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Direct Conversion of Human Skin Fibroblasts into induced Neurons: A Model to Study Age-Related Synaptic Dysfunctions

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2023

Agradecimentos

Gostaria de começar por agradecer à Professora Dr. Luísa Lopes por me ter acolhido no seu laboratório e por ter confiado no meu trabalho desde início. A sua confiança em mim foi essencial para o sucesso deste projeto.

Quero agradecer à minha orientadora Dr. Joana Coelho, pela sua paciência, gentileza e orientação constante. Nos momentos mais difíceis, a Joana esteve ao meu lado, encorajando-me a não desistir e a procurar alternativas. Obrigada por me ter incentivado a ser proativa e por me ter guiado em todos os passos deste projeto, estando sempre disponível para esclarecer as minhas dúvidas e dando dicas valiosas. Graças a si, aprendi a ser uma pessoa mais independente. Não posso deixar de expressar a minha gratidão ao Nico, pela sua ajuda nas experiências de *patch-clamp*, pelos seus ensinamentos, conselhos, apoio e principalmente paciência ao longo deste projeto. Obrigada a todos os restantes membros do laboratório por toda a ajuda prestada ao longo deste projeto.

Agradeço também ao meu orientador, Professor Dr. Hugo Ferreira, por aceitar o desafio de me orientar e pelo apoio que me deu ao longo deste projeto.

E claro, não poderia deixar de mencionar aqueles que tornaram esta jornada possível; aos meus pais e irmãos, que me apoiaram desde início e me ajudaram a ultrapassar as fases menos boas. Agradeço toda a educação e amor incondicional. Obrigada, Mãe, por estares sempre aqui para mim. Agradeço também aos meus amigos de 4 patas, os meus coelhinhos Wally e Draco.

A todos os amigos que acompanharam de perto estes anos de vida académica, oferecendo-me a sua amizade e apoio quando eu precisava de encorajamento e orientação. Ao Pedro, o meu primeiro colega de casa, que trouxe conforto ao meu primeiro ano universitário. Um obrigado especial a todas as minhas amigas do "Caty Moranguinho" e ainda à Ana e Lia; sem vocês estes 5 anos não teriam sido os mesmos.

Aos meus amigos de Vendas Novas, "CBO", "McFlurry" e "Arruaceiros", um agradecimento especial por todas as histórias que partilhamos e por todos os momentos que me fizeram esquecer os períodos mais conturbados deste percurso universitário.

Finalmente, ao João, por todo o amor e apoio.

Mais uma vez, obrigada a todos os que tornaram possível a minha jornada académica e o sucesso deste projeto.

Investigação suportada pela Fundação para a Ciência e Tecnologia (FCT), através do projeto PTDC/MED-NEU/3890/2020.

Abstract

Age-related synaptic dysfunctions comprise a range of pathophysiological mechanisms that are correlated with cognitive decline. It has been demonstrated, both in animal models and human samples, that there is an upsurge of A_{2A} receptors within aging. In animals, this aberrant expression may be the explanation for the mGluR-5 dependent overactivation of NMDA receptors, enhanced calcium influx, dysfunctionalities in synaptic plasticity and increased glutamate release. Modeling aging in differentiated human neurons has been limited, and alternative models are needed to better understand the underlying mechanisms.

In this study, we followed a direct conversion protocol that, according to the literature, retain important aging signatures, unlike iPSC-based reprogramming. Using a protocol from Dr. Jerome Mertens and the Gage lab., we generated induced neurons (iNs) from three skin fibroblast samples, including two from adult (< 65 years) and one from an old (> 65 years) individual. We have optimized and adapted this protocol that combines the delivery of two transcription factors (TFs) and a cocktail of small molecules, by identifying the appropriate cell density and plating method for further experiments such as RT-qPCR, immunocytochemistry, and electrophysiology. We also characterized iNs according to specific cell markers and electrophysiologic properties.

Our results demonstrated a decrease in fibroblast marker vimentin and increased expression of the neuronal marker βIII-tubulin at 21 days, as well as VGLUT1 expression in both adult- and aged-derived iNs, confirmed by RT-qPCR and immunocytochemistry. However, we did notice that iNs at 21 days were still immature, as they lacked expression of some synaptic markers and the ability to fire action potentials.

Overall, direct conversion represents a promising model for studying age-related synaptic dysfunctions in humans. Optimal protocol adaptation and characterization of iNs made in this study can facilitate further research to better assess the underlying mechanisms of age-related synaptic dysfunctions. Futures studies will focus on maturation prospects so that iNs acquire functional synapses, including co-culturing with astrocytes, that are reported in the literature to enhance synapse formation and subsequent neuronal maturation.

Keywords: Aging, Human, Fibroblasts, Neurons, Direct Conversion

Resumo

O aumento do envelhecimento na população mundial é atualmente um dos desafios mais significativos para a comunidade científica, uma vez que traz consequências transversais para toda a sociedade. Este processo de envelhecimento implica uma deterioração progressiva de todos os tecidos e órgãos, o que leva a um aumento da incidência de doenças associadas ao envelhecimento. Nomeadamente, há um conjunto de alterações que levam à diminuição da função cerebral, ou ao declínio cognitivo, podendo estas ser alterações nos neurotransmissores, alterações nas próprias células nervosas, acumulação de substâncias tóxicas ao longo do tempo ou até alterações herdadas [1].

Uma das alterações mais significativas no declínio cognitivo observada no envelhecimento consiste na disfunção sináptica, o que não implica necessariamente que haja perda neuronal, como demonstrado em vários estudos. Não obstante, algumas alterações subjacentes à disfunção sináptica poderão levar de facto a perda neuronal, observada em doenças neurodegenerativas como a doença de Alzheimer ou a doença de Parkinson [2]. Isto sugere que estudar os mecanismos por detrás da disfunção sináptica poderá ajudar a estabelecer um histórico para doenças neurodegenerativas, uma vez que estas podem resultar do agravamento de mecanismos patofisiológicos prematuros.

Sabe-se que durante o envelhecimento há um aumento da expressão dos recetores de adenosina A_{2A}, quer em modelos animais quer em amostras humanas [3]. Apesar da sua baixa expressão em condições fisiológicas no hipocampo, estes recetores têm um papel importante na modulação da transmissão sináptica nessa estrutura, ao regularem a função de outros recetores metabotrópicos e ionotrópicos [4], [5]. Uma elevada expressão de recetores A_{2A} em ratos transgénicos foi suficiente para desencadear défices sinápticos e cognitivos [6]. Esta super-expressão de recetores A_{2A} levou a um aumento na libertação do neurotransmissor glutamato e a um aumento da ativação dos recetores ionotrópicos NMDA, dependente dos recetores metabotrópicos de glutamato 5, que por sua vez provocou um aumento nas concentrações de cálcio intracelular [6]. O aumento da ativação destes recetores e o consequente aumento do influxo de cálcio provocou um aumento de sinapses não funcionais, ou silenciosas, levando a alterações nos mecanismos de plasticidade sináptica e deficiências em fenómenos como a potenciação de longa duração (LTP) ou a depressão de longa duração (LTD) [6].

Apesar dos modelos animais terem permitido grandes desenvolvimentos científicos, muitas vezes os mecanismos observados não são reprodutíveis para a fisiologia humana, reforçando as diferenças anatómicas e genómicas entre espécies [7]. Desta forma, os mecanismos iniciais que levam à alteração do normal funcionamento das sinapses ao longo do envelhecimento precisam de ser estudados em modelos humanos. Contudo, o estudo das propriedades sinápticas do cérebro humano é praticamente inacessível, quer por questões técnicas e éticas subjacentes, quer pelo facto das amostras *post-mortem* adquiridas apenas refletirem os estágios finais da doença, não simulando, desta forma, a evolução dos mecanismos patofisiológicos inerentes a essas estruturas [8]. Uma das técnicas atualmente mais usada e consolidada nos laboratórios para obter neurónios humanos consiste no uso de células estaminais pluripotentes induzidas (iPSCs), uma técnica também designada por reprogramação indireta. As iPSCs correspondem a células que pertenciam previamente a um determinado tipo celular, e foram reprogramadas para células indiferenciadas, com o potencial de originar qualquer tipo de célula [9]. Contudo, ao serem reprogramadas, estas células são rejuvenescidas ao nível epigenético e transcriptómico [10]. Assim sendo, este método de reprogramação pode não ser o mais indicado para modelar o envelhecimento. Mesmo que sejam usadas técnicas artificiais para simular o envelhecimento em iPSCs, a indução destas alterações tem como limitação o facto de não conseguir reproduzir na totalidade tudo o que acontece no envelhecimento natural [10]. A conversão direta, ou transdiferenciação, é uma técnica alternativa de reprogramação celular que consiste em reformular um tipo celular diretamente noutro, sem recorrer a iPSCs [11]. Uma vez que um tipo celular é diretamente convertido noutro, estas células não rejuvenescem, ao contrário das iPSCs, o que torna esta técnica uma grande vantagem para o estudo das características do envelhecimento. De facto, é descrito na literatura que as células obtidas através de conversão direta preservam várias características de envelhecimento presentes nas células iniciais do dador, como por exemplo a preservação da idade epigenética, transcriptómica, metabólica e mitocondrial [9].

Desta forma, com o objetivo de estudar as disfunções sinápticas induzidas pelo envelhecimento, recorremos a um protocolo de conversão direta previamente desenvolvido e validado pelo laboratório do Dr. Jerome Mertens [12]. Usámos como células iniciais fibroblastos da pele de três dadores com idades distintas, incluindo dois adultos (< 65 anos) e um idoso (> 65 anos), esperando no final obter neurónios induzidos (iNs). O protocolo consistiu numa primeira fase na qual os fibroblastos foram transduzidos através de um lentivírus com um sistema Tet-On, de forma a incorporar dois fatores de transcrição: ASCL1 e NGN2. Estes fatores de transcrição são descritos na literatura por induzirem e promoverem diferenciação neuronal. Apesar de adquirirem os fatores de transcrição, os fibroblastos transduzidos não têm a capacidade por si só de se converterem em neurónios, uma vez que apenas a presença do antibiótico doxiciclina no meio celular irá ativar a expressão génica. De seguida, após algumas passagens, estes fibroblastos transduzidos foram submetidos a uma segunda fase de diferenciação, na qual foram expostos a um *cocktail* de pequenas moléculas, o qual também incluiu doxiciclina, durante um período de 21 dias. Estas pequenas moléculas têm a capacidade quer de promover a conversão neuronal, quer de inibir o perfil de fibroblastos. Após a obtenção de iNs ao fim de 21 dias, seguiu-se uma caracterização destas células através de RT-qPCR, imunocitoquímica e *patch-clamp*.

Apesar deste protocolo já ter sido desenvolvido por outros grupos, a sua reprodutibilidade no nosso laboratório teve de ser garantida. Além disso, foi necessário fazer um conjunto de adaptações e otimizações de forma a assegurar não só uma obtenção eficaz e viável de iNs, mas também das melhores estratégias a desenvolver para adquirir uma caracterização dos mesmos. Isto incluiu o estabelecimento de uma densidade ótima de plaqueamento de fibroblastos transduzidos para iniciar o período de diferenciação de 21 dias, e a escolha do plaqueamento mais adequado conforme a técnica a desenvolver para proceder à caracterização dos iNs. Por exemplo, o modo de plaqueamento de fibroblastos transduzidos para começar o processo de diferenciação variou, conforme a caracterização pretendida fosse por RT-qPCR, imunocitoquímica ou *patch-clamp*. Para todas as técnicas, chegou-se à conclusão de que re-plaquear as células após 21 dias comprometeria a sua densidade final e viabilidade. Desta forma, a estratégia passou por completar todo o processo de diferenciação em placas que por si só já teriam propriedades que possibilitassem a caracterização das células. Para as técnicas de imunocitoquímica e de *patch-clamp*, as placas usadas necessitaram de ter um material que permitisse a visualização das células através do microscópio. Uma vez que a diferenciação dos fibroblastos transduzidos para neurónios não é tão eficiente quando realizada em vidro, foram usadas placas com um índice de refração semelhante a este, possibilitando a visualização das células.

Após estabelecer as melhores estratégias quer para a diferenciação neuronal quer para a caracterização das células, avaliou-se a morfologia das células e a existência de marcadores específicos de fibroblastos e neurónios ao fim de 21 dias, através de RT-qPCR e imunocitoquímica, para as amostras correspondentes aos três dadores. Comprovou-se que a expressão de vimentina, um marcador específico de fibroblastos, diminuiu após 21 dias de diferenciação nos iNs, e que estes expressavam β-tubulina III, um marcador neuronal, e VGLUT1, um marcador de neurónios glutamatérgicos, mas não MAP2 ou NeuN, sendo estes últimos marcadores de neurónios mais maturos. No que diz respeito à expressão de proteínas sinápticas, os iNs expressavam SYT1, mas não PSD-95 ou SAP-102. A morfologia das células também se alterou, sendo que os iNs apresentavam uma morfologia semelhante a neurónios, com corpo celular e axónios. Analisou-se ainda a atividade elétrica dos iNs derivados do dador mais novo, comprovando-se que estes não têm ainda a capacidade de disparar potenciais de ação. Contudo, ao monitorizar o potencial de membrana de outro iN, foram observados alguns potenciais pós-sinápticos excitatórios espontâneos putativos, sugerindo uma possível existência de sinapses entre os iNs.

Os resultados sugerem que apesar do protocolo de conversão direta permitir de facto a diferenciação de fibroblastos humanos em neurónios, quer para a amostra de dadores novos e dadores velhos, ao fim de 21 dias os iNs são ainda imaturos. Sendo o objetivo final estudar disfunções sinápticas associadas ao envelhecimento, é crucial explorar perspetivas de maturação para estas células de forma que consigam estabelecer sinapses funcionais e estudar os principais mecanismos pato-fisiológicos. Não obstante, este projeto cumpriu os objetivos de estabelecer um protocolo reprodutível para o nosso laboratório, otimizando-o e adotando estratégias exequíveis para a caracterização dos iNs através de RT-qPCR, imunocitoquímica e patch-clamp. As experiências futuras irão incidir na adição de uma fase de

maturação, através da co-cultura dos iNs com astrócitos, que estão descritos na literatura por promover a maturação das sinapses neuronais, e também no estudo de marcadores de danos de ADN e de senescência, a fim de comprovar que os neurónios induzidos mantêm de facto características associadas ao envelhecimento.

Palavras-chave: Envelhecimento, Humano, Fibroblastos, Neurónios, Conversão Direta

List of Figures

List of Tables

List of Supplementary Data

List of Acronyms

A_1R – Adenosine A1 Receptor

$A_{2A}R$ – Adenosine A_{2A} Receptor

Aβ – β-Amyloid Peptide

AD – Alzheimer's disease

ADORA2A – Adenosine A_{2A} Receptor encoding gene

ALK – Activin-like kinase

ALS - Amyotrophic lateral sclerosis

AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor

ASCL1 - Achaete-Scute Homolog 1

BRN2 – Brain-2

cAMP – cyclic adenosine monophosphate

ChAT – Choline acetyltransferase

C-MYC – cellular myelocytomatosis oncogene

CNS – Central Nervous System

db-cAMP – dibutryl cyclic adenosine monophosphate

DMEM – Dulbecco's Modified Eagle Medium

DMSO - Dimethyl Sulfoxide

DNAm – DNA methylation

endoASCL1 – endogenous ASCL1

FACS – Fluorescense-activated cell sorting

FBS – Fetal Bovine Serum

fEPSP – field excitatory post-synaptic potential

FUS – Fused in sarcoma

GABA – Gamma-Aminobutyric Acid

GAD67 – Glutamate Decarboxylase 67

GCDR – Gentle Cell Dissociation Reagent

GSK-3β - Glycogen Synthase Kinase-3β

HD – Huntington's disease

HPLC – High Performance Liquid Chromatography

ICC – Immunocytochemistry

iGluR – ionotropic Glutamate Receptor

iNs – induced Neurons

iPSCs – induced Pluripotent Stem Cells

KLF4 - Krüppel-like factor 4

LFS – Low-frequency stimulation

LTD – Long-term Depression

LTP – Long-term Potentiation

MAGUK – Membrane-Associated Guanylate Kinase

MAP2 – Microtubule-associated Protein 2

MET – Mesenchymal-to-Epithelial Transition

mGluR – metabotropic Glutamate Receptor

mGluR5 – metabotropic Glutamate Receptor 5

mHTT - mutant huntingtin

MYOD1 – Myogenic Differentiation 1

MYT1L – Myelin transcription factor 1-like

NC – Neuron Conversion

NEAA – Non-Essential Amino Acids

NEUROD1 – Neurogenic Differentiation 1

NES – Nestin

NeuN – Neuronal Nuclear

NGN2 – Neurogenin 2

NT – Non-transduced

NMDAR - N-methyl-D-aspartate Receptor

OCT4 – Octamer-binding transcription factor 4

PBS - Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PD – Parkinson's disease

PGK – Phosphoglycerate kinase

PI – Propidium Iodide

PKA – Protein kinase A

PORN – Poly-dl-ornithine

PSD – Post-Synaptic Density

PSD-95 – Post-Synaptic Density Protein 95

RCF – Relative Centrifugal Force

ROCKi – Rho-Associated, Coiled-Coil Containing Protein Kinase inhibitor

ROS – Reactive Oxygen Species

rtTA – reverse tetracycline-controlled transactivator

S100B – S100 calcium-binding protein B

SAP-102 – Synapse-Associated Protein 102

sEPSP – spontaneous excitatory post-synaptic potential

SYT1 – Synaptotagmin 1

SLC17A7 – Solute Carrier Family 17 Member 7

SMAD – Small Mothers Against Decapentaplegic

SNARE – Soluble NSF Attachment Protein Receptor

SOD1 – Superoxide dismutase 1

SOX2 – SRY(sext-determining region)-box 2

TC – Tissue culture

TF – Transcription Factor

TFM – The Fibroblasts Medium

TGF-β - Transforming Growth Factor-β

transASCL1 – transduced ASCL1

TRE – Tetracycline-Responsive Element

TUBB3 - βIII-tubulin

VIM – Vimentin

VGLUT1 – Vesicular Glutamate Transporter 1

WPRE - Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element

List of Contents

1. Introduction

1.1. Aging

The world's population has been constantly increasing, as it has already passed the 8 billion milestone in November 2022. The increase in longevity contributes to this growth, as a result of better health care and hygiene, healthier lifestyles, diet and improved medical care [13]. In fact, it is expected that by 2050, the world's population of people aged 60 years and older will double to 2.1 billion [14]. As individuals age, several physiological functions progressively decline, making them more susceptible to diseases and negatively impacting their quality of life. Despite the improvements in healthcare that have led to increased lifespans, it is crucial for biomedical research to actively anticipate and address these age-related changes within the context of this growing trend. By doing so, research can significantly contribute to better understanding and managing age-related conditions [15].

It is of greater importance to investigate the mechanisms underlying aging. Namely, to give greater emphasis to complex age-associated perturbations at the molecular level rather than focusing on assessing decline in organ or tissue function and associating it to changes in genetic, epigenetic, or metabolic states [16]. In the spectrum of age-related diseases, the onset of neurodegeneration and the associated cognitive decline is of significant concern due to its profound impact on both the health span and quality of life of individuals. In fact, aging has been widely associated with cognitive decline and is the primary risk factor for most neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) [2].

Many studies examining cellular substrates of age-related cognitive decline have focused on the hippocampus since it plays a crucial role in regulating learning, memory encoding and memory consolidation, and because this brain region is especially vulnerable to the process of aging, as reviewed in [17]. In fact, age-related cognitive decline is believed to be associated with several neurobiological changes observed in the aging hippocampus, such as increased oxidative stress, neuroinflammation, reduced neurogenesis and decreased synaptic plasticity [18], leading to an increased interest in this structure. In particular, in age-related neurodegeneration, cognitive decline has a stronger correlation to early synapse loss, rather than neuronal loss in patients [19]. Additionally, some of the synaptic deficits observed in cognitive impairment may be partly explained by alterations in the expression of several proteins that play key roles in synaptogenesis and synaptic stabilization [20].

It is then critical to explore the main characteristics of regular synapses as well as the main components and mechanisms involved in synaptic dysfunction.

1.2. Synaptic dysfunction

Neurons rely on synapses as vital constituents to facilitate an ordered flow of information throughout the brain. Particularly, modulation of synapse activity represents a major strategy to control brain homeostasis. However, continuous alterations in synapse physiology can lead to significant abnormalities that may manifest as brain disorders. In fact, increasing evidence shows that synaptic dysfunction plays a critical role in several neurological diseases, including neurodegenerative diseases such as AD or PD, as reviewed in [21].

Synaptic dysfunction arises from two main factors: modifications in the fundamental synaptic molecular mechanisms or variations in biochemical processes of the immediate surroundings. This suggests that the molecular components of synapses may play a crucial role in contributing to or causing brain malfunction, as reviewed in [21]. Therefore, it is of utmost importance to understand possible causes for synapse failure, namely, the molecular fundamentals that lead to synaptic dysfunction. First, it is essential to understand the mechanisms associated with synaptic transmission under physiological conditions.

