D).

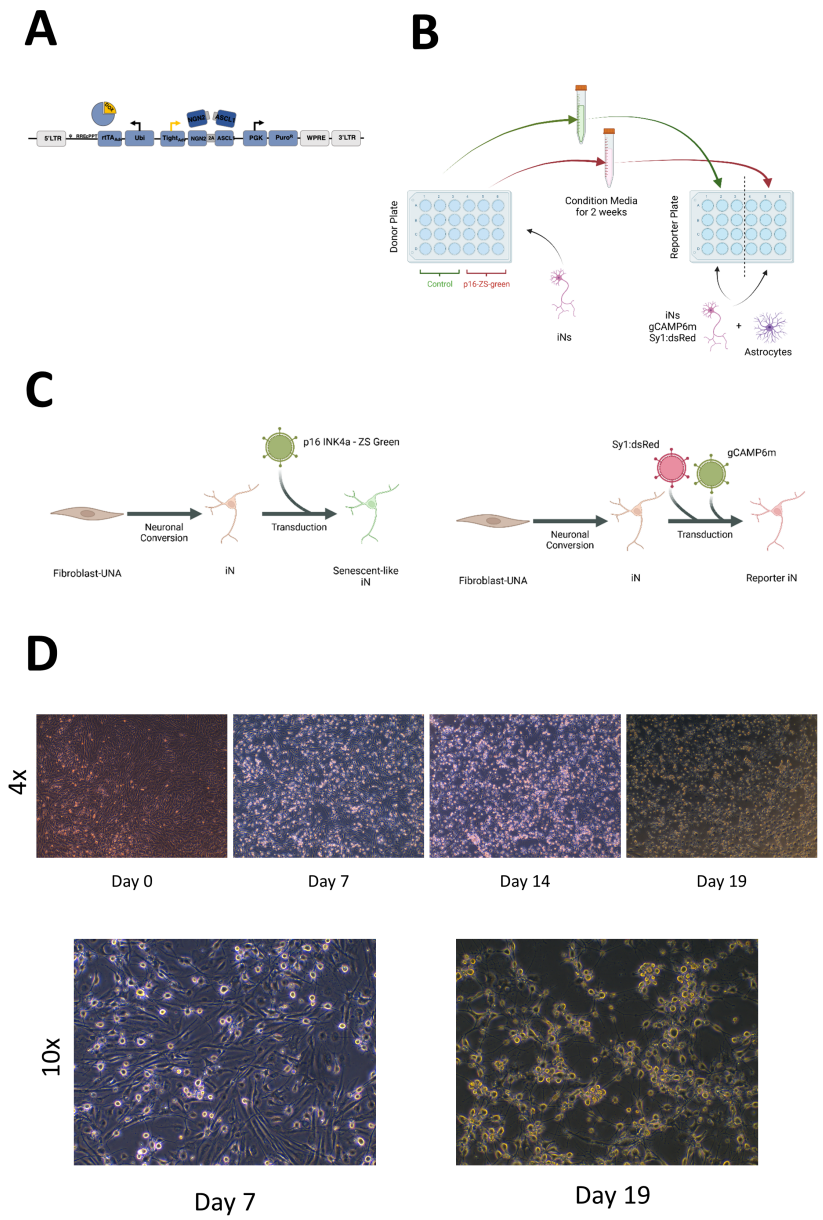

Figure 1: Construct map of UNA for neuronal conversion of fibroblasts to induced neurons (A). Work-flow of the condition media experiment (B). Establishment of p16$^{\mathrm{INK4a}}$ over-expressing donor cells by transduction (left). Establishment of iNs for the reporter group, over-expressing gCAMP6m and Sy1:dsRed (right) (C). Morphological changes of cells during three weeks of iN conversion (D).

2.2 $p16^{INK4a}$ Over-expression in iNs Effects iN Functionality

To assess the effect of SASP on neuronal activity we measured Ca^{2+} transients by using a gCAMP6m reporter. Because intracellular calcium transients reflect neuronal electric events, calcium imaging can be used to quantify neuronal activity [117].

