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# Adult mammalian central nervous system regeneration

By

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# 1.1 Preface:

The Wisconsin Initiative for Science Literacy (WISL) is an initiative to disseminate scientific research to the general public. It encourages Ph.D students to write a chapter of their thesis for a general audience. I am thrilled to participate in this initiative as I have found science communication to be an essential aspect of my time in graduate school, clinical work, and my future career as a physician-scientist. In the last four years of working as a graduate student in the Department of Neuroscience, at the University of Wisconsin-Madison, I have contributed to the understanding of central nervous system regeneration. Specifically, the project described in this chapter focuses on understanding the underlying processes of spinal cord injury with the goal of finding potential therapeutic targets.

Here I want to emphasize that rarely does one study or advancement lead to a clinical therapeutic breakthrough. The findings of every study are years of work condensed into a single drop of in the bucket. While an individual drop is not enough to tip the scale, eventually when there are enough drops in the bucket, it will tip the scale leading to a medical breakthrough such as the discovery of HIV treatments. In the grand scheme of spinal cord injury and axon regeneration research, our findings in this study are just one drop in the bucket toward finding a treatment for spinal cord injuries. However, to achieve this, every single drop is critical.

# 1.2 Introduction

Spinal cord injury is a devastating condition often resulting in partial or complete paralysis. This is mainly due to the inability of the spinal cord to repair itself after injury. The goal of one arm of my thesis projects is to find molecular players that can increase spinal cord repair after injury. In this chapter, we will first discuss the anatomy and function of the central nervous system (CNS), the structures and functions of the cells that make up the CNS, and finally we will discuss the major finding from my thesis about a molecule that can alter the ability of the spinal cord to repair itself.

# 1.3 Background

## What is the central nervous system?

The brain and spinal cord are both part of the CNS. The brain receives information, mainly sensory, delivered by the spinal cord, interprets it, and then sends information, mainly motor, back through the spinal cord to various parts of the body. An analogy would be the brain acting as the city center and the spinal cord contains information highways to and from the control center and the periphery of the city.

The cells of the brain and spinal cord are organized based on their function. (Fig. 1A). Consider the brain as a hospital building. Inside, there are areas designated for specific functions, such as the family medicine department, the labor ward, or the emergency department. These distinct sections within the hospital correspond to specific areas within the brain important for coordinating unique and specific functions. For example, there is a region of the brain called the motor cortex, which is the main area for coordinating motor functions such as flexing a bicep muscle (Fig. 1A-B).

In this area, cells cluster based on the body part they control. Thus, all the cells coordinating movements for your fingers will be spatially located in a different area than the cells coordinating movements for your biceps. Interestingly, the organization of the motor cortex reflects the distance between your body parts and the brain. An example of that would be the cells that coordinate toe movements are closer to the motor cells of your ankle than they are to the motor cells of your neck (Fig. 1B).

Diverse cells in the brain and spinal cord are assigned various functions within the CNS. The main cell types originating from the brain and the spinal cord are neurons, astrocytes, microglia, and oligodendrocytes. **Neurons** are the workhorse of the brain, analogous to the doctors and nurses in the hospital metaphor. They communicate with each other and make executive decisions through sending electric signals down the axons. Even though there are different subtypes of neurons with diverse functions, they all have similar anatomy (Fig. 1C).

Parts of a Neuron (Fig. 1C):

1. **Soma**: The cell body holding the majority of the cell's organs, including the nucleus which contains the genetic information of the cell deoxyribonucleic acid (DNA).
2. **Dendrites**: The smaller branches extending from the soma that **receive** information from surrounding neurons.
3. **Axon**: The largest branch from the soma that **delivers** information to the neighboring cells**.**
4. **Neurite**: The collective term when talking about both dendrites and axons.

The other three types of brain cells surrounding the neurons help support proper neuron function. **Oligodendrocytes** are brain cells that insulate the axons. This insulation around the axon, called myelin, is important to increase the speed of the electric current, or information, that travels down the axon. They are analogous to the telephones in the hospital, to increase the efficiency of communication between neurons. **Microglia** are cells that patrol the brain against infectious organisms, diseases, and clean up debris. They are the janitors and security guards of the hospital building. **Astrocytes** are the main cells that help support surrounding neurons. They have many different hats to wear, but in general they are important for supplying neurons with nutrients, aiding malfunctioning oligodendrocytes, and helping microglia to clean up debris. Astrocytes are the administrators and support staff of the hospital to maintain proper brain function.