1.2.1. Synaptic transmission – an overview

Synapse structure and function

Synapses can be divided in two general groups: electrical and chemical synapses. The first ones allow for the direct transfer of charged ions and small molecules through pores known as gap junctions [21]. On the other hand, the information in chemical synapses is unidirectionally transferred from one neuron (pre-synaptic terminal) to another (post-synaptic terminal) through chemical mediators known as neurotransmitters [21]. **Figure 1.1** allows to distinguish these two types of synapses through the structural elements of each one. Chemical synapses are much more prevalent in mammals whereas electrical synapses are more common in invertebrate and non-mammalian nervous systems, however, they are often found in neuroglial cells in mammals [22].

An action potential refers to a sudden and fast propagating change of the resting membrane potential, and the property of neurons to generate it is termed excitability [23]. The process of inducing the release of neurotransmitters from vesicles located in the pre-synaptic terminal into the synaptic cleft is dependent on the transmission of action potentials that move along axons. Neurotransmitters then activate specific ionotropic receptors, leading to the conversion of chemical signals into electrical impulses, that depolarize the post-synaptic cell and enable downstream transmission [21].

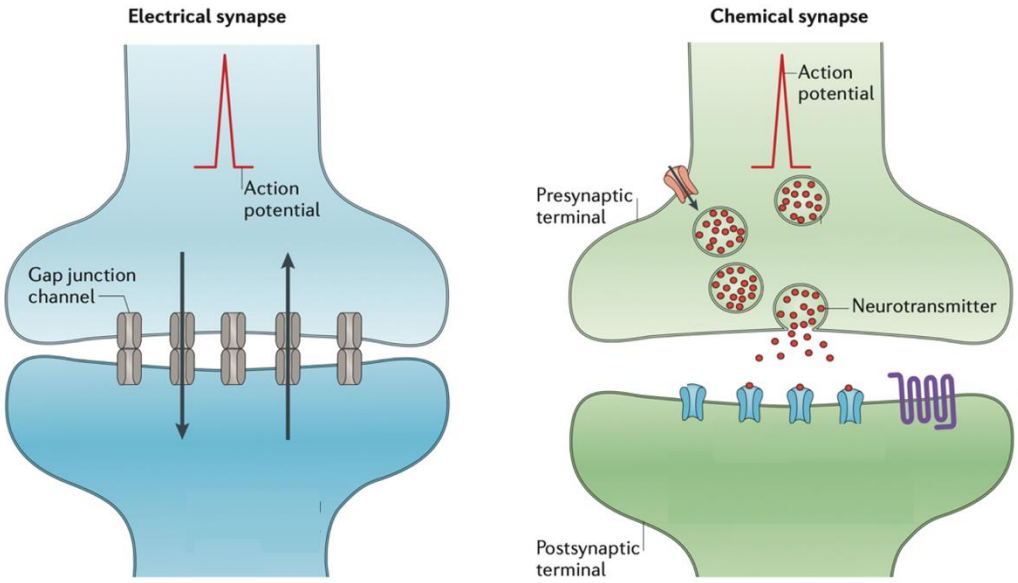


Figure 1.1 - Structural differences between electrical (left) and chemical (right) synapses. In electrical synapses, the connections between neurons are established through gap junctions, that allow direct ionic and small metabolite communication. On the other hand, chemical synapses consist of cell-cell junctions in which a group of specialized proteins manipulate neurotransmitter-mediated signaling transmission. Adapted from [176].

Pre- and post-synaptic structures

Electrical activity arriving at the post-synaptic cell may correspond to excitatory or inhibitory signals that increase or decrease the likelihood of firing action potentials in target cells, respectively. Hippocampal neurons mainly release glutamate, the main excitatory neurotransmitter in the Central Nervous System (CNS), or gamma-aminobutyric acid (GABA), the most abundant inhibitory neurotransmitter in CNS [24]. From now on, we will mainly focus on glutamatergic synapses since some hallmarks of neurodegenerative diseases seem to target excitatory synapses [25].

The main difference regarding the structure of the pre-synaptic terminal at excitatory and inhibitory synapses consists in neurotransmitter-synthesizing enzymes and transporters, as reviewed in [21]. The pre-synaptic elements are easily identified by the presence of neurotransmitter-containing vesicles near

membrane proteins necessary for exocytosis. These proteins include Soluble NSF Attachment Protein Receptor (SNARE) protein complex and voltage-dependent channels that mediate the influx of calcium (Ca^{2+}) [3]. The signal to release neurotransmitters consists of a rise in intracellular Ca^{2+} levels, a result of the opening of voltage-dependent Ca^{2+} channels, activated upon action potential-dependent depolarization of the pre-synaptic membrane (**Figure 1.2**) [21]. This increase in Ca^{2+} levels is detected by the synaptic vesicle protein synaptotagmin I (SYT1), leading to conformational changes in SNARE protein complex which enables the synaptic vesicle to fuse with the plasma membrane [21]. As a result, neurotransmitters can be released into the synaptic cleft.

On the other hand, excitatory post-synaptic terminal is predominately located on dendritic spines, which consist of protrusions from dendrites, and contains the post-synaptic density (PSD) which is identified by its electron-dense structure at the post-synaptic site [26]. The PSD is a complex structure made up of multiple layers of densely packed proteins located beneath the post-synaptic membrane. It serves as a scaffold that anchors several cell surface proteins, including glutamate receptors and cell-adhesion molecules [27]. Examples of scaffold proteins comprise the membrane-associated guanylate kinase (MAGUK) family which include post-synaptic density protein 95 (PSD-95) and synapse associated protein 102 (SAP-102) [21]. PSD-95 plays a critical role in synaptic plasticity and in the stabilization of receptors, as well as the regulation of receptor function, whereas SAP-102 plays a critical role in mediating glutamate receptors trafficking during synaptogenesis [28].

Glutamate is stored in synaptic vesicles within pre-synaptic terminals. Upon release into the synaptic cleft, it activates several different receptors within the post-synaptic membrane of excitatory synapses (**Figure 1.2**). This activation is responsible for regulating multiple neuronal functions including neuronal migration, excitability, and plasticity [29]. SLC17A7, also known as Vesicular Glutamate Transporter 1 (VGLUT1), is a sodium-dependent phosphate transporter that is specifically expressed in neuron-dense areas of the brain. It is responsible for transporting glutamate from the cytoplasm of pre-synaptic neurons to synaptic vesicles, which are then released into the synaptic cleft for neurotransmission [30]. Due to its function, it is used as a reliable marker for glutamatergic neurons. Glutamate receptors at the post-synaptic terminals are responsible for receiving the glutamate molecules that have been released from the pre-synaptic terminal. There are several different types of glutamatergic receptors, but the two main classes are the voltage-sensitive ionotropic glutamate receptors (iGluRs) and the ligand-sensitive metabotropic glutamate receptors (mGluRs) (**Figure 1.2**) [3].

The group of iGluRs mediate the fast excitatory transmission by opening in response to the binding of glutamate and include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-, N-methyl-D-aspartate (NMDA-), and kainate-type receptors [3]. While AMPA receptors (AMPARs) mediate Na^{+} (sodium) influx and consequently post-synaptic membrane depolarization [3], NMDA receptors (NMDARs) require binding of glutamate and glycine, and the presence of a post-synaptic depolarization to open their ion channels, allowing the influx of Ca^{2+} ions into the cell. This Ca^{2+} influx may trigger several downstream signaling events that can lead to changes in synaptic strength and neuronal excitability [31]. Kainate receptors also mediate synaptic transmission through the entering of Na^{+} and Ca^{2+} ions, but at a smaller extent [3].

On the other hand, mGluRs are coupled to G-proteins and mediate slower synaptic transmission by activating intracellular signaling pathways in response to glutamate binding. They are subdivided into three groups, depending on their coupled G-protein, and their activation also modulates neuronal excitability and synaptic transmission [3], [32]. Group I mGluRs include mGluR1 and mGluR5, which are mostly expressed on the post-synaptic membrane [32].

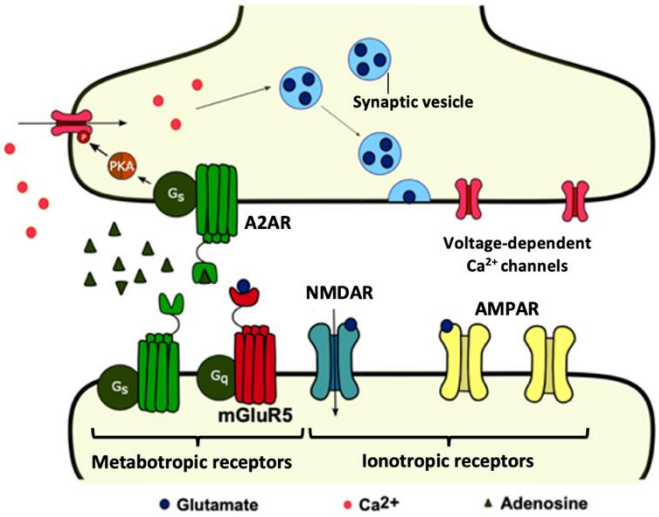


Figure 1.2 - Schematic diagram of an excitatory synapse. At the pre-synaptic site, there are voltage-dependent calcium channels sensitive to Ca^{2+} ions, that trigger the release of neurotransmitters (in this case, glutamate), stored in synaptic vesicles. Post-synaptic elements include both metabotropic (mGluR5) and ionotropic (NMDAR and AMPAR) receptors. NMDAR have an important role in intracellular signaling and synaptic plasticity, and AMPAR are responsible for the fast immediate post-synaptic response to glutamate release. Adenosine A2A receptors are G-protein coupled receptors and have both pre-synaptic and post-synaptic neuromodulator effect. Adapted from [6].

Synaptic plasticity

The ability of synapses to modify their structure and tonus after persistent electrical activity is termed synaptic plasticity and can occur both in pre- and post-synaptic elements. Synaptic plasticity allows synapses to adapt to different contexts, thus enabling learning and memory processes, as reviewed in [21].

Long-term potentiation (LTP) induction following a continuous and strong stimulation is the most studied form of synaptic plasticity and it promotes the activation of NMDAR and subsequent Ca^{2+} influx [21], [33]. Apart from LTP, there is evidence for additional forms of long-term synaptic plasticity. Long-term depression (LTD) can be induced by low-frequency stimulation (LFS) and promotes the activation of NMDARs [21]. Both LTP and LTD mechanisms have been well described and studied mechanisms in animal and human models [34].

To ensure that neuronal activity remains within a healthy range, neurotransmitter release efficiency is adjusted based on overall changes in network activity, a process known as homeostatic plasticity. This process acts as a compensatory stabilizing mechanism that uses negative feedback and modulates the expression of pre-synaptic scaffold proteins ensuring fast but adjustable release of neurotransmitters [35].

The importance of adenosine A_{2A} receptors

Adenosine constitutes an important neuromodulator distributed throughout the CNS that regulates neurotransmitter release and post-synaptic excitability [36]. This neuromodulator role of adenosine is mediated by plasma membrane metabotropic receptors including A_1 and A_{2A} receptors (A_1R and $A_{2A}R$, respectively), the most relevant in the CNS. A_1R is primarily found at the post-synapse in some brain regions including the cortex, cerebellum, hippocampus, and dorsal horn of spinal cord [37]. On the other hand, $A_{2A}R$ is mainly located in the pre-synaptic terminals of neurons in regions like the striatum, olfactory tubercle and cortex [37], [38]. Although $A_{2A}R$ are expressed at lower levels at the hippocampus [39], these receptors have an important role in modulating synaptic transmission in this structure, especially because they help to maintain the balance between excitatory and inhibitory inputs, which is essential for proper hippocampal function [5]. This is due to the fact that in glutamatergic synapses, under physiological conditions and basal activity, adenosine preferentially stimulates A_1R which leads to an inhibition of glutamatergic synaptic transmission in the hippocampus. On the other hand,

adenosine can also activate $A_{2A}R$, leading to a decrease of A_1R binding and consequently an inhibition of A_1R actions (decreased inhibition of glutamatergic synaptic transmission), as reviewed in [3].

Despite prior beliefs that aging is associated with neuronal loss, recent studies across different species have shown that the number of cells is actually preserved in several brain areas, including the hippocampus [40]–[43]. As this number does not change, it is hypothesized that age-associated behavioral impairments may be caused by changes known to occur at the synapse that result in altered mechanisms of plasticity, as reviewed in [44]. This evidence contrasts with the observations in AD or PD, which are characterized with neuronal and synaptic loss mainly in the hippocampus [45] and substantia nigra [46], respectively. As aging is the main risk factor for AD and PD, comprehending the underlying mechanisms behind age-related synaptic dysfunction may help to known to what extent there is an evolution of the underlying causes that ultimate lead to neurodegenerative diseases.

1.2.2. Alterations in synaptic plasticity and in synaptic structures within aging

Evidence suggests that aging is associated to an increase in nonfunctional or silent synapses in the hippocampus, as reviewed in [44]. For instance, it has been stated that the PSD area of axospinous synapses is significantly reduced in aged learning-impaired rats, which may result from structural modifications that lead to a deleterious effect on synaptic function [47]. In addition, in aged memory-impaired animals, there was a decrease in the amplitude of Schaffer collaterals-induced field excitatory post-synaptic potentials (fEPSPs) recorded in CA1 region [48]. Schaffer-collaterals are specialized axonal pathways that project from the CA3 region to the CA1 region of the hippocampus [49], and the decrease in amplitude of fEPSPs recorded in these regions suggests that the connections between neurons may not be as strong as they are in young animals, which contributes to the memory impairments observed in aged animals.

As AMPAR are responsible for post-synaptic membrane depolarization, they play an important role in both LTP and LTD. Consequently, one may think that changes in synaptic plasticity in aging can be related to defects in AMPAR. In fact, evidence suggest that there is a reduction in the functionality of these receptors rather than a decrease in their expression, as the administration of AMPAR positive allosteric modulators, which are drugs that bind to a specific site on AMPAR, restored age-related memory and synaptic potentiation deficits [50]. These modulators of AMPAR are known to enhance fast excitatory neurotransmission in the brain and increase overall neuronal excitability [51].

In aged animals, impaired memory is correlated with alterations in hippocampal synaptic function, and this may relate to alterations in susceptibility to induction of synaptic plasticity, namely in dysfunctionalities in both LTP and LTD, as reviewed in [44]. In fact, some authors reported increased susceptibility to LTD in aged rats [52]. Similarly, a study assessing the impact of typical AD hallmarks on synaptic plasticity has revealed that soluble β-amyloid peptide (Aβ) directly extracted from the cerebral cortex of AD subjects inhibited LTP and enhanced LTD in the normal rodent hippocampus [53]. However, other studies have failed to observe an age-related shift towards LTD in aged animals, and this inconsistency raised questions regarding the relationship between aging and susceptibility to LTP or LTD, as reviewed in [3]. This has been related with disparities in animal strain, stimulation pattern or Ca^{2+}/Mg^{2+} ratio between studies, as reviewed in [3]. In fact, when increasing Ca^{2+} levels in adult rats, LTD was induced, whereas in aged rats, LTD was blocked in high concentrations of Mg^{2+} [52]. These findings correlating LTD susceptibility not only to aging but also to the regulation of Ca^{2+} levels in the recording medium, suggested an age-related Ca^{2+} dysregulation that impacts LTD induction, as reviewed in [3]. Additionally, a recent study has shown that when Ca^{2+} post-synaptic intracellular levels were increased, there was a shift in synaptic plasticity, in aged animals, when applying LFS, i.e., instead of causing LTD, the same stimulus resulted in LTP [54]. Further, it has also suggested that increased Ca^{2+} influx may result from an increase in NMDAR activation [54]. Specifically, it has been observed that aged animals exhibit NMDAR overactivation in response to glutamate or glycine stimulation, apparently as a compensatory mechanism for a decrease in this

receptor density [54], [55]. This overactivation may be a contributing factor to the elevated levels of Ca^{2+} observed in aging neurons.

Another point that is important to comprehend is why aging is a complex process that affects individuals differently: while some older adults maintain their cognitive abilities as they age, other may experience cognitive decline [30]. This variability in cognitive trajectory within people is determined by a combination of genetic and environmental factors that include premorbid cognitive abilities, educational level, lifestyle choices and environmental exposures [56], [57]. Maintaining a stable cognitive trajectory during advanced age is not only desirable but also a reflection of cognitive resilience [56]. On the other hand, any decline in cognitive performance can be an early indicative of a severe neurodegenerative disease [58]. To explore potential molecular mechanisms or components that contribute to individual differences in cognitive trajectory, Wingo and colleagues conducted a large-scale proteome-wide-association study on cognitive trajectory in a large sample of initially cognitively unimpaired older-adult brain donors [30]. In agreement with reported decrease in the density of synaptic elements, it was shown that overexpression of PSD-95 was associated with cognitive stability [30]. Additionally, authors also found that higher levels of SYT1, a pre-synaptic element, were associated with less cognitive decline over time [30]. It was then demonstrated that there is an association between cognitive resilience with preservation of synaptic proteins, shedding light on the importance of these elements in maintaining normal cognitive function in advanced aged.

Upsurge in A_{2A} receptors and the role of mGluR5

It has been well established that upon aging and AD, there is an increase in the expression and signaling of $A_{2A}R$ in the hippocampus, as reviewed in [3]. In fact, both rodent and human brains have shown an upsurge in $A_{2A}R$, as a study conducted on human samples from subjects of different ages and AD has reported an increase in $A_{2A}R$ expression, with a higher expression level observed in samples from AD patients [54]. The increase in $A_{2A}R$ facilitates the release of neurotransmitters in glutamatergic synapses, and this is thought to happen through a process where $A_{2A}R$ activation leads to an increase in the molecule cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA) [59], [60]. PKA then phosphorylates L-type calcium channels, causing them to open and allowing Ca^{2+} ions to enter the neuron, triggering the release of glutamate [61].

The expression of $A_{2A}R$ at normal physiological levels seems to be protective, but overexpression of these receptors at a similar magnitude to that observed in human aging, triggered synaptic and cognitive deficits in transgenic rats [54]. This was accompanied by an mGluR5-dependent NMDAR overactivation which led to an increased Ca^{2+} influx. This overactivation seemed dependent on mGluR5, since mGluR5 blockade prevented the $A_{2A}R$-mediated LTD-to-LTP shift, previously described, and the abnormal NMDAR activity in basal transmission [54]. However, the way in which NMDARs are regulated by mGluR5s is still not fully understood, namely, whether mGluR5 acts on NMDAR through a single pathway that is present in all synapses, or through multiple distinct pathways that are specific to different regions of the brain, as reviewed in [3]. This is an important question because it would help to understand how neuronal circuits function and how they may be disrupted in several disorders. If mGluR5-dependent activation of NMDAR is a generalized mechanism that occurs in all synapses, it would suggest an important role for these receptors in neural communication. On the other hand, if the mechanism is region-specific, it would imply that different brain regions may use distinct strategies to regulate synaptic transmission.

Importantly, $A_{2A}R$ pharmacological blockade or genetic deletion seems to prevent synaptic and memory impairments in aged rodents and several AD models, as reviewed in [3]. For instance, when cultured hippocampal neurons of either mice or rats were exposed to $A\beta_{1-42}$, a toxic fragment found in AD patients, as reviewed in [62], it was observed loss of synaptic markers, modification of the viability of nerve terminals and degeneration of synapses [63]. But when treated with SCH58261, a selective antagonist for $A_{2A}R$, these effects were prevented, suggesting that $A_{2A}R$ play a crucial role in the development of Aβ-induced synaptotoxicity [63]. Additionally, deletion of the gene encoding for $A_{2A}R$ – ADORA2A - also exhibited beneficial effects on synaptic dysfunction in a mouse model of tauopathy, Thy-Tau22, characterized by the deposition of abnormal Tau protein in the brain [64]. Particularly, the

deletion was sufficient to prevent memory defects, hippocampal plasticity impairments as well as Tau hyperphosphorylation, associated with cognitive impairments in several dementing disorders [64], [65]. Also in this study, the use of $A_{2A}R$ antagonist MSX-3 allowed the rescue of hippocampal-dependent memory in Thy-Tau 22 mice, indicating once more the crucial involvement of $A_{2A}R$ in synaptic and memory dysfunction [64]. Likewise, caffeine, a non-selective adenosine receptor antagonist, is also reported to delay cognitive decline in the aged population and reduce the risk of developing AD [66].

As it was already discussed, under physiological conditions adenosine preferentially stimulates A_1R [67] inhibiting glutamatergic neurotransmission by a decrease in NMDAR-mediated responses [68]. The increasing expression of $A_{2A}R$ will then include a shift in the balance of adenosine signaling from predominantly inhibitory to increased facilitatory, as it will bind more frequently to $A_{2A}R$ [60].

Therefore, due to its pathological effects, it is essential to clarify the mechanisms by which $A_{2A}R$ expression increases upon aging and AD on the hippocampus. One of the hypotheses resides in the fact that there may be an association between a polymorphism of ADORA2A with hippocampal volume, and consequently synaptic loss in mild cognitive impairment and AD [69]. This occurs in a non-coding region, and it was hypothesized, although not studied, that it could result in alterations in $A_{2A}R$ expression, as reviewed in [3].

In short, an increase in $A_{2A}R$ upon aging in both human and animal models seems to be the triggering factor to several synaptic dysfunctions. In animal models it has been shown that the increase in these receptors is sufficient to trigger an age-like synaptic phenotype, with increased glutamate release, leading to mGluR5-dependent NMDAR overactivation that promotes an increase in Ca^{2+} influx (**Figure 1.3**). The calcium dyshomeostasis and alterations in the receptors consequently lead to shifts in synaptic plasticity, altering the susceptibility to induce either LTD or LTP.