At this stage we divided mature iNs in two groups. The first group, termed the *donor* group consisted of the aforementioned $p16^{INK4a}$ over-expressing iNs and a not-transduced control group. On the other site, the second group, labelled as the *reporter*-group was transduced with gCAMP6m to detect neuronal activation (Fig.1 C). Because the gCAMP reporter constructs have been reported to display leakiness [118], we also labelled iNs with Sy1:dsRed, and selected cells for co-localisation of both reporters to only measure signals, which really came from converted neurons. Finally, Sy1:dsRed and gCAMP6m transduced iNs were co-cultured with human cortical astrocytes to simulate a more complex human brain environment, and to support the viability and functionality of the iNs. These reporter co-cultures were treated with condition media of the donor groups for two weeks, and the neuronal activity was measured by time-series imaging using Ca^{2+} transients.

Following treatment the human iN-astrocyte models treated with condition media from $p16^{INK4a}$ over-expressing senescent-like iNs showed elevated levels of calcium transients based on an increase in observed gCAMP6m events, compared to the control group receiving condition media from wild-type iNs (Fig.2 C). Overall, the increase in gCAMP6m activity indicates a higher level of neuronal activity or neuronal hyperactivity in cells exposed to condition media of senescent-like cells.

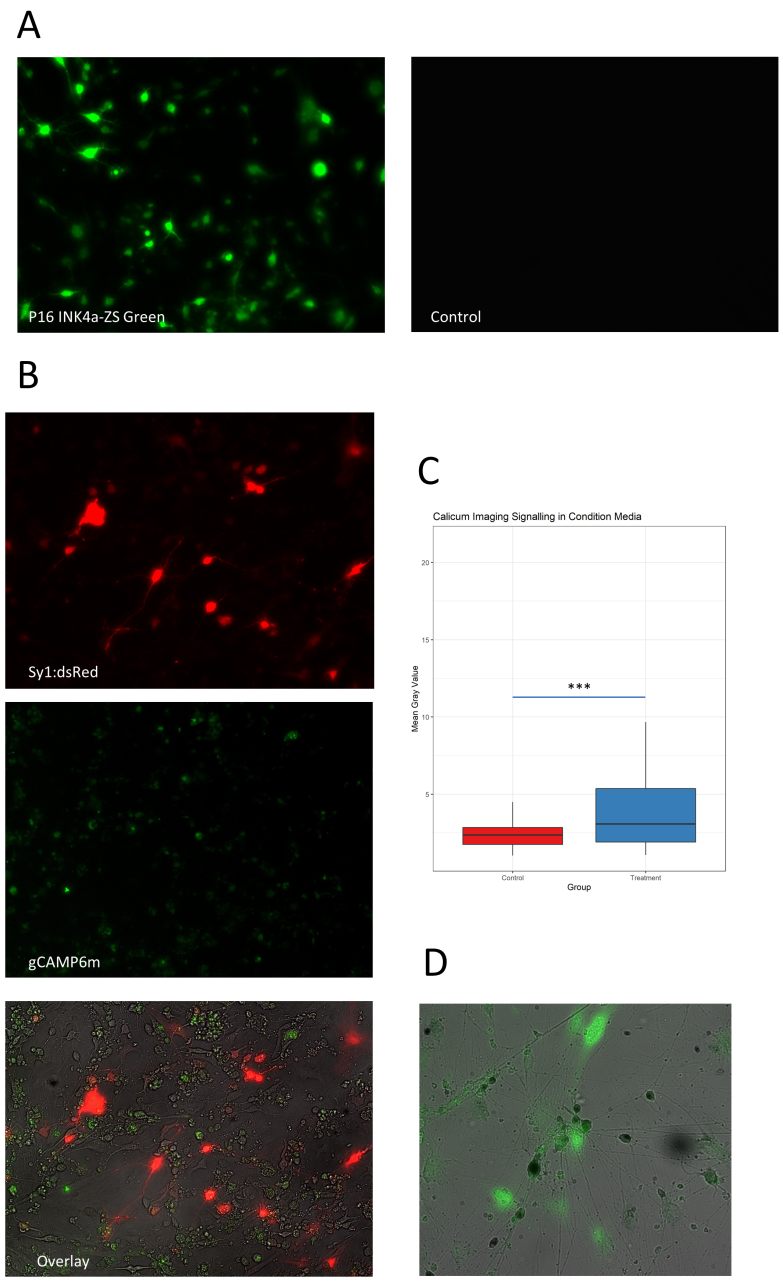

Figure 2: Expression of $p16^{INK4a}$ in donor media iNs (left), and absence in control donor media iNs (right) (A). Expression of the neuronal marker Sy1:dsRed and gCAMP6m in reporter iN and astrocyte co-cultures (top and middle respectively); overlay of fluorescence markers and captured bright-field (bottom) (B). Effect of condition media treatment from either senescent-like INs or control iNs on gCAMp6/neuron activation (C). Co-localization of p16 and SA-β-Gal (D). Significance Levels: $* < 0.05$; $** < 0.01$; $*** < 0.001$