How does the body make cells with different functions, and how do cells change their behavior to respond to different environments? The diversity of cell function in different environments is mainly due to the proteins that they express. What are these proteins being expressed by the

cells? The central dogma of biology states that genetic information is encoded in the DNA, which lives in the nucleus of the cell (the command center) and is identical in every cell within an organism (Fig. 1D). DNA contains the instructions in the form of genetic information to make all the proteins a cell would need. To manufacture proteins based on the instructions encoded in the DNA, portions of the DNA get copied into smaller pieces of ribonucleic acid (RNA) by proteins called RNA polymerases, in a process called transcription. This RNA polymerase reads the DNA while making a piece of RNA with the exact information that is present on that piece of DNA. This piece of RNA then leaves the nucleus, enters the cytoplasm (the workroom), and gets read by another protein, called the ribosome. The ribosome reads the RNA on one end while assembling the protein on the other end encoded in that piece of RNA (Fig. 1D). This is how a cell uses its genetic information to make many different types of proteins to carry out the functions of the cell.

## What is the spinal cord?

The spinal cord is the highway that relays information between the brain and the body. It contains axons that extend from a subset of the neurons in the brain, such as neurons from the motor cortex. The adult spinal cord is surrounded by a column of 26 bones, each called vertebrae, stacked on top of each other, making the vertebral column. The hole through the center of the vertebrae is called the vertebral foramen. The spinal cord passes through the vertebral foramen from the brain at the base of the skull through the spinal column, down to the tailbone (Fig. 2A).

To understand how the brain communicates with the body through the spinal cord, let’s walk through how the brain communicates to the bicep to flex. An electrical signal starts from the soma of a neuron in the motor cortex, called the primary neuron. That electric signal travels through that primary neuron’s axon, down the spinal cord, to synapse on the secondary neuron that lives in the spinal cord, which is part of the peripheral nervous system (PNS). Neurons communicate with each other through synapses, which are junctions where the end of the preceding axon (releases chemicals to bind to the receptors on the surface of the dendrites of the latter neuron (Fig. 1C). It is like a relay race, where the primary neuron (neuron trying to send the information) hands off the baton to the secondary neuron (neuron receiving the information). The baton in this case is made of specific chemicals released by the neuron sending the information. Once the primary neuron synapses with the secondary neuron, information will be sent down its axon, which exits the spinal cord through holes in the vertebrae in bundles of axons called spinal nerves (Fig. 2A). These spinal nerves will travel to the final destination, such as the biceps, to instruct the bicep muscle to flex.

In the case of spinal cord injury, axons in the spinal cord are cut, and the neurons cannot pass the baton anymore. This results in no communication between the brain and the target organ, which can cause paralysis.

## 1.4 What limits CNS regeneration?

Mammalian CNS, unlike the PNS, cannot repair itself after injury. This explains why nerves damaged when a finger is cut off (PNS) can be reconnected, but when the spinal cord is damaged (CNS), those neurons fail to reconnect leading to life-long paralysis. This is like having a portion of a highway completely broken, where cars cannot cross. If cars cannot cross, then communication cannot be sent down this highway to its final destination. Then, the muscle that is supposed to be the final destination of this axon highway will not receive a signal from the brain, leaving it paralyzed. During CNS axon injury, there are processes both within the injured neuron to tell the axon to retract (intrinsic factors), and signals from adjacent cells and outside of the injured neuron (environmental factors) that form a barrier to prevent axon regeneration. In this section, we will discuss some of these intrinsic and extrinsic factors that limit CNS axon healing.

## Extrinsic inhibitory CNS factors

To understand how the environment affects axon regeneration, we first need to discuss the environment that surrounds the axons. All CNS cells are embedded in the extracellular matrix (ECM), which is a scaffold of proteins and fibrous mesh (Fig. 3A). While the ECM is stiff enough to maintain the shape of the organ, it is also flexible and has microscopic holes to allow cells to migrate.