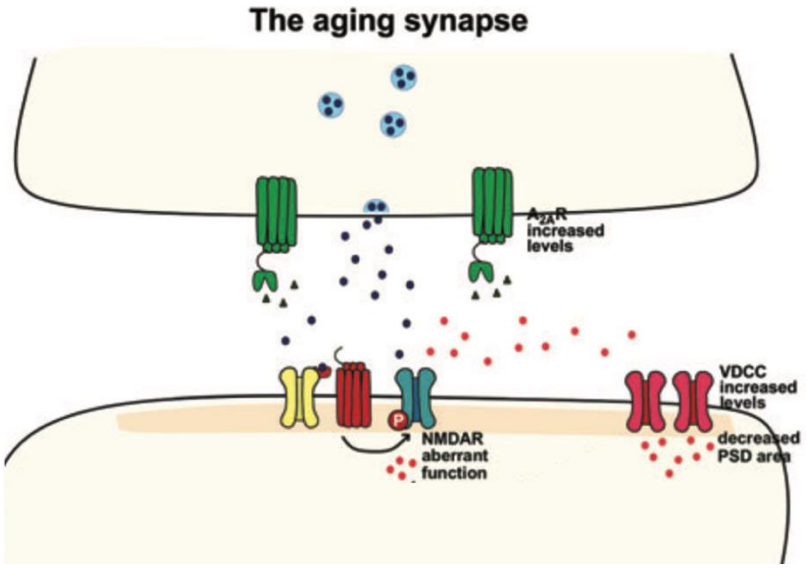


Figure 1.3 - Pathophysiological alterations in the aging synapse. There is an upsurge in A_{2A} receptors, leading to an increased glutamate release and further mGluR5-dependent NMDAR overactivation. Besides that, it is reported a decrease in PSD area as a consequence of a diminished expression of post-synaptic receptors and an increase in voltage-dependent calcium channels (VDCC). Adapted from [3].

It is, therefore, critical to study whether these early synaptic dysfunction mechanisms identified in animal models are also observed in human models to understand whether the $A_{2A}R$/mGluR5/NMDAR interaction is relevant in human pathophysiology. Particularly, since in humans it has been suggested that cognitive decline may be associated with the loss of some glutamatergic proteins at the synapse, it is necessary to address how these dysfunctions may trigger and correlate with a given pathological phenotype. Understanding these early mechanisms of disease pathogenesis will help in the development of novel therapeutic strategies for addressing synaptic and memory dysfunction upon aging and AD.

However, studying these mechanisms in humans in conditioned by access to and establishment of a model that overcomes ethical issues and, unlike postmortem samples, does not only reflect the end-stages of disease.

1.3. Direct conversion as a model to study age-related diseases

As human longevity increases, ensuring the quality of life of individuals has become a crucial concern. To accomplish this, studying age-related diseases is essential to comprehend their pathogenesis, and develop novel therapeutic strategies. However, the problem lies in the fact that our understanding of several age-related diseases is limited to the generation of effective human models in laboratory, as reviewed in [10]. It is essential to develop advanced human models that can accurately mimic these diseases to deepen our understanding of the underlying early mechanisms and explore potential therapies that can improve health outcomes in aging individuals.

Most neurons in the human body are formed during embryonic development and can live for decades or even an entire lifetime. As a result, neurons are considered the longest living cells within the human body. Although a small percentage of neurons may be produced after birth in specific regions of the brain, the majority are generated during prenatal development, as reviewed in [70]. Neurons from primary human sources are highly valuable for research, but their acquisition and maintenance present significant challenges, as reviewed in [10]. This is primarily due to the limited accessibility to human brain tissue for research purposes, which is often attributed to ethical and legal issues [71]. In addition, obtaining a viable living neuron sample from existing tissue samples is challenging due to several factors including post-mortem changes, ischemia, and degradation due to fixation and storage [72], [73]. Furthermore, post-mortem samples only reflect the end stage of the disease, which makes it difficult to understand the early mechanisms involved in the onset of the disease [8]. As a result, directly studying human primary neurons is challenging, which can limit research initiatives and the development of potential treatments for neurological disorders.

To overcome the limitations of the study with human models, animal models have been extensively used by researchers to understand the complex mechanisms underlying several pathologies and have provided meaningful insights into the inner behavior of neurons and the brain, as reviewed in [10]. However, when it comes to modeling brain disorders, animal models have limitations since they may not necessarily reflect the specific mechanisms of human disease, indicating the inherent differences between rodent and human genomes and neuronal subtypes, as reviewed in [7]. By recognizing human neuron's complex nature, generating human neurons to model diseases has gained significant attention and it constitutes a promising approach to unlock new insights into the pathology and treatment of brain disorders.

As neurons age, they tend to build up lesions in their genes. Consequently, this accumulation leads to a reduction in the transcriptional output of these genes, causing alterations in neuron's metabolism to attain cell viability but that could further impact the neuron's functionality, as reviewed in [10]. Furthermore, mitochondria also become more compromised with age, resulting in increased production of free reactive oxygen species (ROS), which is harmful to DNA [74]. In addition, neurons must maintain their cellular identity for longer than other postmitotic cells as they cannot be replaced by cell division, so they must continuously function and retain their specific identity for the proper functioning of the brain, as reviewed in [75]. The accumulation of molecular changes over time can ultimately lead to altered gene regulation and transcriptional noise, which can impair neuronal function and contribute to the development of neurodegenerative diseases [76]. These characteristics are crucial to consider when studying age-related diseases, to better comprehend the mechanisms underlying neuronal dysfunction.

Age-related diseases, such as neurodegenerative disorders, are multifaceted and influenced by a combination of genetic and environmental factors. Therefore, to better understand these age-related diseases, it is crucial to develop models that allow not only the study of neurons but also reflect patient's specific age-related molecular signatures and genotypes, as reviewed in [10]. Primary cell samples obtained from donors have unique features that have accumulated over the patient's lifespan and/or

disease development, as reviewed in [10]. Based on this, ideal strategies include the conversion of somatic cell types, easily obtained not only technically but also ethically, into neurons, without losing important characteristics for the study of age-related diseases.

Induced pluripotent stem cells reprogramming

Many laboratories have developed techniques to study neurons *in vitro*, and one commonly used approach involves the use of induced pluripotent stem cells (iPSCs), a process also known as indirect reprogramming. Using iPSCs involves resetting the epigenetic state of directly collected somatic human cells to an embryonic-like state, and this is achieved through the transient expression of the Yamanaka transcription factors (TFs): OCT4, KLF4, SOX2 and C-MYC [77]. By activating these genes, somatic cells can be reprogrammed to an induced pluripotent state, allowing for their differentiation into several cell types, including neural cells for research purposes, as reviewed in [9].

The epigenetic landscape established by Waddington [78] is a popular model for explaining cell fate changes during normal development and iPSC differentiation (**Figure 1.4**). This model represents the concept that cell fate is not fixed, but rather flexible and reversible. While in normal development the differentiation of each cell is restricted to a cell type, by using indirect reprogramming, somatic cells can be reprogrammed to iPSCs which can then be differentiated into other somatic cell types, demonstrating the plasticity of cell fate [79].

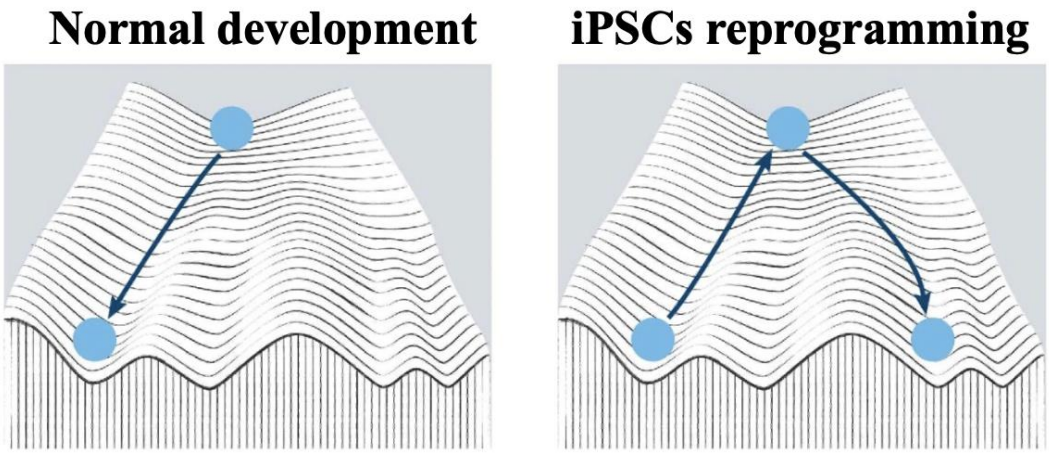


Figure 1.4 - Waddington's landscape model of normal development (left) and iPSCs reprogramming (right). Normal development is depicted as a ball rolling down from the top of Waddington's Mountain to the bottom of a valley, demonstrating the natural restriction of cell differentiation potential during normal development. On the other hand, iPSCs can be generated by reprogramming diverse types of somatic cells (each one representing a valley) and these can be differentiated into other somatic cell types. Adapted from [79].

The advances in iPSC technology have led to the differentiation of a diverse range of well-defined patient-derived neural cells that include neural stem cells, dopaminergic neurons, glutamatergic neurons, GABAergic neurons, motor neurons, astrocytes, oligodendrocytes, and many others, as reviewed in [10]. While iPSCs offer insight into early developmental processes, they are limited in their ability to investigate neuronal function and plasticity, important components for understanding age-related synaptic dysfunction, as reviewed in [7]. Further, it has been demonstrated that iPSCs not only restore the length of telomeres but also lead to rejuvenation at several other levels. Specifically, iPSCs have been found to rejuvenate at the level of epigenetic methylated DNA, the transcriptome, and in several functional phenotypes, including mitochondrial function, cellular senescence, DNA damage and others, as reviewed in [10]. In fact, most iPSC-based models for studying age-related diseases require the use of techniques to simulate aging. Examples of artificial induction of aging include the overexpression of progerin, a protein associated with premature aging, shortening of telomeres, and additional stressors applied to normal culture conditions to elicit disease-specific phenotypes, as reviewed in [80]. However, artificially inducing aging in iPSCs does not accurately replicate the physiological process of natural

aging. Instead, it can create a model of accelerated aging that can display different molecular pathways compared to those observed in aged cells, recapitulating only certain aspects of aging, as reviewed in [10].

Direct conversion

Direct conversion, also known as direct reprogramming or transdifferentiation, refers to the transition of a fully differentiated cell type into another, without transitioning through an intermediary pluripotent state [11]. This technique arises as an alternative to iPSCs reprogramming since it maintains age-associated features of donor cells and circumvents the rejuvenating embryonic state, as reviewed in [9]. Using the epigenetic landscape of Waddington to describe direct conversion, this technique is depicted as a direct path from a valley, representing one fully differentiated cell type, to another valley, representing a different fully differentiated cell type [81] (**Figure 1.5**).

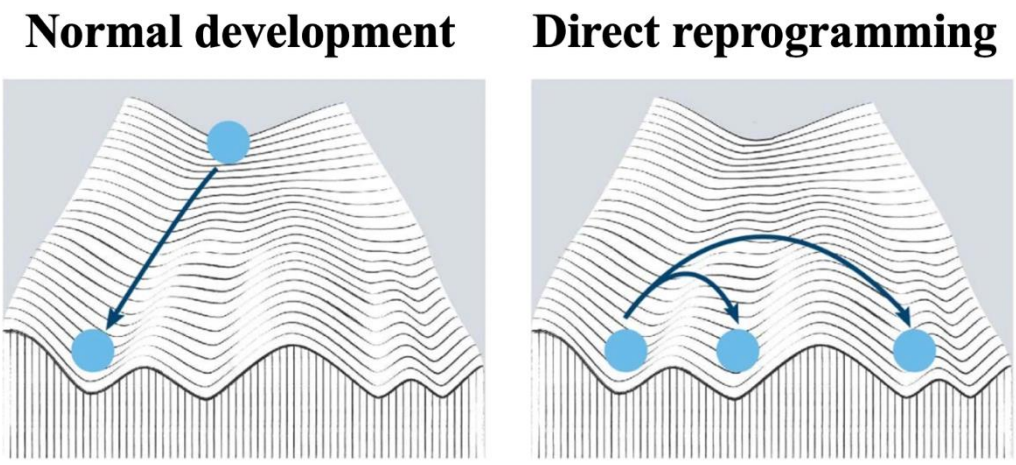


Figure 1.5 - Waddington's landscape model of normal development (left) and direct reprogramming (right). Normal development is depicted as a ball rolling down from the top of Waddington's Mountain to the bottom of a valley, demonstrating the natural restriction of cell differentiation potential during normal development. Direct reprogramming is represented as one ball being pushed in a parallel and direct way to another valley at the bottom of the hill, hence to another cell fate between one mature cell type to another. Adapted from [79].

Direct conversion is a versatile approach for differentiating one cell type to another, which can be achieved through different strategies, including overexpression of transcription factors (TFs), microRNAs or chemical cues, as reviewed in [10]. This approach has led to successful conversion to diverse cell types, including cardiomyocytes [82], hepatocytes [83], pancreatic cells [84] or neurons [85], demonstrating its potential applicability across multiple fields of research.

The first study that managed to efficiently differentiate one cell type to another through direct conversion was performed in the 1980s and allowed the conversion of mouse embryonic fibroblasts into myoblasts by overexpression of MYOD1 - a TF normally expressed in skeletal muscle cells [86]. Only 30 years later, in 2010, the first study with induced neurons generated by direct conversion (iNs) was performed, from mouse embryonic and postnatal fibroblasts as the starting cell type [85]. This was possible by overexpressing a set of pro-neural TFs: BRN2, ASCL1 and MYT1L, widely known as BAM factors. To achieve a simplified and effective protocol for generating iNs, multiple strategies were explored shortly after the initial study. The main focus was to produce iNs from different cell types, with an emphasis on human donors. In fact, in 2011, one such protocol generated functional iNs from fetal human fibroblasts by combining BAM TFs with NEUROD1 [87].

Generating iNs from human cells through direct conversion protocols provides a valuable opportunity to study the effects of aging on cells and the development of neurodegenerative diseases. Through this method, researchers can investigate the cellular changes that occur during the aging process and explore potential mechanisms associated with the onset and progression of neurodegenerative

diseases. The subsequent chapters will primarily focus on the conversion process applied to generate iNs.

1.3.1. Transcription factors

To generate neurons from another cell line it is necessary to induce alterations in the epigenetic profile of the cells, which refer to modifications that occur on the DNA and its associated proteins, but don't alter the DNA sequence itself [88]. This process involves several methods such as genetic modification through a defined activation of TFs, as reviewed in [10]. TFs are proteins that help turn specific genes "on" or "off" by binding to nearby DNA [89]. By overexpressing these TFs, it is possible to replace the transcriptional profile of the original cell population and initiate the neuronal transcriptional program. This process effectively converts the cell to a new identity, i.e., that of a neuron, by directly inducing the expression of neuronal genes, as reviewed in [90]. To ensure a more efficient and directed gene expression, specific TFs are selected based on their known involvement in the development of the target cell type and their high expression levels within the target cells, as reviewed in [91].

Unlike stem and progenitor cells which have a more plastic and flexible epigenetic landscape, fully differentiated somatic cells have a highly regulated and distinct epigenetic profile, making specific regions of the genome inaccessible for most TFs, as reviewed in [80]. Therefore, when overexpressing TFs to allow for direct conversion to iNs, TFs need to have the ability to bind to these previously inaccessible "neuronal regions" of the genome in non-neuronal cell types, as reviewed in [80]. Based on this, TFs can be subdivided into two groups: pioneer and non-pioneer transcription factors. Pioneer TFs have a distinctive property that allows them to enable the opening of closed chromatin sites, giving them access to these sites facilitating the implementation of cell-specific fates [92] (**Figure 1.6**). Examples of pioneering TFs for iN conversion include ASCL1, NGN2 and NEUROD1, as reviewed in [80]. Therefore, the presence of at least one of these pioneer TFs is required to access the normally closed chromatin surrounding neuronal gene *loci* for effective neuronal conversion, as reviewed in [80]. In addition, non-pioneer TFs cannot access these chromatin regions and are mostly recruited by pioneer TFs to either suppress the transcriptome of the starting cell type, or because they are pro-neuronal, thus contributing to establishing a new neuronal identity [93] (**Figure 1.6**). Examples of non-pioneer TFs include ZFP238, SOX8 and DLX3 [94]. Further evidence based on the BAM-conversion (described above) have demonstrated that the pioneer TF ASCL1 alone is able to initiate the process of converting fibroblasts into neurons and can access regions of closed chromatin [95]. ASCL1 selectively bind to specific sites and subsequently recruits BRN2 and MYT1L [95]. Although BRN2 and MYT1L are unable to induce conversion by their own, their involvement enhances the efficiency of the conversion process, following a hierarchical pattern of behavior [94]. BRN2, classified as a pioneer TF, helps opening chromatin and activate neuronal genes, and MYT1L, a non-pioneer TF, represses non-neuronal fates including myogenic genes [96], and works downstream of ASCL1 and BRN2 to further refine and stabilize the neuronal phenotype [94].

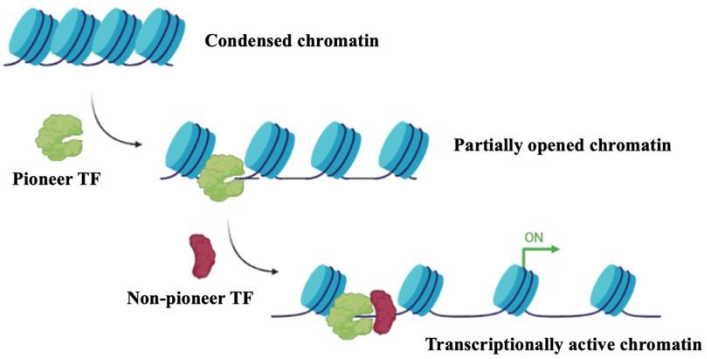


Figure 1.6 - Schematic diagram representing pioneer and non-pioneer TFs activity. Pioneer TFs identify and attach to particular DNA target locations in highly condensed chromatin, modifying it and making it more accessible for non-pioneer TFs to activate gene transcription. Adapted from [177].

1.3.1.1. Lentiviral vector

After establishing the specific TFs required for the direct conversion process, the next step is to design and produce a viral expression construct for each TF, as reviewed in [91]. The purpose of this construct is to introduce and increase the amount of each TF in target cells. This process, known as overexpression, is crucial to promote the direct conversion into iNs. The efficient delivery of these TFs is essential in this process, and lentiviral vectors are a commonly used tool for this task due to their high efficiency in transducing a wide range of cells and their relative ease to use [97]. Lentiviral vectors can stably integrate their genetic material into the genome of the target cell, allowing for long-term expression of the delivered TFs [98]. In addition, lentiviruses can efficiently transport genetic material into cells, including non-dividing cells, and integrate it into the host genome, unlike most of retroviruses, that are limited to transducing only dividing cells [99], [100]. Therefore, most direct conversion to iNs protocols rely on the use of lentiviral vectors, as reviewed in [10].

The use of a 2A peptide cleavage sequence is a popular approach to build lentiviruses for gene delivery [101]. In this strategy, the TFs are introduced into cells as transgenes to regulate gene expression and they are fused together using a 2A peptide sequence. In the context of direct conversion to neurons protocols, the combination of ASCL1 and NGN2 is typically fused via a 2A peptide sequence in order to deliver both transgenes to the target cells simultaneously [102]–[104].

As it is important to control and regulate the gene expression in direct conversion process, the doxycycline (dox)-inducible tetracycline (Tet)-On system is typically used in combination with lentiviral vectors [100]. The Tet-On system is composed of two main components – the reverse tetracycline-controlled transactivator (rtTA) protein, and the tightly regulated tetracycline-responsive element (TRE) promoter which contains multiple copies of the tetracycline operator (tetO) sequence and drives the expression of the gene of interest (G.O.I.) [105]. In the absence of doxycycline, rtTA is unable to bind to the tetO sequence, and the transcription of the gene of interest is repressed (**Figure 1.7 A**). However, in the presence of doxycycline, rtTA undergoes a conformational change, enabling it to bind to the tetO sequence and initiating transcription of the gene of interest [106] (**Figure 1.7 A**). Therefore, the transcription of TFs can be switched "on" or "off" according to the administration and removal of doxycycline, respectively [107]. The rtTA protein is usually expressed under the control of an additional promoter, to ensure adequate production of the rtTA protein for the Tet-On system to work effectively [105]. Typically, this promoter is ubiquitous as it supports high levels of gene expression, as reviewed in [91].

Figure 1.7 B represents an all-in-one dox-inducible Tet-On system used to delivery both NGN2 and ASCL1, called UNA [108]. This system includes a cassete containing rtTA, driven by a ubiquitous promoter (Ubi) and the iN cassete consisting of the TFs NGN2 and ASCL1 fused via a 2A peptide sequence, which is under the control of a tight TRE promoter (Tight). Furthermore, it also contains a puromycin-resistance gene that is driven by phosphoglycerate kinase (PGK) promoter to yield the UNA construct [108]. The addition of the antibiotic puromycin will allow transduced cells to be selected and survive, while those that were not efficiently transduced will die. Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) is a regulatory element that is commonly used in lentiviral vectors to improve transgene expression and enhancing transduction efficiency [109].