3 Discussion

Here we show evidence, that in a healthy human *in-vitro* brain model exposure to SASP modifies neuronal signalling. By measuring the calcium ion transients of induced neurons after continuous exposure to condition media, taken from senescent-like iNs, we could detect neuronal hyperactivity in treated cells.

Our observations resemble evidence from murine models, where neuronal hyperactivity emerges as both a hallmark of ageing and AD [119, 120, 121]. In wild-type mice, neuronal activity positively correlates with increasing age.

Accordingly, senescent cells typically also accumulate with age. Although we do not yet have enough evidence to claim whether this is true for postmitotic cells as well, many hallmarks of senescence have been observed to increase with age in postmitotic senescent cells. We could observe that iNs, which over-express $p16^{INK4a}$, also express SA-β-Gal. The same co-localization of $p16^{INK4a}$ and SA-β-Gal, could be found *in-vitro* in rat hippocampal and cortical neurons, whereas their expression positively correlates with the SASP and age [87].

An even larger increase in neuronal activity was found in AD mice models, where the accumulation of Amyloid-β aggregates leads to the inhibition of glutamate re-uptake. Following inhibition, glutamate accumulates in the post-synaptic space, where it can cause neuronal depolarization. Hence, it might be possible that neuronal hyperactivity is linked to glutamate neurotoxicity in AD [120].

The role of glutaminergic neurons on neuronal hyperactivity is emphasized by our results as the direct conversion of somatic cells into iNs using Ngn2/Ascl1 and small molecules like we used for our model, will primarily convert them into glutamatergic (vGlut1+) neurons, and to a smaller extend into GABAergic neurons [110, 122].

Calcium homeostasis plays a critical role in neurons as they are very sensitive to even small changes in calcium levels. Importantly, neuronal activity is not only affected by calcium, but calcium-associated neuron activity also correlates with age and neurodegeneration. Accordingly, deregulation of calcium signalling is associated with senescence [123, 124].

Senescent cells exhibit increased levels of intracellular calcium, and in turn, calcium signalling might be an instrumental effector of cellular senescence. An increase in intracellular calcium can be observed in response to senescence-inducing stress stimuli, which might cause a higher calcium influx through plasma membrane calcium channels, desensitization of redox-dependent Ca^{2+} channels, or by calcium release from the endoplasmatic reticulum (ER).

This accumulation of cytosolic calcium and the depletion of the ER's calcium stores could also be observed in oncogene-induced senescent cells. From the cytosol, calcium is transferred into the mitochondria where its accumulation interferes with the mitochondrial membrane potential, which in turn causes the production of ROS, and therefore might contribute to the induction of cellular senescence [125, 126, 127].

Overall, levels of intracellular Ca^{2+} are higher in older cells, especially in neurons [128, 129]. In fact, deregulation of calcium signalling has been found in AD, Parkinson's Disease, ALS, and Huntigton's Disease [130].

The alterations of calcium signalling leading to increased calcium influx during aging result in a chronic increase of calcium in presynaptic terminals. This might be caused by altered properties and number of presynaptic calcium channels. Regulation of calcium is especially important in the presynapse as it controls the cell's transmitter release by regulating its release probability and short time plasticity. In turn, presynaptic calcium deregulation is accompanied by age-related deficits, such as a decline in hippocampal long term potentiation and cognitive impairment [131].

Furthermore, intracellular Ca^{2+} homeostasis might also be affected by SASP. Inhibition of mTOR blocks SASP and might attenuate the disruption of calcium homeostasis in senescent cells by promoting the mobilization of cytosolic calcium ions. A calcium overload in old cells, induced by stress stimuli, could then interfere with Ca^{2+} signalling, and promote SASP-related IL-1α processing. IL-1α is important for the induction of SASP by promoting the expression of NF-κB, thus promoting the transcription of SASP-related genes.