CNS neurons have their somas in the brain and extend their axons into the spinal cord. Thus, when the spinal cord is transected, or cut, the axons that are traveling through that vertebral level will get severed and the neurons with cell bodies in that portion of the spinal cord will also get cut open. When this happens the content within the cell spills out into the ECM, which triggers resident astrocytes in the surrounding environment to start cleaning up the cellular debris. This process is called **reactive gliosis**. During this process, cells such as astrocytes release molecular signals that cause severed axons to retract from the area of injury. This signal is one of the ways that the local environment communicates with the injured axon to not grow forward and reconnect with its target, which is necessary for healing.

Another example of the inhibitory environment of adult CNS is the myelin, which is an insulating layer surrounding axons that contains both fat and protein. When axons are injured, myelin proteins bind to surface receptors on axons and signal the axons to stop growing towards the target. Myelin protein binds to proteins on the surfaces of the axon, which communicates with the cell through a set of internal chain reactions. These chain reactions are made up of many proteins, as proteins are the main workhorses of the cell. As a frame of reference, each cell can contain up to 25,000 different proteins at any time.

## Intrinsic inhibitory CNS factors

In addition to the inhibitory environmental factors, numerous intrinsic factors lead to limited CNS regeneration. Intrinsic factors include differences in the availability of certain key proteins in cellular pathways. There is tremendous diversity in the protein population within a single cell. When, where, and how much of a protein is made in a certain cell varies. During adult mammalian CNS aging, the “pro-regenerative” proteins (proteins important for enhancing axon growth) are not expressed, or present in the cell, or not expressed in the right amount. Often the expression of these proteins is modified at the genetic level by transcriptional factors (TFs). TFs are a category of protein that live in the nucleus and bind to DNA to control and aid in the expression of specific genes. TFs interact with unique, target genes to either promote or repress gene expression. TFs are powerful proteins because they can control many different genes’ expression, which then will affect many pathways those genes are involved in.

From mouse studies, scientists have found certain “pro-regenerative” proteins such as TFs are made at reduced amounts during aging, and conversely, certain “anti-regenerative” proteins are made at increasing amounts with aging. These age-dependent changes to the protein expression in the CNS neuron compound the already inhibitory environment that adult mammalian CNS neurons reside in, further limiting healing.

## 1.5 My thesis: Are there human-specific, age-specific factors that drive CNS axon regeneration?

While CNS regeneration is limited in adult mammals, interestingly, scientists found that “young” mammalian CNS can regenerate, but the regeneration potential decreases with development and aging. This suggests that the reduced abilities of the CNS to regenerate are dependent on age. Thus, if we can understand the underlying processes that drive the age-dependent decline in CNS regeneration, then we might be able to use those targets to treat spinal cord injuries by increasing regeneration.

Historically, scientists have used rodent studies to identify factors that limit CNS regeneration. However, when scientists and doctors have tried to develop drugs based on these factors to allow better regeneration, as many as 80% failed to yield therapeutic effects in human clinical trials. Thus, there is still no Food and Drug Administration (FDA) approved treatment for spinal cord injuries. There are numerous challenges in taking findings from rodents and translating them into human treatments. One important component is that the rodent genome is very different from the human genome, which is likely why key pro-regeneration factors discovered in rodents have failed to work in human clinical trials. Thus, we wanted to generate a human neuron model to study axon regeneration in an effort to find a human-specific therapeutic target for spinal cord regeneration.

## How to generate a human neuron model in a petri dish?

There are both technical and ethical limitations to extracting human neurons from patients as it is often not possible to extract human neurons from the human brain. Thus, we had to think of new methods of generating a human neuron model in a petri dish. Scientists found a method to directly convert human skin cells, called fibroblasts, to human neurons. This is called direct reprogramming. Cleverly, scientists have found that by overexpressing (when the cell makes more of a specific gene/protein) two TFs and growing these cells in a specific environment, fibroblasts can convert their identity from a fibroblast to a neuron, called an induced neuron (iN) (Fig. 4A). This means fibroblasts become a neuron in every way: anatomy, function, identity. Cell identity, such as the identity of being a neuron, is the result of the “neuronal” combination of the pathways that are activated or repressed. Further, this method produces iNs that maintain the age of the donor fibroblast, which cannot be done with other human neuron models.