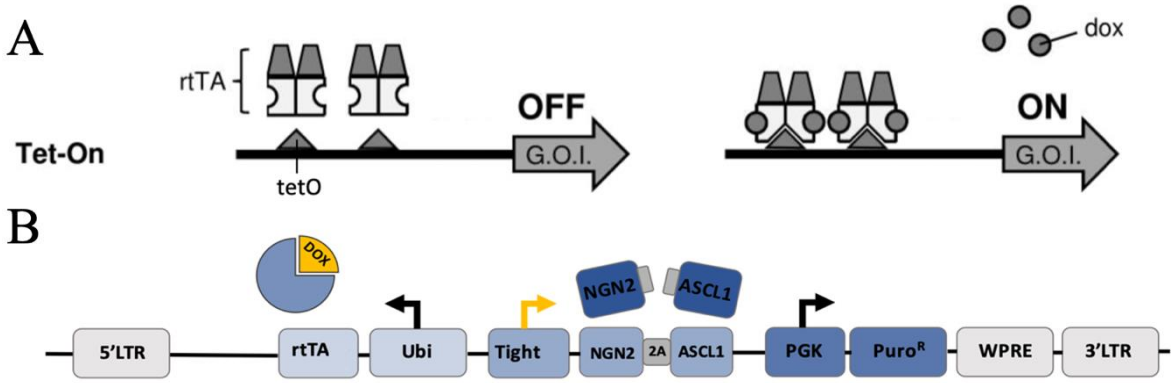


Figure 1.7 - Representation of the Tet-On system and example of a doxycycline (dox)-inducible Tet-On system to deliver the TFs NGN2 and ASCL1. A – In the Tet-On system, upon the addition of doxycycline, rtTA changes its conformation and binds to the tetO sequence in the promoter, enabling gene expression. B – All-in-one viral system used to delivery NGN2 and ASCL1 as TFs, fused via a 2A peptide sequence and controlled by a tight TRE promoter (Tight). rtTA is driven by a ubiquitous promoter (Ubi) and the system also contains a puromycin-resistance gene, driven by the PGK promoter, and WPRE, a regulatory element that allows for more efficient and sustained transgene expression in transduced cells. Adapted from [107], [109].

It is important to keep in mind that the co-overexpression of multiple TFs constitutes a technical challenge and carries the potential risk of causing bias, as reviewed in [80]. Therefore, it is desirable to limit the number of conversion TFs used, and this can be achieved emphasizing the use of pathway modulators, such as small molecules that either repress the identity of the starting population of cells or promote neuronal identity to enhance iNs efficiency and authenticity, as reviewed in [80].

1.3.2. Small molecules

Several chemical compounds, or small molecules, have been identified and are used as enhancers of iN generation and have allowed to better understand the mechanisms of direct conversion, as reviewed in [80]. Developing specific neuronal subpopulations can be achieved through the precise optimization of concentrations, timing, and combinations of these chemicals, as reviewed in [110].

Small molecules are essential to either inhibit or enhance certain pathways important to modulate neuronal differentiation, as reviewed in [110]. For instance, to induce neuronal differentiation, protocols have been taking advantage of the inhibition of glycogen synthase kinase-3β (GSK-3β) and transforming growth factor-β (TGF-β)/small mothers against decapentaplegic (SMAD) signaling, as reviewed in [10]. In fact, inhibition of TGF-β/SMAD signaling has also been associated with increased reprogramming efficiency since it promotes mesenchymal-to-epithelial transition (MET), a phenomenon implicated in the initial stages of iPSC generation [111]. It has been implicated that somehow this MET phenomenon, induced by inhibition of TGF-β/SMAD signaling, may also play a role in direct neuronal conversion, but further research is needed to address this potential correlation, as reviewed in [112]. SMAD pathway inhibition can be achieved through the implementation of activin-like kinase (ALK) inhibitors such as SB-431542, A-83 and noggin [113], [114]. To avoid high concentrations of noggin (500 ng/ml), an expensive compound [115], a strategy widely used consists of combining lower concentrations of noggin (100 ng/ml) plus LDN-193189 (0.5 μM), that inhibits ALK-2, and -3 [112]. In addition, GSK-3β inhibition is reached with the treatment of CHIR99021 [112].

While TGF-β/SMAD and GSK-3β inhibition is thought to destabilize non-neuronal identities and promote neuronal fate-stabilizing, as reviewed in [10], activation of intracellular cAMP has also been shown to greatly promote iN conversion, therefore, dibutryl (db)-cAMP (a derivative of cAMP) has been supplemented in the conversion medium [116]. Furthermore, forskolin was shown to directly stimulate chromatin accessibility for a more efficient NGN2-only conversion, a trait that was once attributed only to pioneer TFs [117].

Table 1.1 summarizes the main application of each of the small molecules described above.

Table 1.1 – Summary of the main small molecules used to enhance neuronal conversion and their specific applications.

Small molecules used to promote iN conversion	Sample applications	References
SB-431542	SMAD pathway inhibition	[113], [118]
A-83	SMAD pathway inhibition	[114]
Noggin	SMAD pathway inhibition	[113]
LDN-193189	SMAD pathway inhibition	[112]
CHIR-99021	GSK-3β inhibition	[112]
db-cAMP	cAMP activation	[116]
Forskolin	cAMP activation	[117], [119]

Primary neurons are sensitive and tend to undergo progressive cell death during culture, which can make the maintenance of healthy cultures challenging [120]. Therefore, in addition to inhibitors and enhancers that promote given pathways, certain supplements that stimulate the survival of neurons in culture are also implemented in the medium. B-27 and N-2 are supplements used in cell culture typically added for growth, expansion and provide long-term viability of neuronal cultures [120], [121]. In addition, laminin is usually added to support growth and differentiation of iNs and adhesion to the culture vessel [122], [123].

1.3.3. Different strategies to obtain different neurons

Carefully designed protocols are essential to generate a specific type of neurons. To differentiate a somatic cell into a desired type of neuron, it is crucial to determine the optimal combination of TFs, small molecules and other factors such as microRNAs or co-cultures, as reviewed in [110]. Depending on the combination, it is possible to obtain specific neuronal subtypes with distinct neurotransmitter and channel properties, that provide a unique platform for studying specific cells.

Based on the desired subtype, neurons can be generated through specific strategies. Neurons can be identified according to the type of neurotransmitter they release (glutamatergic, GABAergic, cholinergic, etc.), their location in the nervous system (medium spiny neurons, etc.), structural differences (multipolar, unipolar, bipolar, etc.) or their functional purpose (sensory, motor or interneurons) [124]. For instance, if the goal is to generate motor neurons to study the contraction of muscle fibers for movement, it matters to explore TFs that act in the embryo to confer a motor neuron identity on committed neural progenitors [125]. On the other hand, if the goal is to study PD, it is interesting to generate dopaminergic neurons, the type of neurons affected by the disease [126].

The most prominent conversion strategies using BAM combination typically give rise to a major population of excitatory glutamatergic neurons [85], [87], while ASCL1/NGN2-based conversion of human fibroblasts into iNs results in a major fraction of glutamatergic iNs, a smaller fraction of GABAergic iNs and rare dopaminergic or serotonergic iNs [104], [127]. In addition, ASCL1/SOX2

strategy to induce iNs generated from pericytes resulted in a mixed culture of GABAergic and glutamatergic iNs [128], and ASCL1 induced oligodendrocytes cells from adult neural stem cells in the dentate gyrus of mice [129]. Therefore, TFs do not exclusively define the identity of the generated neurons and it is also important to consider the epigenetic identity of the starting cells and the subsequent signaling cues present during the conversion, as reviewed in [80]. Additionally, not every pioneer TF can equally differentiate every starting cell type, as ASCL1 induce conversion of fibroblasts, but not keratinocytes, into iNs [90]. This suggests that the starting cell population is also an important decision, since not all cell types are equally amenable to a given reprogramming event [130]. The cell starting type usually employed by many researchers consists in fibroblasts since they correspond to a readily available cell type that allow the generation of large quantities of patient-specific cells, as reviewed in [91]. Moreover, fibroblasts can be obtained from skin biopsies without causing significant damage to the patient, making it an outpatient procedure that is relatively safe and non-invasive [131].

Through direct conversion, it is then possible to generate disease-specific neuronal subtypes such as medium spiny neurons for Huntington's disease (HD) [132], serotonergic neurons for modeling depressive and anxiety disorders [103], [133], peripheral sensory neurons for studying pain-related diseases [134], motor neurons resembling an amyotrophic lateral sclerosis (ALS)-related phenotype [125], [135], [136], dopaminergic neurons to model PD [137], [138] or cortical neurons to model diseases affecting cerebral cortex [139]. The methods used to generate the subtype-specific iNs described previously are listed in **Table 1.2**, and this does not suggest that no other combinations can give rise to the same subtypes of neurons. Rather, it emphasizes that the induction of functional lineage subtypes is highly manipulable and has significant potential to create models for neurological diseases that depend on specific neuron-subtypes. The choice of the exact combination will rely on several factors, including the intended goal, the efficiency demonstrated in previous studies, the accessibility for obtaining the necessary elements to the conversion process and, of course, the associated costs.

Table 1.2 - Neuronal conversion of human fibroblasts and astrocytes to subtype-specific iNs. Adapted from [80].

Starting cell type	iNs generation methods	Neuronal subtypes	Reference
Human fibroblasts	miR-9/9*-124 together with CTIP2, DLX1, DLX2 and MYT1L	Medium spiny neurons	[132]
Human fibroblasts	ASCL1, NGN2, NKX2.2, FEV, GATA2 and LMX1B	Serotonergic neurons	[103], [133]
Human fibroblasts	NGN1/NGN2 and BRN3A	Sensory neurons	[134]
Human fibroblasts	NGN2, SOX11, ISL1, LHX3 together with forskolin, dorsomorphin and basic fibroblast growth factor	Motor neurons	[125]
Human fibroblasts	ASCL1, NGN2, BRN2, MYT1L, HB9, ISL1 and LHX3		[135]
Human fibroblasts	miR-9/9* and p53 shRNA together with ISL1 and LHX3		[136]

Human fibroblasts	miR-124 together with ASCL1, NURR1 and LMX1A	Dopaminergic neurons	[137]
Human astrocytes	ASCL1, NEUROD1 and LMX1A		[138]
Human fibroblasts	BRN2, MYT1l and FEZF2	Cortical neurons	[139]

If the aim of a given study is essentially to generate iNs from aged donors to study specific mechanisms of age-related diseases, the choice of TFs combination is perhaps more important. In fact, there is evidence from previous studies demonstrating low efficiency of direct conversion protocols when applying ASCL1/BRN2 combination, as fetal human fibroblasts were easily converted to iNs, while adult fibroblasts were resistant [140]. On the other hand, ASCL1/NGN2 combination does not seem to differently affect iNs conversion efficiency for aged samples [102], [104], [127].

1.3.3.1. ASCL1/NGN2 combination with a cocktail of small molecules

The discovery that ASCL1 and NGN2 act as pro-neuronal pioneer TFs and can induce neuronal identity in non-neuronal cells is a significant breakthrough in direct conversion protocols. This combination has been implemented by several research groups as it results in high conversion efficiencies in both adult and aged human fibroblasts [102], [104], [127].

The transcription factor NGN2 is known to induce neuronal differentiation in about 90% of human fibroblasts [119], and ASCL1 alone can also induce neuron-like cells from fibroblasts [85]. In fact, these two TFs are well-established master regulators of the nervous system development, and mutations associated with them are related with embryonic lethality, as reviewed in [80]. During fibroblast-to-iN conversion, ASCL1 plays a critical role by functioning as a transcriptional activator inducing neuronal- and muscle-related gene expression, a few hours after induction [94]. In addition, until day 5, ASCL1 is responsible for 80% of chromatin changes that occur during the conversion process, leading to an upregulation of genes related to neuronal processes, neuronal network formation and early genes involved in neuronal maturation [94]. NGN2 is expressed throughout the developing nervous system and has several roles that contribute to the specialization of cells. In particular, it plays a crucial role in directing the commitment of progenitors to neurons by inhibiting glial fate, initiating the expression of pan-neuronal genes, promoting cell cycle exit, supporting neuronal migration, and facilitating the expression of neuron subtype-specific genes, while inhibiting the development into GABAergic interneurons, as reviewed in [110]. The exact targets of these TFs are not very clear, since some studies show that NGN2 binds most of the ASCL1 binding sites in fibroblasts, while others suggest that ASCL1 and NGN2 have divergent binding patterns, as reviewed in [80].

In 2012, Ladewig and colleagues were able to achieve the conversion of postnatal human fibroblasts into functional neurons by combining ASCL1 and NGN2, delivered through a doxycycline-inducible Tet-On system, with small molecule-based inhibition of GSK-3β and SMAD signaling and enhancers of cAMP (described above) [112]. Another study using the same protocol was able to generate iNs from skin fibroblasts of human donors across a broad range of ages and detected an age-dependent loss of nucleocytoplasmic compartmentalization in fibroblasts and corresponding iNs [104]. In agreement with this idea, Mertens and colleagues (2021) used this protocol to obtain iNs from Alzheimer's patients' fibroblasts that showed neuronal defects, reinforcing that this specific protocol of ASCL1/NGN2 combination with small molecules is an attractive model to study age-related mechanisms in iNs [127]. All these protocols share a common first stage of differentiation, where fibroblasts were exposed to the combination of TFs and small molecules and showed typical neuronal markers after 21 days of

differentiation, e.g., βIII-tubulin. Then follows the maturation stage, where the already-differentiated iNs were plated on a layer of supporting cells, such as astrocytes, as they are thought to have an essential role in synaptogenesis [85].

Analysis of generated iNs typically consists of performing immunofluorescence or quantitative PCR detection of selective neuronal markers to ensure that the desired neuronal genes are being expressed and that there is little to no expression of genes typical of the starting cell type. If the characteristics found are consistent with the desired cell type, cells may then be tested in terms of functionality to determine whether they have other properties of the desired cell type, such as electrical responses. Additionally, studies such as genome-wide analysis may also be employed to compare the transcriptional profile, histone, or DNA-modification profile of the generated iNs to the starting cell type, as reviewed in [91].

1.3.4. Advantages of direct conversion over iPSCs reprogramming

Differentiation protocols for human pluripotent stem cells including human iPSCs have been developed and optimized over a much longer period than those for iNs. However, the field of direct conversion technologies has rapidly expanded in recent years, with several new protocols being published. This technology is now considered a subdiscipline of the stem cell field, offering alternative approaches to generating desired cell types from human patients, as reviewed in [80]. Direct conversion aims to serve as an asset to multiple areas of study such as human biology research, disease modeling, drug development and screening and cell replacement strategies, as reviewed in [80]. In fact, iN technology has also shown promise *in vivo* as a strategy to replace damaged cells following brain injury by direct conversion of non-neuronal cell types into neurons directly within the nervous system [141].

One of the key advantages of direct conversion corresponds to the rapidity of the protocol in comparison to iPSCs protocols and the associated costs, two relevant factors in scientific research. The initial step of generating clones in iPSCs protocols usually takes 2-3 months, as reviewed in [10]. Then, differentiating the iPSCs into neurons takes 6-15 weeks, although this time frame may vary depending on the differentiation protocol that is used as well as the desired level of maturity, as reviewed in [10]. On the other hand, direct conversion protocol takes about 2-4 weeks to differentiate cells into iNs, and 2-4 weeks to further mature iNs by co-culturing with astrocytes [87], [104]. Additionally, direct conversion protocols usually involve one conversion medium for the conversion phase and one maturation medium for the maturation phase, which is much simpler when comparing to the number of different recipes of media needed in iPSCs protocols, as reviewed in [10].

Relevancy to study age-related diseases

As mentioned before, to efficiently study age-related diseases, it is crucial to develop patient-specific models that allow the study of neurons in a meaningful and predictive manner. It is then essential to ensure that the genetic identity of the generated cells reflects that of the donor's cells in the body. Moreover, the biological age of the generated neurons is an important factor and should match the biological age of the cell's donor.

There are some published studies using both iPSC reprogramming and direct conversion applied to cells from donors with different ages and with a pathological phenotype. Tang and colleagues (2017) have generated induced motor neurons and iPSC-derived motor neurons from three young (0-3 years) and three old (53-71 years) healthy donors, and from four familial amyotrophic lateral sclerosis patients carrying SOD1 and FUS mutations [142]. The results regarding iPSC-derived motor neurons were consistent with a rejuvenated phenotype, as they presented a dramatically decreased expression of classical senescence-associated markers, resetting many aspects of aging-associated features from fibroblast donors. On the other hand, induced motor neurons generated from direct conversion presented increased signs of DNA damage, loss of heterochromatin and reduced nuclear organization [142]. Another study, modeling Huntington's disease (HD) through direct conversion, showed that induced striatal medium spiny neurons from HD patient fibroblasts exhibited mutant huntingtin gene (mHTT)

aggregates, DNA damage and mitochondrial dysfunction [132]. In contrast, iPSC-derived neurons did not exhibit mHTT aggregates even after the addition of cellular stressors [132]. A more recent study developed by Mertens and colleagues (2021) compared iNs obtained from Alzheimer's patients' fibroblasts and iPSC-derived neurons, and results of genome-wide DNA methylation (DNAm) profiles showed that iNs derived from AD patients share DNAm with adult and old chronological ages of their donors, while iPSC-derived neurons reflected pre-natal DNAm ages [127].

Direct conversion of fibroblasts into iNs provides a valuable tool for studying age-related diseases due to several reasons. Firstly, research has demonstrated that iNs maintain the same epigenetic age as their parental fibroblasts, indicating that epigenetic changes associated with aging can be transferred from fibroblasts to neurons. This suggests that most age-related epigenetic marks can be preserved during the conversion process. Secondly, at the transcriptome level, iNs exhibit strong age-related signatures that share similarities to transcriptomic aging signatures observed in postmortem human brain samples. This implies that the age-related gene expression patterns seen in the brain can be recapitulated in iNs generated through direct conversion. These transcriptomic aging signatures provide valuable insights into the molecular changes associated with aging and age-related diseases. Furthermore, direct conversion of cells into iNs does not involve cell division, which allows for the preservation of nuclear pore-associated aging features. Studies have shown that iNs generated through direct conversion exhibit a marked decline in nucleocytoplasmic compartmentalization, which is an age-related alteration. This preservation of nuclear pore aging features in iNs provides a unique opportunity to study the impact of these alterations on neuronal function and age-related diseases. In addition, other aspects of cellular aging, such as metabolic and mitochondrial aging are also conserved in iNs. This suggests that iNs can serve as a model system to investigate the impact of aging on cellular metabolism and mitochondrial function, which are critical factors in age-related diseases.

Overall, iNs provide a unique opportunity to explore the molecular mechanisms underlying aging and develop potential therapeutic interventions for age-related neurological disorders.

1.3.5. Limitations

Direct conversion has significantly revolutionized cell reprogramming owing to its simplistic and efficient nature. However, this technique does come with certain drawbacks that need to be considered. One of the primary limitations is associated with the source of the cells, as direct conversion protocols have been limited to pericytes and skin fibroblasts, and the first ones are relatively hard to obtain from patients, as reviewed in [10].

Furthermore, another limiting step is the reproducibility of cell reprogramming protocols, as cells have been found to respond differently to the same neuronal differentiation protocols [90]. However, this challenge can be addressed by optimizing the protocol according to the specific objectives and needs of each research group. By tailoring the protocols to the specific cell types and research questions, scientists can work towards developing more reproducible and reliable direct conversion techniques in the future.

Another limitation of direct conversion is that it does not involve a highly expandable intermediate stage, such as in iPSCs protocol, and the generated neurons are postmitotic. This means that the number of neurons obtained depends entirely on the proliferation of the starting cell type, usually fibroblasts, which have a limited ability to expand and become senescent after a few passages, as reviewed in [10]. Consequently, the number of starting cells limits direct conversion protocols, making it challenging to scale up enough cells for human treatment purposes, as reviewed in [91]. To overcome this issue, direct conversion protocols require a high number of starting cells to obtain a higher number of iNs. A higher number of starting cells is usually achieved through immortalization or long-term storage in liquid nitrogen, but it constitutes the risk of introducing artifacts that may invalidate their use as an aging model, as reviewed in [80] and [144].

In addition, as described before, most iN protocols rely on the use of lentiviral vectors, which carry safety concerns when considering clinical applications due to potential integration-induced artifacts, as reviewed in [10]. This is an important consideration in studies that aim to transplant differentiated cells

into human patients, since the TFs are permanently integrated into the host genome, as reviewed in [91]. Furthermore, although small molecules are expected to be less invasive than using TFs, they can have potent neuroprotective abilities, so they also might mask important indicators of disease- and epigenetic age-related signatures in iN-based disease models, as reviewed in [80]. Although small molecules are described to either inhibit or enhance specific pathways in cell fate determination, the exact mechanism of action of the identified compounds is not fully understood, as reviewed in [91]. As a result, further research is necessary to clarify the mechanisms of action of these small molecules, which may uncover new information about how cells determine their fate.

The combination of elements used, and the starting cell type are fundamental to generate a specific subtype of iNs, as mentioned before. However, certain combinations give rise to a heterogenous population of iNs, as ASCL1/NG2 strategy combined with a cocktail of small molecules generates a major fraction of glutamatergic iNs, a smaller fraction of GABAergic iNs and rare dopaminergic iNs [104]. While this is a well-established method, the emergence of distinct subtypes of neurons can create challenges if the aim is to study certain mechanisms that require a single subtype of neurons. To overcome this, fluorescence-activated cell sorting (FACS) can be used to target and isolate cell groups, providing an alternative strategy to obtain the desired subtype of iNs for disease modeling and drug screening.