Upstream, the mTOR complex regulates the translation of IL-1α and MAPKAPK2. mTOR furthermore contributes to several other age-associated changes in calcium mobilization, which further emphasizes its already very prominent role as a core target for ageing research [127, 132, 133, 134].

SASP exposure might also affect astrocytes, which then would have an impact on neuronal signalling by interfering with astrocyte-neuron communication. Senescent human astrocytes have been observed to acquire the SASP, and to show neurotoxic effects, which could be important for age and disease-associated neurodegeneration [135]. Furthermore, senescent astrocytes appear to be enriched in various neurodegenerative diseases as postmortem studies of ALS patients show [136].

Although there is still little knowledge about neuron-glia networks, increased calcium signalling in astrocytes is associated with a decline in neuronal spontaneous activity [137]. An increase in calcium activity can also be observed *ex-vivo* in aged astrocytes as well as in astrocytes with Aβ pathology. In the latter, astrocytes affected by Aβ pathology show higher frequencies and duration of calcium transients near Aβ plaques [119]. Additionally, human astrocytes can acquire the SASP, and subsequently exhibit neurotoxic effects, through the activation of the oxidative stress response, following exposure to extracellular Tau. Astrocytes found in the proximity of Aβ plaques also happen to produce IL-6, which further strengthens a link between SASP, Aβ, and AD [94, 138] Reciprocally, SASP factors enhance the accumulation of Aβ, Tau hyperphosphorylation, and NFT deposition [139]. In fact, impaired astrocytes exhibiting neuroinflammatory properties are involved in both the initiation and progression of AD [140, 141, 142].

Accordingly, removal of senescent astrocytes reduces the malign effects of SASP *in-vitro* [143, 136, 135], and has been hypothesized to inhibit the for-

mation of NFTs as well as neurodegeneration in AD models [93, 144, 139]. Importantly, inhibition of IL-6 could ameliorate the neurotoxic effects, which might indicate that IL-6 has especially severe effects on neuron vitality [135].

Furthermore, astrocytes also regulate the burden of glutamate excitotoxicity as astrocytes take up glutamate and convert it to glutamine via the enzyme glutamine synthase. However, this process depends on age as the enzyme's activity, as well as its expression, decreases with age, and in senescent cells. Thus, senescent astrocytes might be a contributing factor for neurotoxicity [145, 146, 147, 139].

Another important component regulating the Ca^{2+} influx into neurons are NMDARs, which upon activation increase of intracellular calcium levels. Accordingly, neuronal impairment in response to senescence, and cells exposed to SASP's inflammatory micro-environment could partly be caused by over-activation of NMDARs, and aberrant cellular calcium influx. Over-activation of NMDARs can be a result of persistent neuroinflammation from neuronal abnormalities that cause cell death in aged mice [148]. Interestingly, NMDARs have also been linked to glutamate excitotoxicity, which can cause delayed neuronal degeneration from excessive Ca^{2+} influx. In fact, calcium and glutamate-induced calcium accumulation in cortical neurons also correlates with neuronal cell death [149, 150]. The functional decline and neuronal cell death observed from NMDAR related calcium deregulation even resembles the progressive decline in cognitive function as seen in AD patients [151].

However, while extrasynaptic NMDARs are associated with cell death, activation of synaptic NMDARs might initiate plasticity, and stimulate cell survival [151]. Intervention experiments with the clinically tested NMDAR antagonist Memantine, which targets extrasynaptic NMDARs, shows beneficial effects in cases of moderate to severe AD [151, 152]. Recently, it has also been observed that knockdown of $p16^{INK4a}$ decreases expression of the pro-inflammatory SASP factors IL6 and CXCL8 in OIS and DNA damage-induced senescent cell-lines.

Furthermore, several of the prominent factors secreted during SASP have been linked to NMDAR activity. The pro-inflammatory cytokine IL-1β can cause an increase in intracellular calcium through NMDARs. This can be prevented by the administration of IL-1 antagonists, which can inhibit NMDAR-mediated synaptic transmission, and further inhibit the uptake of glutamate by astrocytes [153, 154, 155, 156]. Furthermore, IL-6 can help neurons to evade glutamate-induced cell death, whereas evasion of apoptosis is a central hallmark of senescent cells. On the other side, persistent exposure to IL-6 has been found to disrupt neuronal functionality [153, 157, 158, 159].