This process of direct reprogramming takes 4 weeks from the start of the overexpression of the TFs in the fibroblasts to having at least 50% of fibroblasts converted to iNs in the dish (Fig. 4B). The medium, that is, the nutrient bath we grow the cells in, has a combination of molecules that encourage fibroblasts to become neurons. In addition to generating a human model in the dish, we also generated a model that captures differences in axon regeneration abilities across the human life cycle. Previous mouse studies show that young mice regenerate better than adult mice after injury due to a combination of environmental factors and cell-intrinsic factors. We wanted to investigate if this correlation between age and regeneration also exists in humans. We used our human cells in a petri dish model to study changes in intrinsic factors that occur with aging. Specifically, we accomplished this by directly reprogramming human fibroblast cells from 11 different age groups including, 8 gestational weeks (GW), 12GW, 20GW, neonate (0-1 year-old (YO)), 36 YO, 37YO, 58YO, 62YO, 70YO, and 72YO into iNs. Using iNs of different ages, we investigated “do we see any age-related differences in axon growth abilities?”

## What did we learn from this neuron model that can help regeneration?

To identify if there were age-dependent changes in the potential for iN regeneration after injury, we looked at neurite growth. Mouse models have shown that there is an age-related decline in neurite length, so we wanted to know if this was also true in human neurons. I and my collaborator made Fib-iNs from all 11 ages and measured the total neurite length of different aged Fib-ins and observed that the 8 and 12GW iNs grew significantly longer neurites (Fig. 4C). Further, we identified the 20GW time point as the transition period where the neurites change from growing long neurites to short neurites. This was surprising because we thought that neurite growth would slowly decline with age, rather than drop rapidly mid-gestation. We then asked why 8 & 12GW iNs could grow long neurites, but all the other ages could not. From this point on, I will refer to the

8 and 12GW iNs as the “long-growing,” as they can grow long neurites, and neonatal to 72YO iNs as “short-growing,” because they grew short neurites.

To try to understand what makes long-growing iNs different from short-growing iNs, we used a technique called RNA sequencing on all the iNs. RNA sequencing is a technique that allows scientists to look at all the RNAs expressed in the cell and their levels of abundance. Because RNAs serve as the instructions for making proteins, scientists often use RNA sequencing to look at what messages the cell is producing at a specific time point to try to infer the type and amount of the respective proteins present. Using this technique, we were able to identify the RNAs made in these different aged iNs, with the aim of trying to figure out which ones are important for increasing neurite growth.

Shockingly, we found over 3000 RNAs that were expressed at different levels between the long-growing iNs and the short-growing iNs, out of a total of ~20,000 RNAs. An example of what we define as RNAs with different levels of expression would be in 8GW iNs, gene “A” makes 3 copies of gene “A” RNA, and in 72YO iNs, gene “A” makes 10 copies of gene “A” RNA. This difference in the number of copies of gene “A” RNA produced in these two different ages is defined as differential expression of gene “A.” Thus, we would say gene “A” has a higher expression in 72YO iNs.

We wanted to perform a “gene screen.” The goal of a gene screen is to test the underlying assumption that the genes tested are important for driving the differences in neurite growth behavior amongst iNs of different ages. However, sometimes genetic differences are simply correlated with a phenotype (observable characteristic), e.g. short vs long growing neurites, rather than the cause of that phenotype. Thus, to determine which genes control neurite growth, we have to manipulate the protein levels of those genes and observe the resulting changes in neurite growth. To identify which genes were most likely related to neurite growth, our collaborator performed a computational analysis, which helped us narrow our list from 3000 genes to ~50 genes.

So how do scientists alter protein levels in cells? One option is to increase the expression of the protein (overexpression) and the other option is to decrease the expression (knockdown) of the protein (Fig. 4D). To overexpress a protein, we deliver extra copies of a target gene in the DNA to increase the amount of target RNA produced, which in turn generates more target protein. To knockdown the target gene, we deliver to the cell a special type of RNA (shRNA), that can bind to the target RNA and destroy it. This prevents the target RNA from being made into protein. Even though all the manipulations are at the DNA or RNA level, the ultimate goal is to change the respective protein level to be able to see a phenotype (Fig. 4D).

In this gene screen of 50 target genes, I overexpressed certain genes and knocked down the other ones, which led us to an interesting discovery. When I knocked down a protein called AT-rich interactive domain-containing protein 1A (ARID1A) in the long-growing iNs, suddenly they stop growing long neurites (Fig. 4E). Through this gene screen, I identified a new protein that is important for controlling human neurite growth.