2. Motivation and Aim of the Study

The main purpose of this project is to establish an efficient model for studying age-related synaptic dysfunctions in humans. Previous studies in animal models have shown that an overactivation of NMDA/mGlu5 glutamate receptors and calcium overload, associated with increased A_{2A} receptors levels, may contribute to cognitive decline. Consequently, it is of utmost importance to understand whether these mechanisms correlate in human models or, on the other hand, human cognitive decline stems from other pathophysiological mechanisms.

To do so, we will follow a previous validated direct conversion protocol developed by Dr. Jerome Mertens and the Gage lab. [12] which involves the delivery of two transcription factors (ASCL1 and NGN2) and a 21-day phase where the cells are exposed to a cocktail of small molecules. We will use skin fibroblasts samples obtained from patients with different ages, including two adult donors (< 65 years) and one aged donor (> 65 years), all cognitively healthy. By following the protocol, we aim to generate induced neurons (iNs).

Although the protocol has already been validated by Dr. Mertens, it is necessary to evaluate its reproducibility for our specific objective and, consequently, make any necessary adjustments accordingly. Therefore, the development of this master's thesis will involve the optimization and adaptation of the protocol, while ensuring the characterization of the generated iNs. We will confirm whether iNs exhibit a glutamatergic phenotype using RT-qPCR and immunocytochemistry techniques. Additionally, we will perform whole-cell patch clamp experiments to assess whether iNs can fire action potentials.

It is then expected that the protocol will be reproducible in our laboratory so that the ultimate goal of studying age-related synaptic dysfunction can be achieved.

3. Materials and Methods

3.1. Cell culture

This subsection intends to describe all the procedures performed regarding cell culture. Overall, this protocol can be divided in two main steps: 1) fibroblasts expansion, and 2) direct conversion of these fibroblasts into induced neurons (iNs). Direct conversion can be further subdivided into the lentiviral transduction stage, and the differentiation stage.

Throughout the conversion process, cells underwent a series of steps until they reached their final differentiated state, and therefore their designations varied during the process. The initial state of the cells corresponded to typical fibroblasts, or non-transduced fibroblasts (NT-fibroblasts). Fibroblasts that were lentivirally transduced were designated as UNA-fibroblasts, and iNs corresponded to cells that were differentiated after a period of 21 days. Both fibroblasts (non-transduced and transduced) behaved like proliferative cells, which is why their expansion was necessary, and with that a series of procedures described in detail in chapter **3.1.1**. Direct conversion procedures are described in chapter **3.1.2.**

3.1.1. Fibroblasts expansion

Primary human skin fibroblasts were obtained from donors with different ages, cognitively healthy, provided by the iMM Biobank (**Table 3.1**). Namely, for this study we used two samples of adult-derived fibroblasts (donors with a chronological age < 65 years) and one sample of aged-derived fibroblasts (donor with a chronological age > 65 years). Protocols were approved by ethics commission.

Table 3.1 - Characterization of human skin fibroblast samples.

ID Sample	Gender	Age (years)
181754	Male	25
181661	Female	40
211392	Male	70

The samples were cryopreserved in tubes with a density of 2.0×10^6 cells/ml.

To start a new culture, cryopreserved tubes were placed in 37 ºC water bath, dried with paper tissue, and disinfected with 70% ethanol in order to avoid contaminations. Thawed cells were transferred to 15 ml tubes containing 8 ml of wash medium, Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, cat. no. 21969035) to dilute the solution of cryopreserved cells, and then centrifuged for 5 minutes at 1000 x relative centrifugal force (rcf). The supernatant was discarded, and the pellet was resuspended in pre-warmed growth medium TFM or TFM-P, depending on the state of the cells (see **Supplementary Table 1**), and seeded in the intended vessel. To expand fibroblasts, 6-well plates, T-25 and T-75 flasks were typically used.

The medium of all cells was changed every third day and the vessels were maintained in a humidified atmosphere of 5% of CO_2 at 37 ºC.

Passages of fibroblasts

When fibroblasts reached about 80-100% confluence, they were expanded in order to avoid clustering of cells due to contact inhibition. The confluence of cells was assessed through visual inspection resorting to bright-field microscopy. To split fibroblasts, cells were washed with 1x Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific, cat. no. 70011044) and detached with TrypLE Select Enzyme (Thermo Fisher Scientific, cat. no. 12605028) for 15 minutes. To dilute and stop the action of TrypLE properly, cells were washed with DMEM (4x the volume of TrypLE considered).

The suspension was centrifuged for 5 minutes at 1000 x rcf and the supernatant was discarded. The cell pellet was resuspended in pre-warmed growth medium, and the cells were seeded in the new vessel at the best splitting ratio. Typically, this ratio was 1:2 or 1:3, depending on the overall proliferating rate of the cells, as the growth speed varied dramatically between different cell lines.

Fibroblast freezing

In order to make the most of a cell sample, and in particular to anticipate a situation of contamination, it is of utmost importance to make stocks of cells at some time points, for later use. For that purpose, when fibroblasts reached nearly 100% confluence, cells were washed with 1x PBS, detached with TrypLE for 15 minutes and washed with DMEM. Then, they were centrifuged for 5 minutes at 1000 x rcf and the pellet was resuspended in freezing medium containing 90% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, cat. no. 16000044) and 10% Dimethyl Sulfoxide (DMSO) (Merck, cat. no. D2650). The solution with cells was distributed into 1 ml freezing vials and stored in liquid nitrogen.

This procedure was particularly important for UNA-fibroblasts, since by freezing these cells there was no need to perform the lentivirus transduction on a regular basis, one could simply thaw a vial and work with those cells thereafter.

Cell quantification

To get an accurate measurement of how many cells there were in each vessel, cells were counted using a hemocytometer, an instrument for visual counting of cells under a microscope.

Cells were detached with TrypLE, resuspended in DMEM and centrifuged for 5 minutes at 1000 x rcf. The pellet was resuspended in normal growth medium. Considering a dilution factor of 5, 100 µl of the cell suspension were transferred to an Eppendorf containing 400 µl of Trypan Blue (Merck, cat. no. T8154), a vital stain to selectively color dead cells. Following that, 100 µl of the mixture were pippeted and applied to the hemocytometer, in both chambers underneath a coverslip, in order to the liquid to be drawn out by capillary action. Live cells do not take up Trypan Blue, so unstained cells were counted using a hand tally counter.

Once the cells were counted, the average number of cells per square was calculated. The value obtained was then multiplied by 10^4 (conversion factor to ml) and by the dilution factor considered from the Trypan Blue addition. The final value represented the number of live cells per ml in the original cell suspension:

$$\text{Cells/ml} = \text{Average number of cells} \times 10^4 \times \text{Dilution factor} \quad \textbf{(1)}$$

3.1.2. Direct conversion of fibroblasts into iNs

The direct conversion protocol that we used and attempted to reproduce and enhance in our laboratory relied on a combination of two transcription factors, along with a cocktail of small molecules strategy, developed by Mertens and colleagues (2021) [12].

3.1.2.1. Lentiviral transduction

The first step to convert human skin fibroblasts into iNs was to perform the lentiviral transduction. This allowed the cells to co-overexpress later the two pro-neuronal pioneer TFs, namely, NGN2 and ASCL1. These TFs were delivered to the cells using a lentiviral vector system referred as "UNA". The lentiviral vector consisted in a dox-inducible Tet-On system that carried both NGN2 and ASCL1, linked via a 2A peptide sequence (See in detail **1.3.1.1** and **Figure 1.7 B**).

The plasmid was obtained from Dr. Jerome Mertens and the Gage lab., The Salk Institute for Biological Studies, La Jolla, CA, USA, and encapsulated in lentiviral particles at the lab. of Prof. Luís Pereira de Almeida, and the Viral Vector for Gene Transfer Core Facility of the University of Coimbra.

In order for the fibroblasts to be transduced with the lentivirus, aliquots of virus were mixed with normal growth medium (TFM, see **Supplementary Table 1**) and 4-8 ug/ml of Polybrene (Merck, cat. no. TR-1003-G) were added to enhance the efficiency of the lentiviral transduction. One day after the cells were kept in contact with this mixture, the medium was replaced by TFM medium alone. Following 48 to 72 hours later, 1 ug/ml Puromycin was added to TFM in order to select fibroblasts that were transduced (UNA-fibroblasts). This antibiotic was included in the medium (TFM-P, see **Supplementary Table 1**) until the differentiation process could start. It is important to note that the growth medium of UNA-fibroblasts, TFM-P, does not include doxycycline, the antibiotic that will initiate the lentiviral system and allow the TFs to be expressed in the cells.

UNA-fibroblasts grow and expand like typical fibroblasts, so equally they had to be expanded (and backed-up) to be ready for the differentiation process. Thus, all the procedures stated in **3.1.1** were also performed with UNA-fibroblasts.

3.1.2.2. Differentiation

After lentiviral transduction, UNA-fibroblasts might experience cellular stress or genotoxicity that could impact their conversion efficiency [12]. To mitigate these effects and allow for the selection of the transduced cells, it was recommended to passage the cells at least 3-5 times before initiating differentiation [12]. This allowed for the establishment of a stable population of transduced fibroblasts, ensuring consistency and reproducibility for downstream experiments. After that, UNA-fibroblasts were detached from the vessel's surface and pooled into several densities: i. 25,000 cells/ml; ii. 50,000 cells/ml; iii. 75,000 cells/ml; iv. 100,000 cells/ml; v. 125,000 cells/ml and vi. 150,000 cells/ml in TFM-P. After 24 hours, the medium was changed to Neuron Conversion (NC) medium (see **Supplementary Table 1**) for 21 days. This medium included doxycycline that allowed the expression of downstream target genes, in this case, the two TFs required for neuronal conversion, laminin, to promote cellular adhesion, in combination with a defined cocktail of small molecules that modulated specific pathways important to neuronal conversion: noggin (inhibition of SMAD pathway), LDN-193189, A83-1 and SB-431542 (ALK inhibitors), CHIR-99021 (GSK-3β inhibitor), forskolin, and db-cAMP (cAMP boosters). In addition, B-27 and N-2 were also included in NC medium to promote neuronal survival.

It is important to note that when undergoing differentiation, UNA-fibroblasts should not be seeded in glass, even with special coatings, as they did not adhere easily to this material. However, certain downstream applications required a vessel with a refraction index similar to that of glass (1.5). To overcome this challenge, UNA-fibroblasts were typically seeded in special tissue culture (TC)-treated vessels to complete 21 days of differentiation, hoping to obtain fully differentiated neuron-like cells.

During the differentiation process, converting iNs did not proliferate and required close cell-cell contact with one another. Therefore, vessels with large surface areas, such as 6-well plates, T-25, or T-75, were not necessary. In fact, for applications such as electrophysiology, smaller surfaces were required to meet specific set-up specifications. Ultimately, the type of vessel used for each experiment was determined by a compromise between the characteristics required for each application and the ease of cell adhesion to the vessel's surface. This will be detailed in the next subchapters.

3.2. Reverse transcriptase qPCR

3.2.1. Cell plating

Total RNA was extracted from both fibroblasts (UNA-fibroblasts and NT-fibroblasts) and iNs (at 21 days of differentiation), all plated in TC-treated vessels. Specifically, fibroblasts were plated in TC-treated 6-well plates, but T-25 and T-75 flasks were also used, depending on the proliferation rate and confluence of cells. Transduced fibroblasts for iNs differentiation were plated in TC-treated 24-well plates (**Table 3.2**).

Table 3.2 - Useful information about the vessels used for the final purpose of extracting RNA.

Cell type	Vessel	Surface area (cm^2)	Growth medium (ml)	Catalog number
Fibroblasts	6-well plate	9.6	≃ 2	VWR, CORN3516
	T-25	25	≃ 5	VWR, 734-2311
	T-75	75	≃ 12	VWR, 734-2313
iNs	24-well plate	1.9	≃ 1	VWR, CORN3527

3.2.2.RT-qPCR expression analysis

RNA extraction

Total RNA was extracted from fibroblasts (both UNA and NT) and iNs using Trizol LS reagent (Invitrogen, cat. no. 15596026). RNA concentration was assessed by Nanodrop 2000 (Thermofisher Scientific, USA) analysis.

Reverse Transcriptase-PCR (RT-PCR)

RNA was reverse transcribed to synthesize complementary DNA (cDNA), using random primers and SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, cat. no. 18090050), and negative controls were made without the enzyme.

Real Time PCR (qPCR)

Quantitative differences in gene expression were determined by real time PCR (qPCR) using SYBR Green PCR Master mix (Thermo Fisher Scientific, cat. no. 4364344), 5 µM of each primer and 8 ng/µl of total cDNA. The reactions were performed in Rotor-Gene 6000 Real Time Rotary Analyzer (Corbett Research) and in 7500Fast – 96-well (Applied Biosystems), using gene specific primers, designed in National Center for Biotechnology Information (NCBI) Primer-Basic Local Alignment Search (BLAST) tool. The thermal cycler conditions were 10 minutes at 95 ºC, 40 cycles of a two-step PCR, 95 ºC for 15 seconds followed by 60 ºC for 60 seconds with a final thermal ramp from 72 ºC to 95 ºC.

Expression levels were normalized to GAPDH. The sequences of used primers (all from Invitrogen, HPLC purified) are listed in

Supplementary Table 2.

3.3. Immunocytochemistry (ICC)

3.3.1. Cell plating

For imaging using fluorescence microscopy it was required to use a vessel's material with refractive indexes close to 1.5. Thus, fibroblasts (both UNA and NT) and iNs were plated in ibidi µ-Plate 24 Well Black ID 14 mm (**Table 3.3**). The surface of these plates consists in a thin plastic coverslip with the standard requirements for microscope and imaging techniques, providing optimal cell adherence combined with excellent optical properties.

Table 3.3 - Useful information about the vessels used for the final purpose of immunocytochemistry.

Cell type	Type of vessel	Surface area (cm^2)	Growth medium (ml)	Refractive index	Catalog number
Fibroblasts and iNs	**24-well plate**	1.54	≃ 1	1.52	ibidi, 82426

3.3.2. Immunostaining protocol

Cells were fixed with formalin (400 µl/well) for 20 minutes at room temperature and washed 3 times with PBS (15 minutes), followed by a 1 hour blocking with PBS containing 10% FBS. Then, cells were incubated with primary antibodies (**Table 3.4**) for 1 hour, or overnight at 4 ºC in PBS containing 0.05% Tween 20 and 3% FBS. After washing as described above, samples were incubated in 1:500 secondary antibodies (**Table 3.5)** for 1 hour at room temperature. Nuclear staining was performed with Hoechst (12 µg/ml) diluted in PBS. After washing, the cells were mounted with aqueous mounting medium (DAKO, Agilent, cat. no. S302380-2) and let to dry for 24 hours at room temperature.

Cells were observed either with Zeiss Cell Observer or Zeiss LSM 980 Confocal Microscope.

Table 3.4 - Primary antibodies and related conditions used in ICC experiments.

Protein	Primary Antibody	Host	Dilution
Vimentin	Vimentin (V9), Santa Cruz Biotechnology, cat. no. sc-6260	Mouse	1:200
βIII-tubulin	Anti-β-Tubulin III, Merck, cat. no. T2200	Rabbit	1:200
VGLUT1	VGLUT1 antibody Synaptic Systems, cat. no. 135511	Mouse	1:250
GAD67	GAD67 antibody (JM11-11), Novus, cat. no. NBP2-66855	Rabbit	1:500
PSD-95	PSD95 (D27E11), Cell Signaling, cat. no. 3450	Rabbit	1:200

Table 3.5 – Secondary antibodies and dilutions used in ICC experiments.

Secondary Antibody	Dilution
Donkey 488 anti-rabbit, Thermo Fisher Scientific, cat. no. A21206	1:500
Donkey 488 anti-mouse, Thermo Fisher Scientific, cat. no. A21202	1:250
Donkey 568 anti-mouse, Thermo Fisher Scientific A10037	1:500
Donkey 647 anti-rabbit, Thermo Fisher Scientific A31573	1:200

3.4. Electrophysiology (patch-clamp)

3.4.1. Cell plating

In order to record electrical activity from iNs using patch clamp electrophysiology, two methods were used to find the best and suitable cell plating procedure for this purpose, considering that the set-up required some specifications regarding the plating material's refractive index, which should be similar to that of glass, and the dimensions of the culture dish, that should fit the microscope's holder.

Seeding iNs in Geltrex-coated glass slides

Based on this method, converting iNs completed 21 days of differentiation in TC-treated 24-well plates (Corning Costar, cat. no. CLS3527), and then they were collected and seeded in Geltrex-coated coverslips (Thermo Fisher Scientific, cat. no. A1569601). For that purpose, 21 days-converted iNs were detached using 0.5 ml of pre-warmed TrypLE in each well for 7 minutes (or until the cells started to detach) and then washed with a mixture of NC medium without the small molecules, and Y-27632 (Merck, cat. no. Y0503), a ROCK inhibitor (ROCKi) to allow the survival of the cells. After that, iNs were centrifuged at 1000 x rcf 20 ºC for 5 minutes, the supernatant was discarded, and the pellet was gently resuspended in NC medium and Y-27632. iNs suspension was then seeded in previously Geltrex coated-coverslips (300 µl/well) in 24-well plates, and the next day the medium was replaced to NC medium alone.

Seeding UNA-fibroblasts on µ-Dish 35 mm for the differentiation process

As previously stated, converting iNs did not grow well in glass, even with special coatings, making the cell yield low and disturbing further applications. Namely, electrophysiology experiments require a great number of cells to increase the likelihood of making contact with each other, and thus increase the probability of surviving and recording electrical activity from them. This being said, based on this method, UNA-fibroblasts were seeded in µ-Dish 35 mm with low walls (for allowing the recording electrode to reach the iNs), which contains a polymer coverslip bottom that favor cell adhesion (**Table 3.6**). At 21 days of differentiation, iNs plated in these dishes were used to patch-clamp experiments.

Table 3.6 - Useful information about the vessels used for the final purpose of patch-clamp electrophysiology.

Cell type	Vessel	Surface area (cm^2)	Growth medium (ml)	Catalog number
iNs	μ-Dish 35 mm low	3.5	≃ 0.8	ibidi, 80136

4. Results

4.1. Plating cell density influenced the efficiency of differentiation into iNs

As direct conversion did not involve a highly expandable intermediate stage and cells stopped dividing as they started differentiating, the number of iNs depended entirely on the fibroblast proliferation prior to conversion. Therefore, to achieve a suitable number of iNs for downstream applications, starting UNA-fibroblasts should be seeded at higher densities to benefit cell-cell contact, promote some proximity during conversion, and to improve the overall conversion efficiency [12].

We cultured UNA-fibroblasts at different densities (i. 25,000 cells/ml, ii. 50,000 cells/ml, iii. 75,000 cells/ml, iv. 100,000 cells/ml, v. 125,000 cells/ml and vi. 150,000 cells/ml) to begin differentiation process. The objective was to initiate the differentiation process and evaluate its efficiency while finding a compromise between a sufficiently high density for further applications and maintaining the viability of the process. We then took pictures at 14 days of differentiation (**Figure 4.1 A**) and as it is observed, when plated at a density of 100,000 cells/ml or higher, cells started to cluster.

To gain a better understanding regarding the development of these cell clusters, we took pictures at different stages of their formation (**Figure 4.1 B**), and it was observed that over time, these cell aggregates began to detach from the surface of the vessel. To assess whether these clusters were composed of live or dead cells, we performed a cell viability test using with Hoechst (12 µg/ml) – a nuclear staining dye - and Propidium Iodide (PI) (1:3000) (**Figure 4.1 C**). PI is a dye that cannot penetrate viable cell membranes, making it useful for identifying dead cells based on membrane integrity. We found positive PI staining at the sites of cell aggregation, suggesting that the clusters were composed of non-viable cells, and subsequently detached from the surface.