Another possible explanation of neuronal hyperactivity could be a more frequent activation of synaptic NMDA receptors, due to presenilin-mediated dysfunction of intracellular Ca^{2+} stores in presynaptic boutons [121].

In the end, the relationship between $p16^{INK4a}$ and SASP is still unclear. At this point, it is possible that factors other than SASP could evoke the effects observed in our data. In summary, this emphasizes that exposure to SASP alters cell functionality in a human model to resemble aged and AD phenotypes.

4 Methods

4.1 Cell Culture

All cells were incubated at 37 °C, 5% CO_2. Culture media were changed every second day. Specific culture strategies for each used cell type is enumerated below:

1. Fibroblasts were cultured in a medium composed of 15% tetracycline-free Fetal Bovine Serum, 1% MEM Non-Essential Amino Acids Solution (100x) (NEAA, Gibco™), and 74% DMEM 1x (ThermoFisher®).

2. HEK293T cells were cultured in MEF culture medium: 10% Fetal Bovine Serum, 1% NEAA, and 89% DMEM/F-12 (ThermoFisher®).

3. Human cortical astrocytes were acquired from a commercial vendor and cultured in astrocyte medium according to the manufacturer's specifications (ScienCell 1800, 1801).

4.2 Neuronal Conversion

Direct conversion of human fibroblasts to induced neurons was achieved as described in Mertens *et al.* [160]. Fibroblasts were transduced with Ngn2:2A:Ascl1 under the control of the TREtight promoter (Clontech), and a puromycin-resistance gene driven by the PGK promoter. UNA-transduced fibroblasts were selected with puromycin and expanded before they were detached with TrypLE (ThermoFisher), and pooled in a 3:1 ratio to achieve high cell densities. The following day neuronal conversion was induced by changing the culture media to NK media for three weeks. The NK media is composed from DMEM:F12 (x1 Thermo Fisher Scientific), Neurobasal (x1 Thermo Fisher Scientific), and the following supplements: B27 (1x Thermo Fisher Scientific), N2 (1x Thermo Fisher Scientific), Doxycycline ($2\frac{\mu g}{mL}$, Sigma Aldrich), Laminin ($1\frac{\mu g}{mL}$, Thermo Fisher Scientific), di-butyryl cyclic-AMP ($100\frac{\mu g}{mL}$, Sigma Aldrich), human recombinant Noggin ($150\frac{ng}{mL}$, Preprotech), LDN-193189 (500 nM, Fisher Scientific Co), A83-1 (500 nM, Santa Cruz Biotechnology Inc.), CHIR99021 (3μM, LC Laboratories), Forskolin (5 μM, LC Laboratories) SB-431542 (10 μM, Cayman Chemicals).

After three weeks of conversion the mature iNs were transferred to a maturation medium (BP-GBCLL): BrainPhys (1x Stemcell Technologies), B27 (2%), N2 (1%), GDNF ($20\frac{ng}{mL}$, ED), BDNF ($20\frac{ng}{mL}$, RD), db-cAMP ($500\ \frac{\mu g}{mL}$), laminin ($1\frac{\mu g}{mL}$).

4.3 Lentivirus production

Lentiviral systems to integrate transgenic vectors were produced according to a second generational HIV-1 based system, featuring a packaging plasmid (psPAX2),