## What is ARID1A and how does it regulate neurite growth?

ARID1A is a protein that is part of a category of proteins called nucleosome remodelers, which are proteins that help DNA condense or relax (Fig. 4F). What does it mean for DNA to be condensed and why is it condensed? The majority of the time genomic DNA is folded up tightly around proteins called histones, similar to wrapping rope (DNA) on a spool (histones; Fig. 4F). If the DNA in the cell is uncoiled, it is around 2 meters long whereas the diameter of a neuron nucleus is a million times smaller. Thus, only with extremely efficient coiling, can DNA get packed into the nucleus. Furthermore, this coiling is another level of control which dictates which genes can be expressed at a certain time. Proteins, such as **activating nucleosome remodelers**, help uncoil and recruit proteins like TFs to the genes that they are trying to express. There are also nucleosome remodelers that work in the opposing fashion, further coiling DNA and preventing the expression of those target genes (Fig. 4F). ARID1A is an activating nucleosome remodeler, which means it usually binds to histones and instructs those histones to loosen and recruit proteins like RNA polymerase and TFs to transcribe the gene.

From our RNA sequencing dataset and the human brain RNA sequencing dataset, we found ARID1A RNA and protein levels decline with aging, mirroring the decline in iN neurite growth we observed in our human neurons in the petri dish model. Thus, both in the human brain and in our human neuron model, ARID1A protein expression decreases with aging, potentially contributing to the decreased CNS neurite growth in adults.

If knocking down ARID1A in 8GW iNs can reduce neurite length, can overexpressing it in a short-growing iN, such as 36YO, increase neurite length? We were excited to find that overexpressing ARID1A protein in 36YO iNs caused them to grow longer neurites. Taken together, our research suggests that ARID1A, a protein that decreases with age, is important for reversing this age-dependent decline in neurite growth. By increasing ARID1A levels in adult cells, which normally grow short neurites, we can increase their neurite length. In the broader context, this means that this mechanism can potentially be used to increase adult CNS neurite growth in the context of spinal cord injury.

# 1.6 Future directions- where do we go from here?

The goal of the study was to create a human neuron model where we can grow human neurons of different ages in a dish to find molecular drivers in age-dependent decline in human axon regeneration. By developing this human neuron model, we discovered that human neurons experience a decline in intrinsic growth ability early in gestation. This intrinsic growth ability continues to remain low throughout development and aging. We also discovered an age-regulated

protein, ARID1A, to be a key regulator of human neurite regeneration. Our future studies will involve looking at the molecular pathways that ARID1A may alter in young vs old human neurons. Also, we have goals of transplanting these human neurons in mouse brains and spinal cords to look at how environment affects intrinsic neurite growth abilities. These exciting and novel discoveries bring us one step closer to the ultimate goal of translating advancements in molecular neuroscience to treatments for spinal cord injuries.