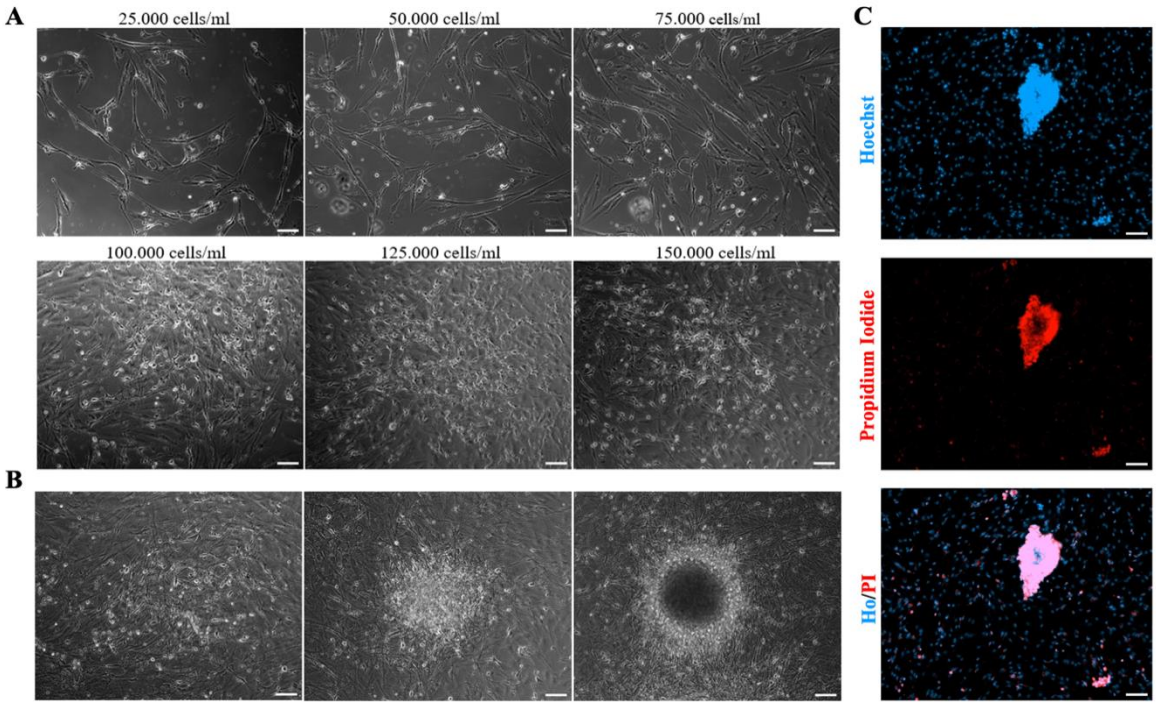


Figure 4.1 - Influence of cell density on neuronal conversion. (A) – Bright field phase-contrast pictures of iNs at 14 days of conversion plated at different densities – cells started to aggregate when submitted to a density of 100,000 cells/ml or higher. (B) – Different stages of cell aggregates evolution upon a high-density culture of iNs (125,000 cells/ml). (C) – Staining of cells plated at high density (100,000 cells/ml) that formed cell aggregates, with Hoechst and Propidium Iodide. All scale bars represent 100 µm.

4.1.1. Plating cell procedure influenced density for patch clamp experiments

To perform electrophysiology patch-clamp experiments, cells were seeded based on two approaches. The first one consisted in detaching cells after they had completed 21 days in TC-treated 24-well plates and re-plating them in Geltrex coated-coverslips. This method provided an easy delivery of iNs for electrophysiology and coverslips matched the set-up profile for patch clamp experiments, however, the cell yield at the end revealed to be low as observed in **Figure 4.2 A**, with cells exhibiting less neuronal-like morphology and contact proximity.

To overcome the low cell density, the second approach consisted of differentiating cells throughout the 21 days directly in µ-Dishes 35 mm low, that already had the required features for the electrophysiology set-up, which resulted in a higher-density iNs culture (**Figure 4.2 B**).

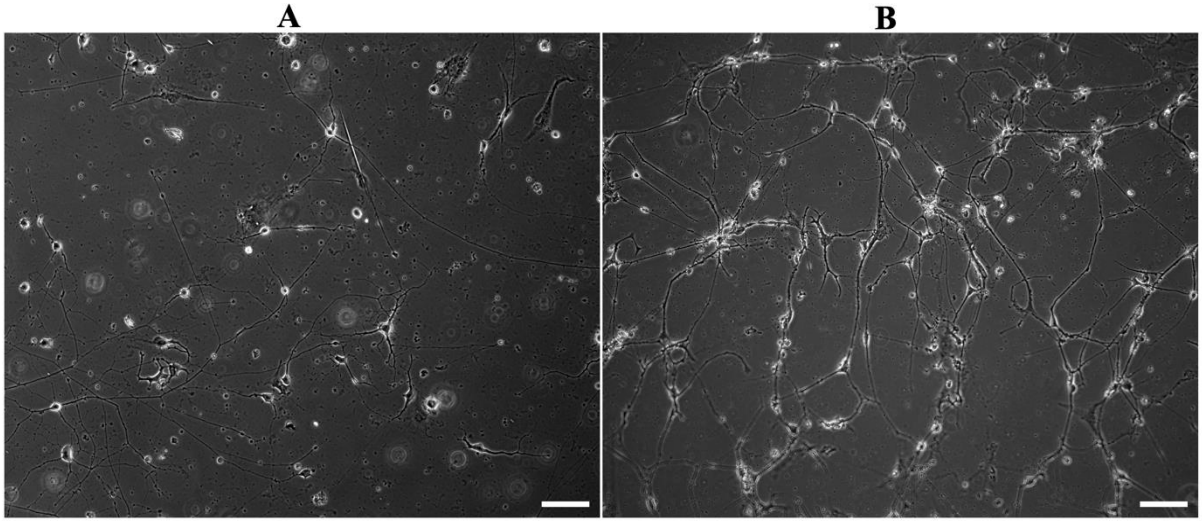


Figure 4.2 - Different plating procedures resulted in distinct neuronal networks. (A) – Cells re-plated in Geltrex-coated slides after 21 days resulted in scattered iNs. (B) – Plating cells directly on µ-Dishes 35 mm provided higher cell density at the end of 21 days, favoring patch clamping experiments. Scale bars represent 100 µm.

4.2. Direct conversion of human fibroblasts from adult donors into iNs

4.2.1. Transduction efficiency

The first step to commit fibroblasts into a neuronal state involved the lentiviral transduction to introduce the two pro-neuronal pioneer TFs: NGN2 and ASCL1. To assess the successful transduction and expression of these TFs, RT-qPCRs were conducted on the three cellular states: NT-fibroblasts, UNA-fibroblasts and iNs, for both cell lineages corresponding to the adult donors (**Figure 4.3 A**). As expected, the expression of both TFs was detected exclusively in iNs. This outcome was attributed to the presence of doxycycline in NC medium, which induced transgene expression.

Furthermore, the expression of both transduced and endogenous ASCL1 was examined in NT-fibroblasts, UNA-fibroblasts and iNs (**Figure 4.3 B**). The results revealed that the transduced ASCL1 gene was selectively expressed in iNs, indicating successful incorporation of the transgene. In contrast, the expression of the endogenous ASCL1 gene was significantly suppressed in all three cellular states.

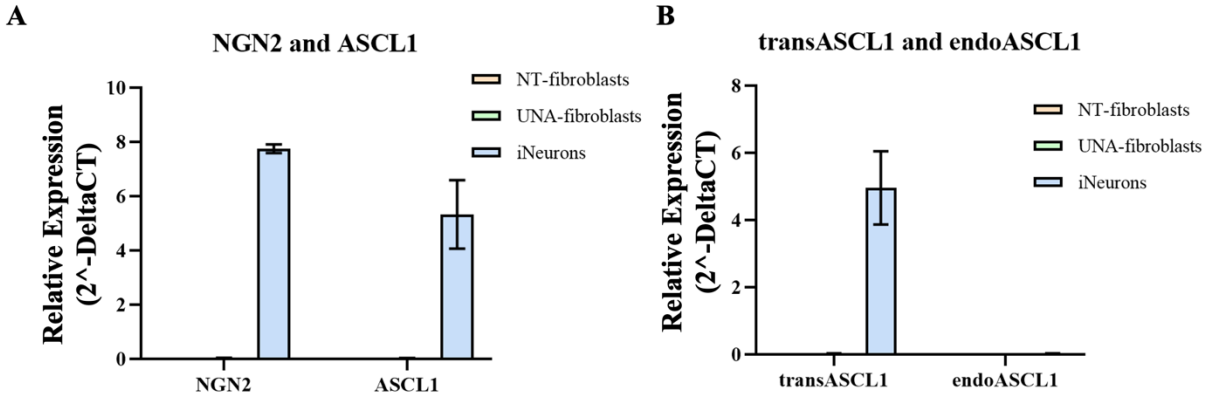


Figure 4.3 - Efficiency of lentiviral transduction of human skin fibroblasts. (A) – Expression levels of both NGN2 and ASCL1 – the transcription factors – in non-transduced (NT) fibroblasts, transduced fibroblasts (UNA-fibroblasts) and iNs at 21 days. (B) – Expression levels of transduced and endogenous ASCL1 genes in NT-fibroblasts, UNA-fibroblasts and iNs at 21 days. Error bars refer to standard error of the mean between values of the two cell lineages.

4.2.2. Markers of neuronal commitment

To monitor the progression of the differentiation process, phase contrast bright-field images were captured over the course of the 21-day differentiation period (**Figure 4.4 A**). Notably, fibroblasts exhibited significant morphological changes, gradually adopting a distinct neuronal morphology as the differentiation process unfolded (**Figure 4.4 A**).

We aimed to investigate the expression levels of specific genes associated with fibroblasts and neurons to gain insights into the differentiation process, by performing RT-qPCR to the two cell samples from adult donors. We analyzed the expression of vimentin (VIM), a well-known fibroblast marker, in all three cellular states. Additionally, we examined the expression of neuronal markers including βIII-tubulin (TUBB3), Microtubule-associated Protein 2 (MAP2) and Neuronal Nuclear (NeuN, or FOX-3), in iNs (**Figure 4.4 B-C**). Throughout the conversion process, the expression of vimentin gradually decreased across the three states of the cells, indicating a shift away from fibroblast characteristics. Notably, iNs exhibited significant expression of βIII-tubulin, indicative of successful differentiation into neuronal lineages. However, the expression levels of mature neuronal markers, such as MAP2 and NeuN, were not significantly detected in iNs (**Figure 4.4 C**).

To gain further insights into the developmental stage of the converting iNs, we assessed the expression of nestin (NES), a marker for neural stem cells, and S100B, to evaluate the faith of the cells and discard differentiation into astrocytes (**Figure 4.4 D-E**). We detected nestin expression in iNs, suggesting the presence of neural stem cell-like characteristics at 21 days. Importantly, the expression of S100B was not detected, indicating that cells were not committing to an astrocyte fate.

To have a more detailed understanding of the cellular morphology throughout the conversion process and to confirm the presence of characteristic markers for each cell state, we conducted immunocytochemistry on NT-fibroblasts and UNA-fibroblasts (**Figure 4.4 F**) as well as iNs at 21 days (**Figure 4.4 G** and **Supplementary Figure 1**). Non-neuronal cells, specifically fibroblasts, exhibited typical fibroblast morphologies along with the positive staining of vimentin. Conversely, after 21 days of differentiation, the morphology of the cells underwent noticeable changes, displaying distinct features of neuronal structures including cell bodies and neurites. Interestingly, within the same culture, there were cells that had not yet committed to a neuronal lineage and retained a morphology resembling "fibroblast-type" cells (indicated by the white arrow in **Figure 4.4 G**).

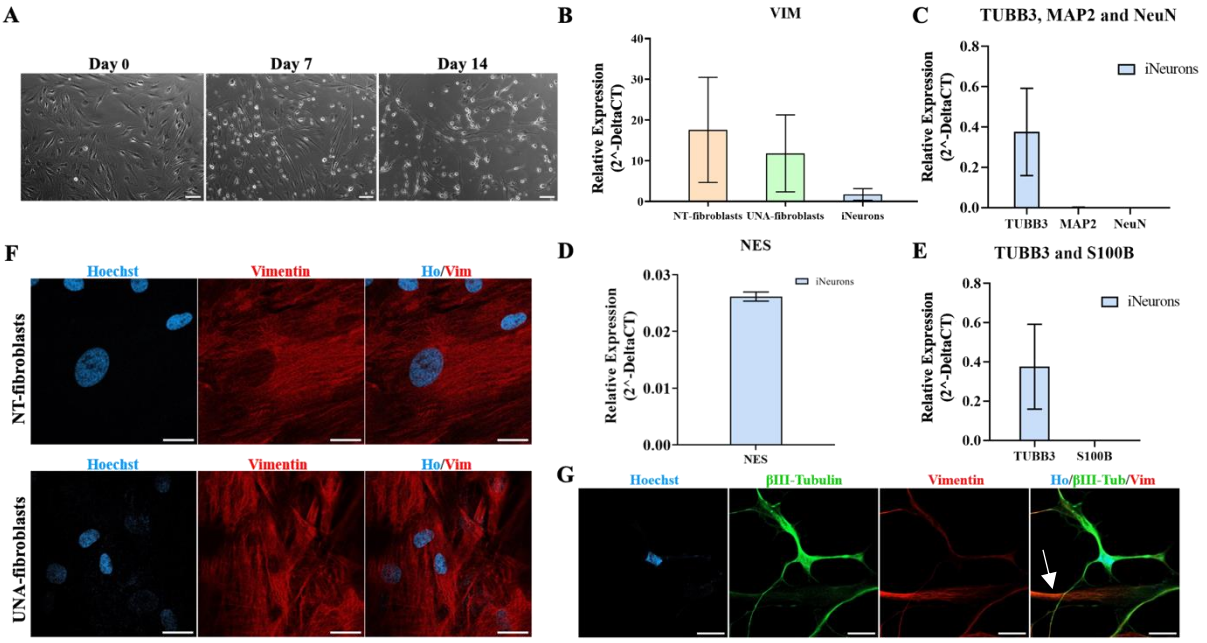


Figure 4.4 - Fibroblasts from adult donors can efficiently differentiate into iNs. (A) – Phase-contrast bright field pictures taken every 7 days throughout the differentiation process. Scale bars represent 100 µm. (B) – Expression levels of vimentin in the 3 states of the cells: NT-fibroblasts, UNA-fibroblasts and iNs at 21 days of differentiation. (C) – Expression levels of βIII-tubulin, MAP2 and NeuN in iNs at 21 days of differentiation. (D)- Expression levels of nestin in iNs at 21 days of differentiation. (E) – Expression levels of βIII-tubulin compared to S100B. (F) – Immunofluorescence images of NT-fibroblasts and UNA-fibroblasts staining for vimentin. Scale bars represent 25 µm. (G) – Immunofluorescence images of iNs at 21 days of differentiation staining for βIII-tubulin and vimentin. White arrow points to a cell with "fibroblast-like" morphology. Scale bars represent 25 µm. All error bars refer to standard error of the mean between values of the 2 cell lineages.

4.2.3. Markers of neuronal subtypes and assessment of synapse function

To investigate the neurotransmitter phenotype of iNs, we examined the expression of VGLUT1, a marker for glutamatergic neurons, glutamate decarboxylase 67 (GAD67), a marker for GABAergic neurons, and choline acetyltransferase (ChAT), a marker for cholinergic neurons. RT-qPCRs were performed on both samples from adult donors to assess the expression levels of these markers (**Figure 4.5 A**). The results revealed that at 21 days, iNs predominantly expressed VGLUT1 (Fold Change ≃1080). In contrast, the expression levels of GAD67 (Fold Change ≃ 11) and ChAT (Fold Change ≃ 1) were relatively low. This indicates that the combination of NGN2 and ASCL1 with the small molecule cocktail primarily resulted in a culture enriched in glutamatergic iNs. Immunocytochemistry was also performed on 21-day differentiated iNs (**Figure 4.5 B and C**). The staining revealed that the cells exhibited positive staining for VGLUT1, which is consistent with the RT-qPCR results, confirming their glutamatergic phenotype. Interestingly, immunocytochemistry also showed co-staining for GAD67, suggesting the presence of a subpopulation of iNs that exhibited both glutamatergic and GABAergic characteristics. Negative controls without the primary antibodies were performed to determine the specificity of the staining observed in the experimental samples with adult donors (**Supplementary Figure 2 A**).

In addition, we aimed to investigate whether there were any markers of synapses present at the end of the 21-day timepoint. To achieve this, we performed RT-qPCR analysis on iNs (**Figure 4.5 D**). Specifically, we examined the expression of SYT1, which is known to regulate neurotransmitter release at the synapse [145], as well as PSD-95 and SAP-102. PSD-95 and SAP-102 are members of the MAGUKs family and are crucial components of the PSD [146]. The results showed that only SYT1 was expressed by iNs, indicating the presence of this essential protein involved in neurotransmitter release

(**Figure 4.5 D**). Additionally, we confirmed the lack of immunocytochemical staining for PSD-95 in iNs (**Figure 4.5 E**).

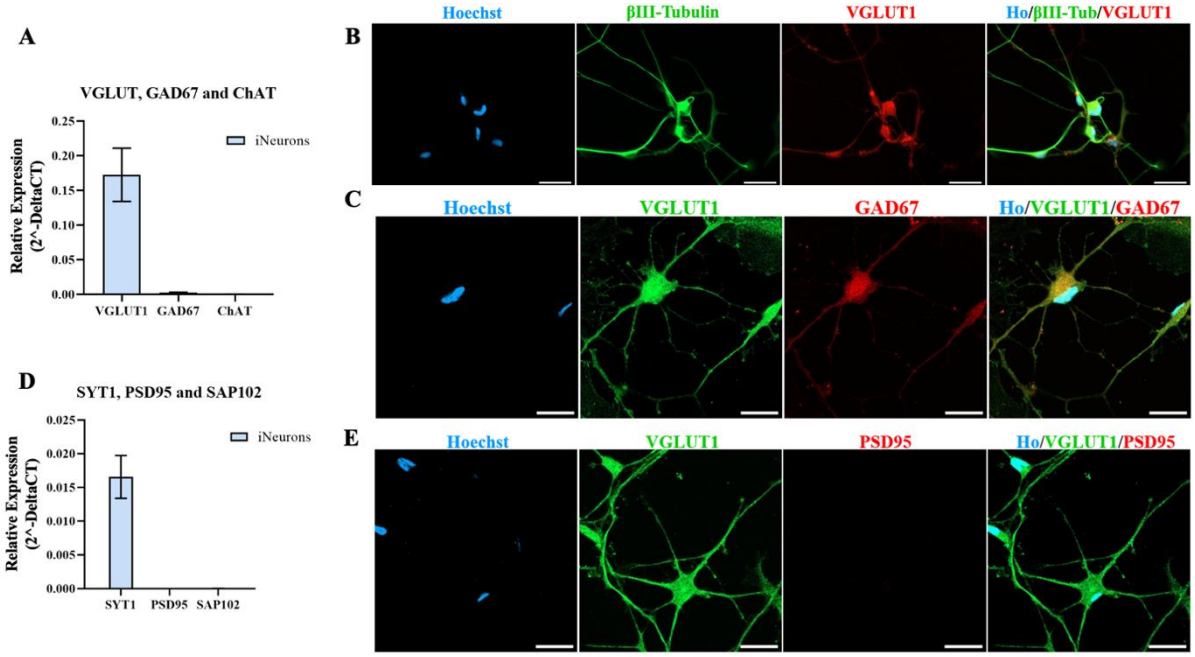


Figure 4.5 - Fibroblasts from adult donors differentiate mostly into glutamatergic iNs and possess few synaptic markers. (A) – Expression levels of VGLUT1, GAD67 and ChAT of iNs at 21 days of differentiation. (B) – Immunofluorescence images of iNs at 21 days of conversion for βIII-tubulin and VGLUT1. Scale bars refer to 50 μm. (C) – Immunofluorescence images of iNs at 21 days of differentiation for VGLUT1 and GAD67. Scale bars refer to 25 μm. (D) – Expression levels of SYT1, PSD-95 and SAP-102 of iNs at 21 days of differentiation. (E) – Immunofluorescence images of iNs at 21 days of differentiation for VGLUT1 and PSD-95. Scale bars refer to 25 μm. All error bars refer to standard error of the mean between values of the 2 cell lineages.

In order to study the intrinsic electrophysiological properties of individual 21 days differentiated iNs, as well as the synaptic connections between them, whole-cell patch-clamp experiments were performed to one cell sample from the younger donor (25 years old, see **Table 3.1**). It was possible to record iNs in this preparation (**Figure 4.6 A and C**). However, iNs failed to fire action potentials when injecting steps of increasing current (**Figure 4.6 B**), indicating once more that they were not fully matured at 21 days. Interestingly, membrane potential recordings of a single iNs showed some putative spontaneous excitatory post-synaptic potentials (sEPSPs) **(Figure 4.6 D)**, suggesting the presence of synapses between iNs in the preparation.

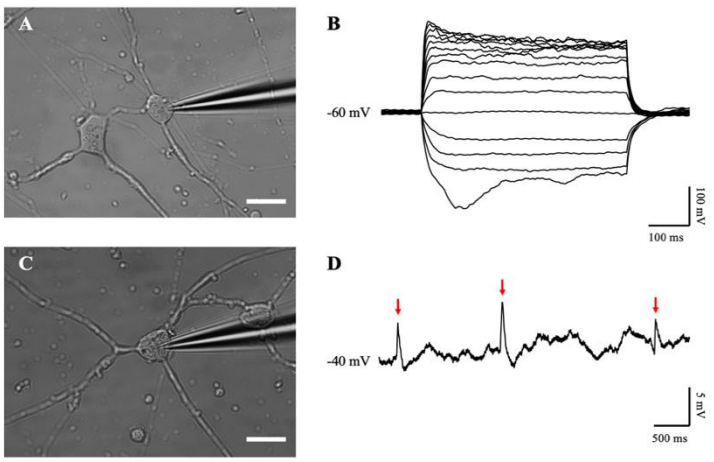


Figure 4.6 - 21 days-differentiated iNs failed to spike but received putative excitatory synaptic inputs. (A) and (C) – Images of iNs recorded using whole-cell patch-clamp. Scale bars represent 20 μm. (B) – Input/output curve of the iN recorded in (A). (D) – Membrane potential recording of the iN in (C). Red arrows point to putative spontaneous excitatory post-synaptic potentials (sEPSPs).