an envelope plasmid (pMD2.G), and the actual lentiviral transfer plasmid of interest. Pseudotyping to enable human fibroblast tropism was done in all cases with vesicular stomatitis virus glycoprotein (VSV-G), encoded within pMD2.G. Fast-growing HEK293T cells were exploited as hosts for viral production, and were passaged to Poly-L-Ornithine coated 15cm culture dishes and cultured with 15mL MEF-medium. After Hek293T cells reached about 70-90% confluency they were transfected, using a custom transfection mix, consisting of reduced serum medium (Opti-MEM^{TM}), Polyethylenimine (PEI) [1.1%($\frac{v}{v}$)], psPAX2 [11 µg µL^{-1}], pMD2.G [5 µg µL^{-1}], and the transfer plasmid [11 µg µL^{-1}]. Before application, the transfection mix was thoroughly mixed and incubated for 5 min at 20 °C. For transfection 1mL of transfection mix was drop-wisely distributed to each 15cm dish and incubated 5-8h at 37 °C and 5% CO_2. After incubation, the transfection medium was aspirated and replaced with standard MEF culture medium. Following three days of culture, the lentiviral containing supernatant was harvested on the final day. Determination of quantitative lentiviral titer was accomplished with Lenti-X^{TM} GoStix^{TM} Plus (Takara BIO INC.). The supernatant of all dishes was pooled and filtered through a 0.22 µm vacuum filter system to remove cell debris. Viral particles were enriched by vacuum ultra-centrifugation at 20,000xg for 2h at 4 °C. Subsequently, the supernatant was carefully removed by aspiration and/or decantation and the virus pellets were shortly air-dried. The yield of two 15cm dishes was re-suspended in 80 µL sterile 1x DPBS + Glucose and quickly frozen in cryovials at −80 °C as 40 µL aliquots.

4.4 Generation of Donor and Reporter Cultures

1. **iN-Astrocyte Reporter Co-Culture:**
   Converted iNs were transduced with Sy1-dsRed and with CMV-gCaMP6m [116] to assess neuronal functionality. Successful conversion was assessed by validating the expression of Sy1-dsRed. In the meantime, cultured astrocytes were sub-cultured onto acid-edged cover-slips, coated with Poly-L-Ornithine and Laminin, and grown to 70-80% confluency. At this point, the previously transduced iNs were carefully split in a ratio of 3:1 on top of the astrocyte-mounted coverslips to achieve high densities of neurons, which enhances viability and functionality. The co-culture was maintained for one week in BP-GBCLL media + 5% Knockout-serum (Thermo Fisher Scientific) so that they could recover, and the iNs got time to grow out their axons and dendrites to establish a functional network.

2. **Senescent PoMiSc Donor iNs:**
   Converted iNs from the same cell line as the reporter co-cultures were transduced with p16^{INK4a} to induce senescence. The expression of p16 was validated by fluorescence.

4.5 SA-β-Gal staining

SA-β-Gal staining was done with the Biopioneer cellular senescence detection kit (CS-001). The culture medium was aspirated and the cells were washed twice with PBS (1x) before they were fixed with 1x fixative solution and incubated for 5-10min at room temperature. Afterward, cells were rinsed twice with PBS 1x and stained with staining solution for 5h at 37 °C.

4.6 Condition Media Treatment

After maturation of the astroctye-iN co-culture, the cells were used for a condition media experiment (Fig. 1 B). All wells of the reporter co-culture were divided into either a treatment or a control group. Each well on the reporter plate was assigned a corresponding well on the donor plate, containing either the senescent-induced iNs for the treatment group or the non-senescent iNs for the control group. The culture media of the reporter cultures was aspirated and substituted with media from their assigned well corresponding to their treatment group. The used media from the donor wells was refreshed with new co-culture media. In total cells were treated for two weeks and changes were performed three times a week.

4.7 Assessment of Neuronal Functionality

After two weeks neuronal functionality was assessed by time-series Calcium-imaging using spinning disk microscopy.

Time-series images were analyzed in Fiji/ImageJ. For each file's z-stack the standard deviation was calculated. From there we selected iNs, which clearly expressed gCAMP6m and Sy1-dsRed by drawing Regions of Interests (ROI) around the cell perimeter. For each field of view/image, a mean gray value multi-measurement was performed. From the generated ROI mean gray values the background mean gray value of the corresponding field of view was subtracted. Next signal intensity was normalized to the basal level by dividing each stack's mean gray value by the minimum mean gray value of that stack. Finally, we removed the first and last 100 images of each stack of 2000 as these might distort the data. Differences between Control and Treatment groups were assessed by Wilcoxon signed-rank test. I used two analysis approaches: 1) Select an equal number of cells in Control and Treatment a priori. 2) Select all good-looking cells in Control and Treatment. As this generates different sample sizes I subset the larger dataset by random. Both methods showed similar and significant results. In total 10,762 images of three cell lines in five experiment set-ups were analyzed.

4.8 Visualisation

Statistical data were processed in R (4.1) and plotted with ggplot2. Schematic drawings were produced in Biorender. Assembly and modification of figures were done using Microsoft PowerPoint.

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