# Figures and legends:

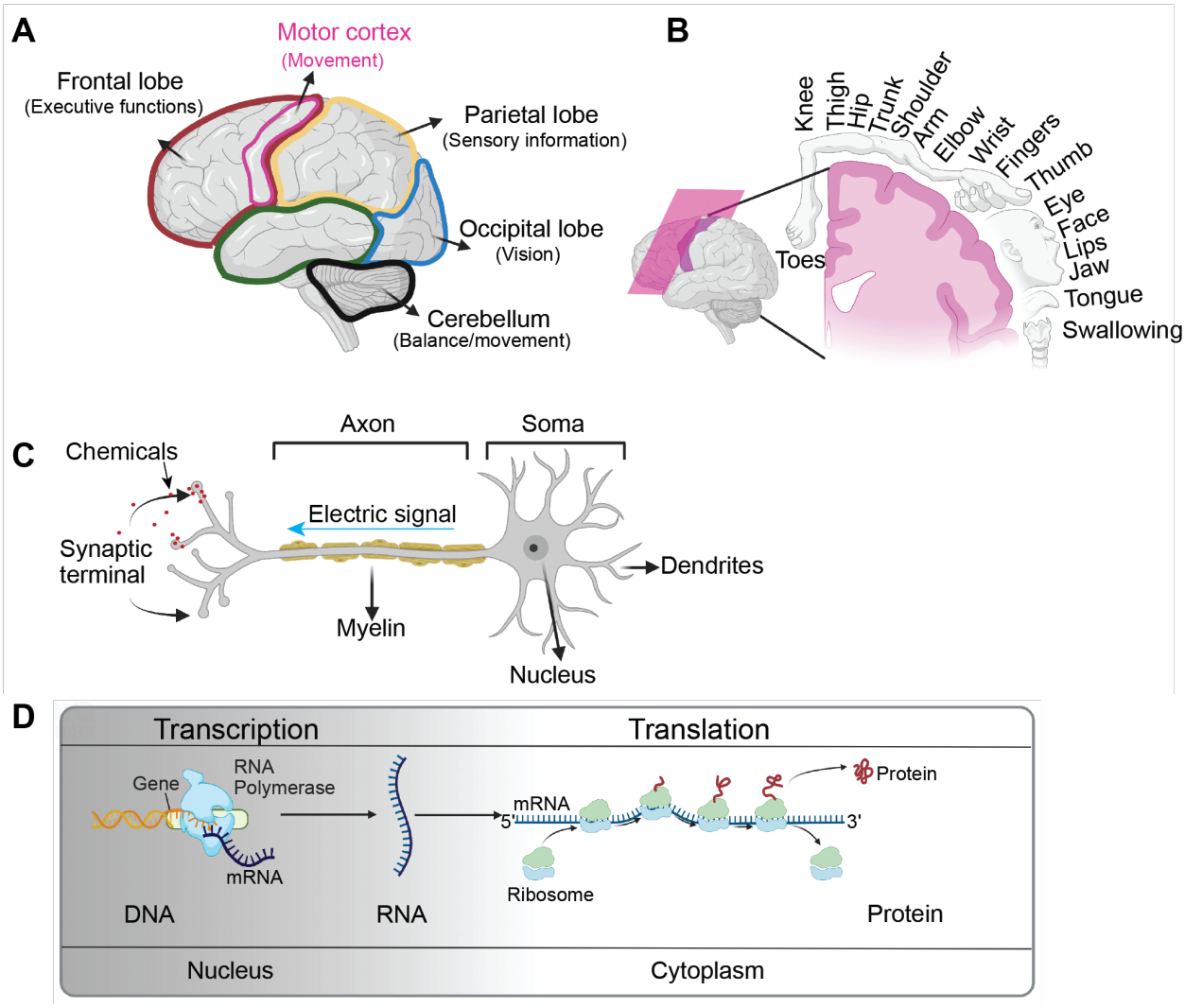


**Figure 1: The central nervous system from the organ to the cellular level. A)** The brain has different regions for specific functions. For example, the frontal lobe is important for coordinating executive functions like planning. Within the frontal lobe, is a region called the motor cortex,

important for controlling movements of different body parts. **B)** The motor cortex looks like a hairband that sits between your ears. From the head-on perspective, areas for controlling the face are closest to the ears, then as you move towards the crown of your head you will encounter motor control areas for muscles that are increasingly farther from the brain. **C)** A neuron has multiple compartments including the soma where the nucleus is located, multiple dendrites, an axon insulated by myelin, and a synaptic terminal where information in the form of chemicals gets transmitted to the dendrites of the next neuron. **D)** DNA (the genetic information) gets transcribed into RNA (the messenger) by RNA polymerase in the nucleus, which then travels into the cytoplasm to get translated into protein (functional parts of the cell) by ribosomes. Artwork created on BioRender.com.

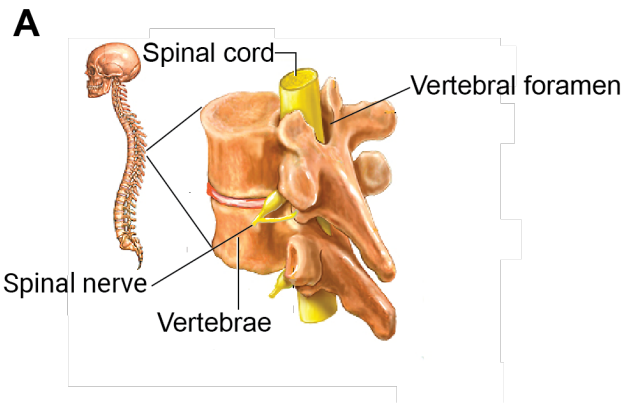


**Figure 2: The spinal cord and spine. A)** Modified schematic of the human spinal cord traveling through the vertebral column and exiting at different levels as the spinal nerve (https://medlineplus.gov/ency/imagepages/19470.htm).