4.3. Direct conversion of human fibroblasts from an aged donor into iNs

To ensure the applicability of the direct conversion protocol to study aging perturbations, it was crucial to assess its effectiveness in aged-derived donor cells.

We examined the transduction efficiency and gene expression patterns of fibroblasts and iNs derived from an aged donor (see **3.1.1**), focusing on TFs and regulation of transduced and endogenous ASCL1 genes, in all three states of the cells. Similar to adult-derived cells, only aged-derived iNs expressed the TFs NGN2 and ASCL1(**Figure 4.7 A**). Transduced ASCL1 was specifically detected in aged-derived iNs (**Figure 4.7 B**).

Furthermore, we analyzed neuronal (**Figure 4.7 C**) and fibroblasts (**Figure 4.7 D**) markers and found that the expression pattern was comparable to adult-derived cells. At 21 days of differentiation, iNs primarily expressed βIII-tubulin, while MAP2 and NeuN were not observed, suggesting an immature stage of differentiation. Vimentin expression decreased throughout the processs and notably, nestin expression confirmed the immature stage of iNs (**Figure 4.7 E**). Additionally, we examined iNs subtype (**Figure 4.7 F**) and whether they could express any pre- (SYT1) or post- (PSD-95) synaptic marker (**Figure 4.7 G**). Aged-derived iNs were predominantly glutamatergic, as indicated by higher expression of VGLUT1 (Fold Change = 1223) compared to GAD67 (Fold Change = 3) and ChAT (Fold Change = 0.5). Among the tested synaptic markers, only SYT1 showed significant relevance.

To further examine the morphology of aged-derived iNs and validate the presence of specific markers, we conducted immunostaining using βIII-tubulin and vimentin (**Figure 4.7 H**), and VGLUT1 and GAD67 (**Figure 4.7 I**). Immunostaining revealed positive staining for βIII-tubulin, confirming the neuronal identity of aged-derived iNs. The presence of vimentin supported their fibroblast-to-neuron conversion. Interestingly, co-staining for VGLUT1 and GAD67 was observed in aged-derived iNs, despite the relatively low GAD67 gene expression, suggesting that a subset of iNs may exhibit co-expression of glutamatergic and GABAergic markers. Negative controls without the primary antibodies were performed to determine the specificity of the staining observed in the experimental samples with aged donors (**Supplementary Figure 2 B**).

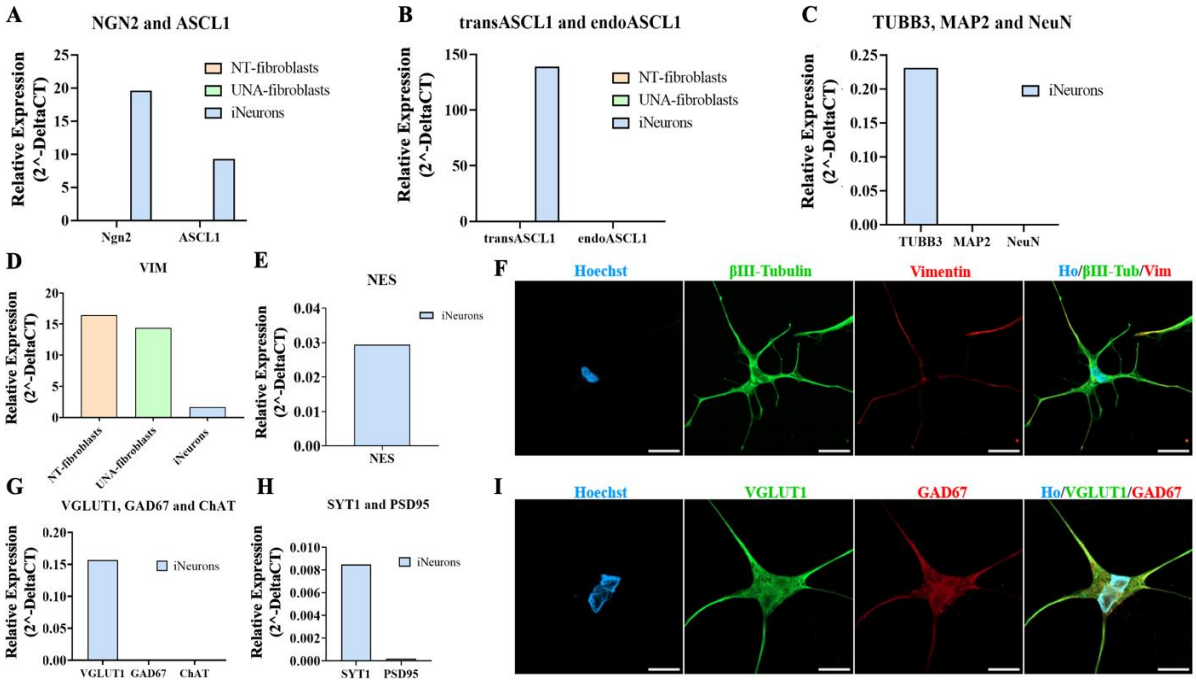


Figure 4.7 - Aged-derived fibroblasts can efficiently be converted into iNs. (A) – Expression levels of NGN2 and ASCL1 in NT-fibroblasts, UNA-fibroblasts and iNs at 21 days of differentiation, derived from an aged donor. (B) - Expression levels of transASCL1 and endoASCL1 in NT-fibroblasts, UNA-fibroblasts and iNs at 21 days of differentiation, derived from an aged donor. (C) - Expression levels of βIII-tubulin, MAP2 and NeuN in iNeurons at 21 days of differentiation, derived from an aged donor. (D) - Expression levels of vimentin in NT-fibroblasts, UNA-fibroblasts and iNs at 21 days of differentiation, derived from an aged donor. (E) - Expression levels of nestin in iNs at 21 days of conversion, derived from an aged donor. (F) - Expression levels of VGLUT1, GAD67 and ChAT in iNs at 21 days of differentiation, derived from an aged donor. (G) - Expression levels of SYT1 and PSD-95 in iNs at 21 days of differentiation, derived from an aged donor. (H) – Immunofluorescent images of aged-derived iNs at 21 days of differentiation for βIII-tubulin and vimentin. (I) - Immunofluorescent images of aged-derived iNs at 21 days of conversion for VGLUT1 and GAD67. Scale bars represent 25 μm.

5. Discussion

The primary objective of this project was to develop an enhanced model for studying age-related synaptic dysfunctions in humans. While there are existing conversion protocols in the literature for generating neurons, their practical implementation in the laboratory often presents undisclosed challenges. Therefore, it was essential for us to optimize a direct conversion protocol [12] that could consistently produce differentiated iNs and effectively use them in a high-throughput assay format.

Through our research, we found that the plating cell density to start the differentiation process had a significant impact on the protocol's effectiveness and suitability for downstream applications. To address this, we established an optimal fixed value for this parameter, ensuring consistent and reliable results. In addition, we set the best plating procedure for the intended application, such as patch-clamp experiments, to maximize the protocol's performance in the desired context.

We successfully obtained iNs from both adult (< 65 years) and aged (> 65 years) human skin fibroblasts, demonstrating the effectiveness of the ASCL1/NGN2 combination along with a cocktail of small molecules, regardless of the donor's age. However, the generated iNs did not exhibit complete maturation, which posed a limitation for our project. Our primary objective was to investigate age-related pathophysiological changes, which require fully developed neuronal subtypes displaying the specific characteristics of interest. Nonetheless, we managed to optimize the overall differentiation protocol, and in the future, we aspire to incorporate a maturation stage in our methodology that enable us to compare the primary mechanisms underlying age-related synaptic dysfunctions observed in animal models.

5.1. Plating cell density influenced the efficiency of differentiation into iNs

To initiate the differentiation process effectively, it was essential to establish an appropriate fibroblast density that ensured efficient neuronal commitment but also facilitated further applications. This was necessary because differentiating iNs did not proliferate due to cell cycle arrest induced by the TFs [147]. Once committed to a neuronal fate, the number of cells relied solely on fibroblast proliferation prior to differentiation. It is worth noting that UNA-fibroblasts exhibited high proliferative potential, and even though induction of differentiation with NC medium led to post-mitotic state entry, this transition is described in the literature to not be immediate [148]. In fact, cell proliferation gradually diminishes over time until reaching a terminally differentiated state [148]. Hence, it was critical to determine an initial plating density that accounted for UNA-fibroblasts' initial proliferative nature in differentiation stage.

To ensure an adequate quantity of iNs for downstream applications, it was necessary to seed initial UNA-fibroblasts at sufficiently high densities, favoring cell-cell contact during conversion, improving iNs survival and the overall conversion efficiency [12].

Through our results, we observed that plating a variable number of UNA-fibroblasts just before initiating the differentiation process had a significant impact on the outcome (**Figure 4.1 A**). Specifically, we found that low cell densities (25,000 cells/ml and 50,000 cells/ml) resulted in cultures of scattered iNs. Consequently, the neuronal networks exhibited low complexity and less development of important contact characteristics. Furthermore, when plating at such low densities, considering the presence of cell death throughout the differentiation process, the overall cell yield at the end of the 21-day period was low. This suggested that low densities were not suitable for efficient iN conversion, which aligns with existing literature findings. Previous studies have indicated that at low densities, cell lack the necessary cell-cell signaling, which negatively impacts the maturation of neuronal networks and hampers synapse formation [149]. Additionally, maintaining healthy differentiated neuron-like cells becomes more challenging due to reduced paracrine trophic support, as reviewed in [150].

On the one hand, when using considerably higher densities (100,000 cells/ml, 125,000 cells/ml and 150,000 cells/ml), we observed the formation of aggregations and cell clusters. These clusters were

likely a result of contact inhibition between cells, which interfered with and limited the process of differentiation. Consequently, the formation of cell clusters at high densities led to the detachment of the entire culture from the vessel surface, despite the presence of laminin in NC medium to provide an attachment substrate for the cells throughout the experiment (**Figure 4.1 B**). This is consistent with previous findings in the literature, where high-density cultures become overcrowded, making it challenging to distinguish individual neurites [148].

It was important to assess whether the cell clusters consisted of dead cells to determine the viability of high-density cultures. Staining the cells with PI, a dye that freely enters the cell membranes of dead or dying cells but is excluded from viable cells, revealed positive staining in the cell clusters (**Figure 4.1 C**). This indicated that the cell clusters were composed of cells with reduced viability, suggesting a potentially toxic environment that compromised the overall cell viability. In fact, studies have shown that at such high densities, nutrients in the medium are rapidly depleted, leading to lower cell viability [148].

Based on these findings, we determined that the cell density of 75,000 cells/ml was the optimal choice for initiating neuronal conversion. This value aligns with previous studies in the literature that used the same protocol, which recommend a cell density range of 57,000-95,000 cells/ml for the initiation of the differentiation process [108], [127]. By selecting this established density, we anticipated successful neuronal conversion while ensuring cell viability and the ability to generate cellular complexity.

5.1.1. Plating cell procedure influenced density for patch clamp experiments

In addition to cell density, the plating procedure played a crucial role in the successful differentiation of fibroblasts into iNs. The choice of materials used for cell culture vessels had a significant impact on several factors such as surface area, cell adhesion, and refractive indexes, and must be carefully considered depending on the further intended application.

For PCR experiments, cells were plated in TC-treated vessels, which provided optimal conditions for cell adhesion and growth during the differentiation process. In contrast, for ICC experiments, materials with refractive indexes suitable for the imaging system were employed to ensure accurate and high-quality imaging of the differentiated cells, while ensuring optimal cell adhesion.

However, when it came to electrophysiology patch-clamp experiments, the plating vessel had to fulfill three specific requirements: 1) the material's refractive index should be appropriate for imaging purposes, 2) the vessels should allow for adequate cellular adhesion throughout the entire duration of the differentiation process and 3) the dimensions of the plating vessel should be compatible with the electrophysiology recording chamber, ensuring a proper fit within the experimental setup.

The most straight forward approach would be to differentiate neurons in TC-treated vessels and subsequently harvest and plate them into glass coverslips that are compatible with the recording chamber and possess the appropriate refractive index for imaging and cell visualization. For this reason, the first approach to deliver cells for patch-clamp experiments involved re-plating converted iNs into Geltrex-coated coverslips after a minimum of 21 days of differentiation in TC-treated vessels. This was the technique suggested and used by Dr. Jerome Mertens and colleagues in one of their studies [104]. However, the harvesting process after the 21-day differentiation period has posed limitations to this approach. Although the final cell density appeared satisfactory, the delicate and specialized neuronal structures of the converted iNs have proven to be extremely sensitive and susceptible to structural damage during the culture process [148]. Attempting to detach iNs using TrypLE resulted in irreparable loss of cellular material, leading to the cultivation of scattered iNs without preserved complexity or visible cell-cell connections (**Figure 4.2 A**). While TrypLE is known for its superior stability compared to crude or purified trypsin (its main constituent) [151], similar outcomes were observed to those reported in a previous study that used trypsin as a dissociating reagent, resulting in dissociated cultures [148]. In fact, the mentioned study compared several dissociating reagents, including gentle cell dissociation reagent (GCDR), versene, accutase and dispase. Although trypsin caused dissociated

cultures, it remained the most effective reagent for detaching a population of a human-derived cell line [148].

Although the method for detaching iNs proved to be problematic for further applications, we explored an alternative approach after iNs had completed 21 days of differentiation. We attempted to use Nunc Multidishes with UpCell Non-Enzymatic Surface (Thermo Fisher, cat. no. 174899), which enable non-enzymatic cell harvesting while maintaining high viability and intact surface proteins [152]. This vessel works by promoting cell adhesion at 37 ºC due to its hydrophobic surface, and facilitating cell detachment when the temperature is reduced below 32 ºC, causing the surface to become hydrophilic. Our intention was to detach iNs from this vessel by lowering the temperature in the cell culture room and then re-plate them on poly-dl-ornithine (PORN)/laminin coated-coverslips. However, when we decreased the temperature to about 23 ºC and waited for an hour (the time recommended for temperatures of 20-25 ºC was 15-40 minutes [153]), periodically monitoring under a microscope to observe the cells, they remained attached to the surface. Although we managed to gently resuspend the cells, once re-plated on PORN/laminin-coated coverslips, they had already lost their complex morphology, and the following day we confirmed their viability had been compromised, leading to cell death.

Since harvesting the cells clearly compromised our ability to study the electrophysiological properties of these neurons, we tried to overcome the effects of re-plating, maintaining a suitable set-up for patch-clamp experiments. To achieve this, we directly seeded UNA-fibroblasts in µ-Dishes 35 mm low, anticipating efficient differentiation with an optimal cell density and complexity. We selected these dishes due to their features, including a polymer coverslip bottom for cell visualization, a TC-treated surface promoting cell adhesion and growth, and low walls suitable for microelectrode placement [154]. As observed in **Figure 4.2 B**, when cells were plated in µ-Dishes 35 mm low, they successfully completed the entire differentiation process, resulting in a higher cell yield after 21 days. Based on these findings, we confidently concluded that this approach represented the optimal method for differentiating cells for patch-clamp experiments.

5.2. Direct conversion of human fibroblast from adult donors into iNs

To evaluate the efficiency of the conversion process, it was crucial to assess not only the successful expression of the TFs mediated by lentiviral transduction but also the effectiveness of the differentiation process itself that occurred in the presence of small molecules and doxycycline.

To begin, we examined the expression of NGN2 and ASCL1 in the three cellular states to understand how their expression changed during the conversion process. While UNA-fibroblasts represented the cells that had already been transduced with the lentivirus, it was important to note that the activation of the lentiviral system and subsequent gene expression depended on the presence of doxycycline. The Tet-On system, used to regulate gene expression, requires the presence of doxycycline as an inducer to activate the lentiviral system and subsequent gene expression. Consequently, it was not surprising that only iNs exhibited expression of both TFs (**Figure 4.3 A**). To confirm that the expression of ASCL1 originated from the lentiviral transduction and not from an endogenous source, we evaluated the presence of transduced ASCL1 (transASCL1) and endogenous ASCL1 (endoASCL1) (**Figure 4.3 B)**. As expected, only transASCL1 was expressed in iNs, providing evidence that the expression of ASCL1 in these cells resulted from the lentiviral transduction process.

After a 21-day timepoint in the presence of NC medium, we reached the initial stage of iN conversion, as described by Mertens and colleagues [12]. Therefore, at the end of this timepoint, we examined whether the expression of fibroblast markers persisted and if the converted cells exhibited the presence of established neuronal markers.

Vimentin, a fibroblast intermediate filament, is a prominent structural protein found in several non-muscle cells, including fibroblasts, endothelial cells, macrophages, and melanocytes [155]. Therefore, the presence of vimentin expression in both NT and UNA fibroblasts, as confirmed by RT q-PCR (**Figure 4.4 B)** and ICC (**Figure 4.4 F**) was not surprising. As the cells underwent differentiation into

iNs over the course of 21 days, a subsequent decrease in vimentin expression was anticipated. However, we can still observe that iNs express vimentin, which may indicate a non-mature stage of the cells. The morphology of fibroblasts (**Figure 4.4 F**) was characteristic, featuring larger, flat, and elongated cells with extended processes and oval-shaped nuclei [156]. Notably, no significant morphological differences were observed between NT-fibroblasts and UNA-fibroblasts since the absence of doxycycline in the medium did not induce any phenotype change in the cells.

βIII-tubulin is a vital protein involved in the organization of microtubules within cells, specifically in neurons. It plays a significant role in the growth of specialized extensions of nerve cells, such as axons and dendrites [157]. In addition, the expression of βIII-tubulin is closely associated with the early stages of neuronal differentiation [158]. In our study, we observed the expression of βIII-tubulin in iNs at 21 days of differentiation (**Figure 4.4 C**), providing evidence that these cells were undergoing differentiation into neurons. Furthermore, positive staining of βIII-tubulin, particularly in structures resembling neurites, further supported this notion (**Figure 4.4 G** and **Supplementary Figure 1**) and enabled us to calculate the efficiency of the direct conversion process in adult-derived samples: ≈76% (**Supplementary Figure 1**). MAP2 is a protein found in neurons throughout the nervous system, contributing to the assembly and stabilization of microtubules [159]. NeuN, on the other hand, is a neuron-specific protein that binds to DNA and is restricted to the nervous system. It serves as a marker for mature neuronal cell types [160]. The absence of expression for both MAP2 and NeuN in iNs indicated that although cells were transitioning into a neuronal state, they had not reached a mature stage by the 21-day timepoint (**Figure 4.4 C**).

Nestin is typically expressed in neural stem cells during the early stages of development and is commonly used as a marker for neuronal stem cells [161], [162]. The expression of nestin in 21-days differentiated iNs (**Figure 4.4 D**) also suggested that the cells were indeed differentiating into neurons but were still in an early developmental stage. Neural stem cells possess the ability to give rise to several cell types, including neurons, astrocytes, and oligodendrocytes [163]. Considering the expression of nestin in our iNs at 21 days, it would be possible that these cells were not only differentiating into neurons (as confirmed by βIII-tubulin expression) but also potentially undergoing differentiation into other cell types, such as astrocytes. To investigate this possibility, we examined the expression of S100B (**Figure 4.4 E**), a gene primarily expressed in more developed astrocytes, and we found that iNs did not express this marker.

To gain insights into the neurotransmitter phenotype of the differentiated iNs, it was crucial to determine the types of neurons they were differentiated into. Our experimental approach was designed to primarily yield glutamatergic neurons, with a smaller fraction of GABAergic neurons [12]. To confirm whether out results aligned with this expectation, we conducted RT-qPCR experiments to examine the neurotransmitter markers in 21-day differentiated iNs (**Figure 4.5 A**). Consistent with previous studies employing similar methodologies [104], [127], our findings revealed that most of iNs were glutamatergic, with a minor fraction expressing GABAergic markers. Furthermore, the expression of cholinergic markers was minimal. Immunocytochemistry analysis provided additional confirmation of the neurotransmitter phenotype of the 21-day differentiated iNs (**Figure 4.5 B)**. However, co-staining for both glutamatergic and GABAergic markers was detected (**Figure 4.5 C**). While we anticipated a small fraction of neurons to exhibit GAD67 staining, we were surprised to observe co-localization of VGLUT1 and GAD67 in the exact same regions. GAD67 is a key enzyme involved in GABA production in neurons [164]. This co-staining pattern may also suggest that at 21 days, the cells had not reached a fully mature state where a distinct neuronal identity had been established. Although there might be some protein expression in these cells, our inference from RT-qPCR data suggested that VGLUT1 is the gene transcribed in greater abundance after 21 days of differentiation, consistent with a predominant glutamatergic phenotype. The specificity of the labeling for VGLUT1 and GAD67 was confirmed through negative controls (**Supplementary Figure 2 A**). In addition, it would be interesting to find out whether this co-localization pattern would be maintained by using other GABA-specific antibodies, since Dr. Mertens and colleagues have been using anti-GABA antibody in their experiments [108].

Since synaptic function and neurotransmitter release are regulated by specific proteins, it was also of greater importance to characterize the generated iNs according to their ability to express synaptic proteins. This analysis would also provide insight into the developmental stage of the iNs at 21 days.