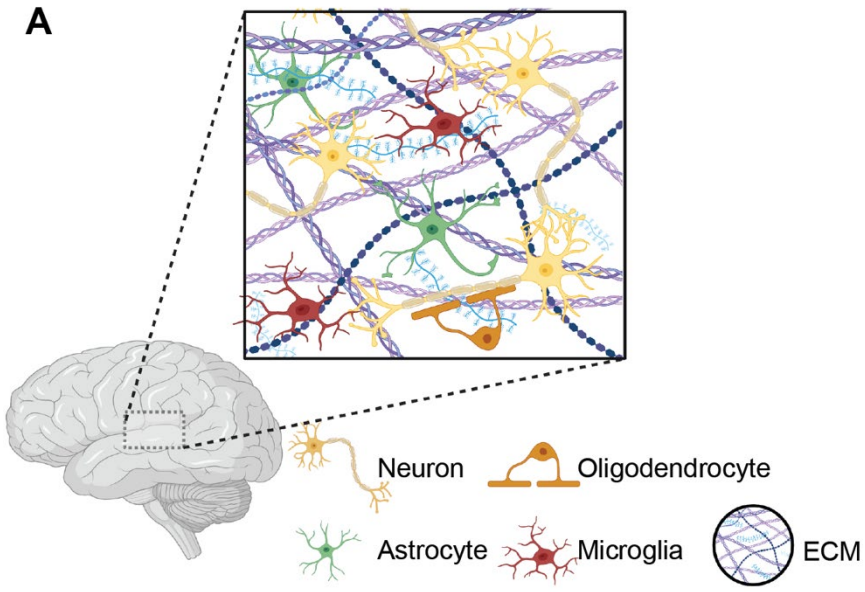

**Figure 3: Composition of the extracellular matrix. A)** Cells of the brain (neurons, microglia, astrocytes, and oligodendrocytes) are suspended in the extracellular matrix (ECM), filled with proteins and fibrous mesh. Artwork created on BioRender.com.