Specifically, we investigated the expression of key synaptic proteins, including SYT1, PSD-95 and SAP-102. The process of synapse development involves distinct molecular events that can be divided into two stages: synaptogenesis and synapse maturation [165]. Synaptogenesis involves the recruitment of pre- and post-synaptic proteins to initiate chemical communication and facilitate fast synaptic transmission [165]. SYT1, a pre-synaptic protein, plays a crucial role in mediating the release of calcium-triggered neurotransmitters [166] and is expressed in several neuron types before the onset of synaptogenesis [167]. PSD-95 and SAP-102 belong to the MAGUKs family, and are essential proteins located in the post-synaptic density. They play vital roles in clustering and anchoring glutamate receptors and other proteins at synapses [168]. SAP-102 is involved in the synaptic trafficking of AMPA and NMDA receptors during synaptogenesis, while PSD-95 is known to have a more prominent role in later stages of synapse maturation rather than during the initial stages of synaptogenesis, as reviewed in [165]. Considering this information, our RT-qPCR and immunocytochemistry data indicated that iNs were in a developmental stage even before the initiation of synaptogenesis. This was supported by the absence of PSD-95 expression and staining (**Figure 4.5 D** and **E**), as well as the lack of SAP-102 expression (**Figure 4.5 D**), that is expected during synaptogenesis. The presence of SYT1 expression (**Figure 4.5 D**) aligned with its known expression before synaptogenesis occurs. Moreover, according to previous studies, pre-synaptic differentiation typically precedes post-synaptic differentiation during synaptogenesis [169].

To functionally characterize and evaluate the electrical properties of 21-day differentiated iNs, we conducted whole-cell patch-clamp experiments. The patch-clamp technique involves the use of a glass micropipette to penetrate the cell membrane and establish a connection between the neuron's cytoplasm and an electrode inside the micropipette [170]. Initially, we examined the ability of iNs to generate action potentials (**Figure 4.6 A**) by gradually increasing the injected current (**Figure 4.6 B**). It was observed that iNs did not have the ability to spike upon any injection of current, as it would be expected from a mature neuron (**Figure 4.6 B**). On the other hand, when monitoring the membrane potential of another iN without applying any current (**Figure 4.6 C**), we detected some putative potential spontaneous excitatory post-synaptic potentials (sEPSPs) (red arrows in **Figure 4.6 D**). Several explanations could account for these observed events. One possibility is that the recorded iN was receiving synaptic inputs and establishing connections with other iNs. However, considering the indications that these cells were still at an immature stage, we would not expect them to have already established functional synaptic transmission between each other. In the literature, it is described that prior to synapse formation, there can be irregular release of neurotransmitters from developing pre-synaptic terminals, and this electrical activity may play a role in the early stages of synaptogenesis, as well as synapse maturation and maintenance [169]. Thus, the observed sEPSPs might result from this irregular release of neurotransmitters, which could contribute to the subsequent maturation of iNs. In fact, previous studies have found that spontaneous GABA-receptor-mediated synaptic transmission are dominant over glutamate-mediated activity during early development, and that GABA-containing synapses are thought to be the first to form, as reviewed in [172]. It has also been suggested that, in neonatal hippocampal neurons, GABA acts on its receptors to provide most of the excitatory drive, while glutamatergic synapses remain relatively quiescent [173]. Considering this, we hypothesize that the observed sEPSPs in our iNs might arise from an irregular release of GABA, which could be required for the developmental processes and maturation of neurons. To confirm whether these putative sEPSPs were indeed a result of irregular GABA release, specific antagonist drugs targeting glutamatergic and GABAergic neurotransmission could be employed.

5.3. Direct conversion of human fibroblasts from an aged donor into iNs

The primary goal of this project was to establish a comparative model for studying age-related dysfunctions, by generating iNs from donors with different ages. To achieve this, we aimed to determine the feasibility of applying the direct conversion protocol to fibroblasts obtained from an older, cognitively healthy donor.

The literature highlights that a higher chronological age of the donor present limitations regarding the rapid expansion and early senescence of aged-derived cells in culture [174]. This suggests that aged

cells may not reach the same level of confluence as adult-derived cells, posing a potential risk when implementing the conversion protocol [174]. This limitation is particularly relevant given the demanding and stressful procedures involved in the conversion process. These procedures included lentivirus transduction and the initiation of the 21-day differentiation process, which required cells to maintain an adequate density, as described above.

Despite the inherent limitations and risk factors involved, we successfully achieved the differentiation of aged-derived fibroblasts into iNs. We observed that the proliferation rate of these cells was slower, and they exhibited a lower maximum confluence compared to younger-derived fibroblasts, resulting in a lower cell density when reaching confluence. Nevertheless, we efficiently transduced aged-derived fibroblasts with the lentivirus (**Figure 4.7 A** and **B**), leading to a similar pattern of TF expression compared to adult-derived fibroblasts (**Figure 4.3**). During the 21-day differentiation process, aged-derived iNs displayed a developmental window similar to that of adult-derived iNs, as βIII-tubulin was prominent, while they did not express either MAP2 or NeuN (**Figure 4.7 C**), indicating an early stage of neuronal differentiation. The morphological changes observed, such as pyramidal somas, condensed nuclei and growth of neurites further indicated neuronal development (**Figure 4.7 F**). As the conversion progressed, vimentin expression decreased while nestin was expressed by aged-derived iNs, suggesting a commitment to neuronal lineage, but indicating a premature stage (**Figure 4.7 D** and **E**). Similar to adult-derived iNs, aged-derived iNs exhibited a predominantly glutamatergic profile (**Figure 4.7 G**) and positive staining for GAD67 (**Figure 4.7 I**). In terms of synaptic markers, only SYT1 was expressed (**Figure 4.7 H**), indicating a developmental window before synaptogenesis, as discussed before.

Although it would have been interesting to perform whole-cell patch-clamp experiments to analyze the behavior of these aged-derived iNs, the extended time required for their expansion and subsequent 21-day differentiation precluded us from conduct these experiments within the available timeframe. Additionally, while we had two samples of adult-derived fibroblasts, unfortunately we were only able to proceed with one sample from an aged donor (chronological age > 65 years). We initially had a second sample from another aged donor; however, we faced an issue with contamination in all the vials containing the cells, preventing us from proceeding with the cell culture. It is important to acknowledge that having a larger sample size of aged donors would have significantly strengthened the robustness of our findings, specifically in representing the older age group. Obtaining data from multiple aged donors would have allowed us to assess the reproducibility and consistency of the results, thereby enhancing the reliability of our conclusions.

This project intends to be a significant advancement in the study of patient-specific pathologies, particularly age-related synaptic dysfunctions. The steps undertaken within the scope of this thesis were important to establish a reproducible model for further research. However, it is crucial to acknowledge that there are still certain aspects that require attention and improvement for this model to effectively serve as a template for studying age-related synaptic dysfunctions in humans.

One significant limitation of our study corresponds to the sample size. It is essential to expand the number of samples of adult and aged-derived skin fibroblasts to achieve a more comprehensive and representative investigation. This is particularly crucial considering the genetic heterogeneity observed in humans. In fact, one of the key observations in our study was the variability between samples in RT-qPCR data (shown by error bars), highlighting the importance to increase the samples to better assess the consistency and reproducibility of our findings. Furthermore, in addition to consider the chronological age and cognitive health of the donors, it is imperative to consider their clinical condition. It is plausible that other underlying disturbances or conditions might influence the behavior and characteristics of the cells. Therefore, incorporating information regarding the clinical condition of the donors will provide a more comprehensive context for interpreting and generalizing the results.

Considering our collective findings from both adult- and aged-derived iNs, we concluded that these cells were at an immature stage, potentially within a developmental window prior to synaptogenesis. This conclusion is supported by several observations in 21-day differentiated iNs: the continued expression of vimentin, the absence of mature neuronal markers such as MAP2 and NeuN, the lack of post-synaptic markers like SAP-102 or PSD-95, the co-expression of both VGLUT1 and GAD67 and

the inability to spike. These findings pose a limitation to our study, as the investigation of age-related synaptic dysfunctions requires the study of several proteins and receptors involved in synaptic transmission. For instance, it would be interesting to compare the levels of $A_{2A}R$ in adult-derived iNs with those in aged-derived iNs to determine if there is an increase, as suggested in the literature. Furthermore, we could explore the potential aberrant functioning of NMDAR, which could lead to an increase in calcium influx, and examine if there is any correlation with $A_{2A}R$ levels. Additionally, considering that overexpression of PSD-95 is associated with cognitive stability and higher levels of SYT1 are linked to less cognitive decline over time [30], it would be valuable to monitor these markers and determine if our findings align with the existing literature.

When comparing our findings with a study by Mertens and colleagues (2015), we observed differences in the maturation timeline and functional properties of iNs [104]. In fact, in their study, iNs expressed MAP2 and NeuN around 6 weeks after conversion, and electrophysiological characterization showed the presence of multiple evoked and spontaneous action potentials after 8 weeks of co-culture with astrocytes [104]. In contrast, our results did not align with these findings which could potentially be attributed to the absence of astrocytes in our culture system. Previous research has indicated that culturing neurons without astrocytes results in fewer functional synapses compared to co-culturing with astrocytes. This suggests that astrocytes secrete essential factors for synaptic development, as reviewed in [175]. In addition to co-culturing with astrocytes for maturation, another approach described the use of a maturation medium containing small molecules that promote neuronal maturation and survival [12]. A further study apparently following the same protocol was able to generate iNs staining for NeuN following 3 weeks of conversion, without switching the medium or co-culturing on astrocytes [108]. This discrepancy further emphasizes the importance of verifying the reproducibility among protocols and optimizing various steps for successful iN generation. Therefore, it is of greatest interest to study maturation prospects for these cells so that they continue to develop and acquire characteristics that identify them as more mature neurons. We hope that by maturating these iNs we can study age-related perturbations.

In addition to include a maturation stage, it would be important to increase the timepoints for following-up the process in RT-qPCR and immunocytochemistry assays. In this study, we collected iNs at 21 days of differentiation, however, expanding the timepoints would provide valuable insights into the dynamic changes occurring during earlier stages of differentiation. For instance, adding a timepoint after 21 days of differentiation (ideally after a maturation stage) would help to clarify whether the protein co-expression of GAD67 and VGLUT1 is maintained or not, as it was hypothesized that this happened due to lack of iNs maturity.

It would also be interesting to confirm whether aged-derived iNs retained transcriptomic and functional signatures of aging, as this is a major advantage of direct conversion over iPSC-based models according to the literature [104]. Through combining state-of-the art electrophysiology, transcriptomics, and computational biology, it would be interesting to discriminate individual cognitive trajectories based on patient-specific synaptic signatures. Specifically, in addition to look for synaptic markers, it would be important to look for DNA damage and senescence markers in aged-derived iNs.

Cell culture contaminations pose significant challenges in experimental studies, and despite adopting preventive measures, they can occasionally occur and disrupt research progress. During the course of this study, we faced some contaminations that caused the loss of valuable time, effort, and even a cell line from an aged donor. These unforeseen circumstances limited our ability to implement a maturation stage and to incorporate additional timepoints throughout the experimental process within the available timeframe for this study.

All gathered, despite all the limitations, through the adaptation and optimization of Dr. Mertens and colleagues' protocol [12], we were able to efficiently convert human skin fibroblasts to neuron-like cells by subject them to a lentiviral transduction to deliver two TFs: ASCL1 and NGN2, combined with a 21-day differentiation with small molecules. This was valid for our samples of adult-derived fibroblasts (n=2) and aged-derived fibroblasts (n=1), with about 8-10 independent cultures being performed for each sample.

6. Conclusion and Further Perspectives

Through the adaptation of Dr. Jerome Mertens and the Gage lab.'s direct conversion protocol [12], that combines the delivery of ASCL1 and NGN2 with a cocktail of small molecules, this study successfully established an efficient and stable model for producing iNs from skin fibroblasts of both adult and aged donors. However, despite this achievement, it was acknowledged that further improvements are necessary for the maturation of iNs. As 21-day differentiated iNs showed no maturation features, different maturation strategies will be explored.

Particularly, co-culturing iNs with different partners such as mouse/rat primary astrocytes, human primary astrocytes or immortalized human astrocytes will be evaluated. In fact, mouse and rat primary astrocytes are already available in culture, and different plating strategies will be investigated to determine the most effective approach.

Furthermore, agreements have already been made to acquire additional human skin fibroblast samples. By optimizing and adapting the protocol used in this study and developing new maturation prospects in a larger sample size, the next goal will be to explore age-related synaptic dysfunctions in iNs and to investigate their comparability to animal models.

In conclusion, this study successfully developed an efficient and stable model for iN production while identifying areas for future improvement. Through the exploration of alternative maturation strategies and an increased sample size, this study aims to shed light on age-related synaptic dysfunctions in iNs and their comparability to animal models.

7. Supplementary Data

Tables

Supplementary Table 1- Identification of culture media used in the process of fibroblasts expansion (TFM and TFM-P) and in the differentiation process (NC medium).

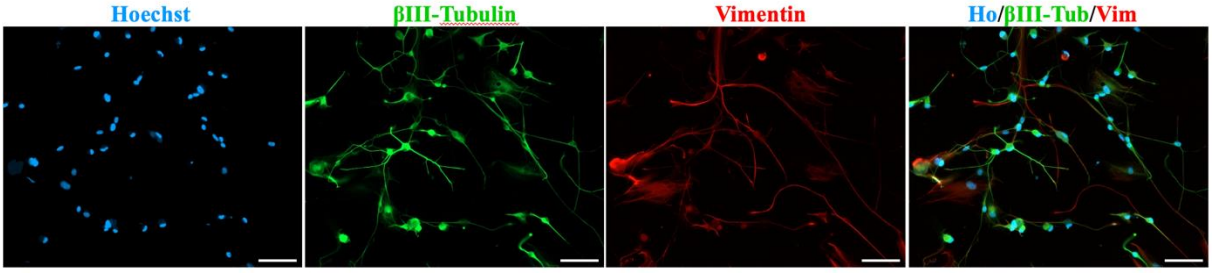
Name	Components	Concentration	Catalog number
TFM	Dulbecco's Modified Eagle Medium (DMEM)	-	Thermo Fisher Scientific, 21969035
	Fetal Bovine Serum (FBS)	10%	Thermo Fisher Scientific, 16000044
	Non-Essential Amino Acid Solution (NEAA)	1%	Thermo Fisher Scientific, 11140050
TFM-P	Dulbecco's Modified Eagle Medium (DMEM)		Thermo Fisher Scientific, 21969035
	Fetal Bovine Serum (FBS)	10%	Thermo Fisher Scientific, 16000044
	Non-Essential Amino Acid Solution (NEAA)	1%	Thermo Fisher Scientific, 11140050
	Puromycine	1 µg/ml	InvivoGen, ant-pr-1
Neuronal conversion (NC) medium	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM:F12)	-	Thermo Fisher Scientific, 11320074
	Neurobasal	-	Thermo Fisher Scientific, 21103049
	B-27	1x	Thermo Fisher Scientific, 17504044
	N-2	1x	Thermo Fisher Scientific, 17502048
	Laminin	1 µg/ml	Merck, 11243217001
	Doxycycline	2 µg/ml	Merck, D9891
	db-cAMP	400 µg/ml	Biogen, TO-1141/10 MG
	Noggin	150 ng/ml	Stem Cell, 78060
	LDN-193189	0.5 µM	Stem Cell, 72147
	A83-1	0.5 µM	Frilabo, sc-203791
	CHIR-99021	3 mM	Stem Cell, 72054
	Forskolin	5 µM	Biogen, TO-1099/10 MG
	SB-431542	10 µM	Stem Cell, 72234

Supplementary Table 2 - RT-qPCR primer-probe sequences.

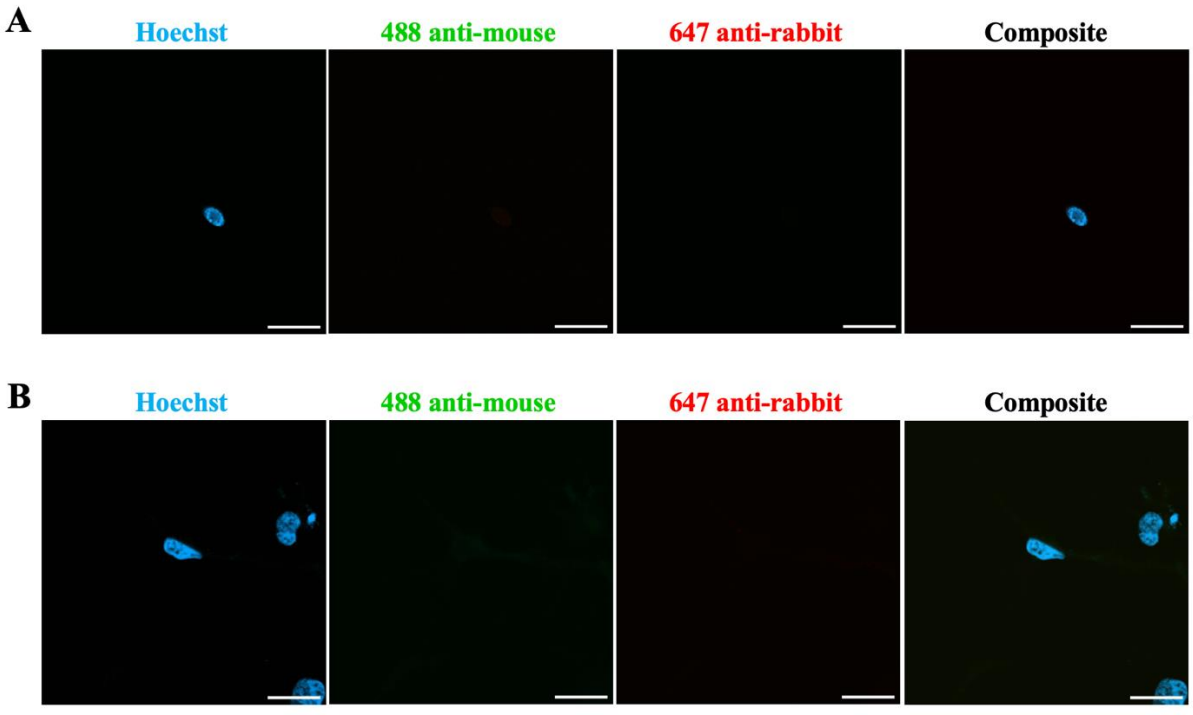
Gene	Target gene	Organism	Forward Primer	Reverse Primer
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Human	GACAGTCAGC CGCATCTTCT	GCGCCCAATA CGACCAAATC
ACTB	Actin-β	Human	ACAGAGCCTC GCCTTTGCC	TGGGGTACTT CAGGGTGAGG
TUBB3	Tubulin β 3 class III	Human	CAACCAGATC GGGGCCAAGT T	CGAGGCACGT ACTTGTGAGA A
NeuN	RNA binding fox-1 homolog 3	Human	CCTTGACTTC GGTCCTGGAG	TCTGGGCTCT CTGTTTGCAG
MAP2	Microtubule associated protein 2	Human	GCTCCCGGAG AAGGATTCTG	AGTGAGGTGC CTTTGCTTCA
VGLUT1	Solute carrier family 17 member 7	Human	AGCTGGGATC CAGAGACTGT	CCGAAAACTC TGTTGGCTGC
GAD67	Glutamate decarboxylase 1	Human	GGGAACTAGC GAGAACGAG G	GGGCGCAGGT TAGTGGTATT
ChAT	Choline O-acetyltransferase	Human	AAATGCTGAG CTAGGGGCAG	ACCCGACTGG AGAAAGCAA G
VIM	Vimentin	Human	GGACCAGCTA ACCAACGACA	AAGGTCAAGA CGTGCCAGAG
NES	Nestin	Human	GTAGCTCCCA GAGAGGGGA A	CTCTAGAGGG CCAGGGACTT
S100B	S100 Calcium Binding Protein B	Human	ACAAGGAAG AGGATGTCTG AGC	GCCGTCTCCA TCATTGTCCA
SYT1	Synaptotagmin 1	Human	GACTGTTCTG CCAAGCAACG	TCCTGATCTTT CATCGTCTTC CC
PSD-95	Post-synaptic Density Protein 95	Human	CTGCATAGCG ACCTCTTCCA	CCCGTTCACC TGCAACTCAT A
SAP-102	Synapse-Asssociated Protein	Human	CATGCACAAG CACCAGCAC	GAGCTGTAGC CCCCATAACC
ASCL1	Achaete-Scute Family BHLH Transcription Factor 1	Human	CCAAGCAAGT CAAGCGACAG	TAGCCAAAGC CGCTGAAGTT
NGN2	Neurogenin 2	Human	TGGGTCTGGT ACACGATTGC	GGGTCTCGAT CTTGGTGAGC

endoASCL1	Endogenous Achaete-Scute Family BHLH Transcription Factor 1	Human	AGCAGGAGCTTC TCGACTTCACCA	TGCAGGTTGTGCG ATCACCCT
transASCL1	Transduced Achaete-Scute Family BHLH Transcription Factor 1	Human	AGCAGGAGCTTC TCGACTTCACCA	AAGCGCATGCTC CAGACTGCC

Figures



Supplementary Figure 1 - Immunofluorescence images of iNs at 21 days of conversion for βIII-tubulin and vimentin. The efficiency of the protocol was calculated by counting the number of cells stained positive for βIII-tubulin and dividing it by the total number of cells. Scale bars represent 100 µm.



Supplementary Figure 2 - Negative controls made exclusively with the secondary antibodies, to test specificity of primary antibodies. (A) – Negative control made within the sample of adult-derived iNs. (B) – Negative control made within the sample of aged-derived iNs. Scale bars represent 25 µm.

8. References

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