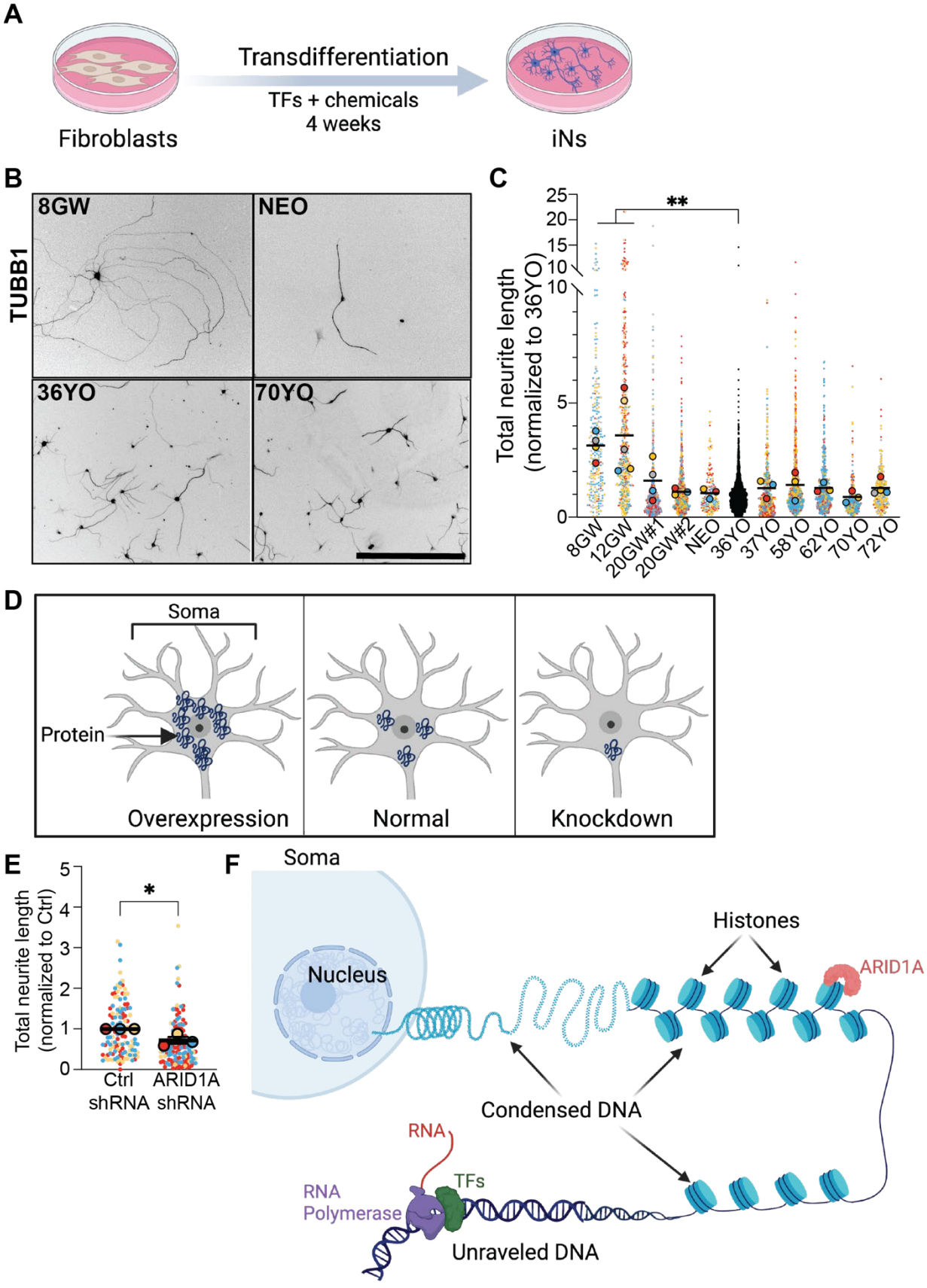
A
Transdifferentiation
TFs + chemicals
4 weeks
Fibroblasts
iNs
B
TUBB1
8GW
NEO
36YO
70YO
C
Total neurite length
(normalized to 36YO)
**
8GW
12GW
20GW#1
20GW#2
NEO
36YO
37YO
58YO
62YO
70YO
72YO
D
Soma
Protein
Overexpression
Normal
Knockdown
E
Total neurite length
(normalized to Ctrl)
*
Ctrl
shRNA
ARID1A
shRNA
F
Soma
Nucleus
Histones
ARID1A
Condensed DNA
RNA
TFs
RNA
Polymerase
Unraveled DNA

**Figure 4: Finding a novel driver of human neurite growth with our human neuron in a petri dish model: A)** Schematic of direct reprogramming/transdifferentiation of fibroblasts to human-induced neurons (iNs). **B)** Pictures of 8 gestational week (GW), neonate (NEO), 36 years-old (YO), and 70YO iNs labeled by a neuron marker beta-tubulin (TUBB1). The black circles are the somas, and the lines coming off the black circles are the neurites. Here the scale bar is showing 500uM for a frame of reference neurite lengths. **C)** Superplot displaying quantification of relative total neurite length, the length of all the neurites added up on each cell, after normalization to the 36YO total neurite length within each experiment as a control for batch differences. Large dots are the average of each experiment, and small dots represent normalized neurite length for each iN within one experiments. Colors correspond to experimental replication. Asterisks indicate that only 8GW and the 12GW iN neurite lengths significantly differ from the 36YOs (**P-value < 0.001). **D)** Protein results of the overexpression or knockdown of a gene. **E)** Superplot showing normalized total neurite lengths of control (Ctrl) shRNA compared to ARID1A shRNA knockdown reveal that by knocking down ARID1A in 8GW iNs, they produced significantly shorter neurites than the control condition in the same age (*P-value < 0.01). **F)** Schematic showing how DNA is condensed through wrapping itself around histones then further condensing to fit in the nucleus. ARID1A is a part of a complex of proteins that help unravel DNA by interacting with the histones. Only unraveled DNA can get transcribed by RNA polymerase to make RNA with the help of transcription factors (TFs). Lear *et al.,* 2023, unpublished data. Artwork created on BioRender.com.

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