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Emergent Behavior of Living Neuronal Networks

Creating circuits and exploring the dynamics of neurons in vitro

Hoerberg, Carl-Johan

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CARL-JOHAN HÖRBERG
DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY

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Creating circuits and exploring the dynamics of neurons *in vitro*

Carl-Johan Hörberg

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DOCTORAL DISSERTATION

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Author(s)
Carl-Johan Hörberg

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Emergent Behavior of Living Neuronal Networks: Creating circuits and exploring the dynamics of neurons *in vitro*

Abstract
In the past two decades there's been an incredible development in the field of *in vitro* neural cultures. Thanks to the developments in embryonic stem cell technology, and the ability to produce pluripotent stem cells from adult human cells, producing human neural cultures has become much more widely accessible, and the ability to recreate physiological processes is increasing at an accelerating rate. Neurons in culture, however, do not spontaneously form network structures which resemble those found in the brain, and perhaps as a consequence, exhibit abnormal spontaneous and stimulus-evoked activity. We tested several techniques which aimed at overcoming this challenge, and to create more physiologically relevant cultures. In paper I, we employed a bioprinting approach to achieve controlled deposition of cells on 3D fiber substrates. In paper II, we examined spontaneous self-assembly of clusters of cells on microelectrode arrays as a way of recreating cortical microenvironment. Cluster formation was influenced by cell density and astrocyte concentration, which had a significant effect on spontaneous electrical activity. Lastly, in paper III and IV, we used soft lithography to create perforated microchannels which could guide neuronal connectivity on microelectrode arrays on a network-wide level. Neurons grown in these structures exhibited clustered spontaneous activity and exhibited plasticity when we stimulated them with patterned emulated sensory information. These techniques are all different means to the same end - to create better representations of the human brain *in vitro*.

Key words
neural cultures, microfluidics, microfabrication, axon guidance, neural networks, microelectrode arrays

Classification system and/or index terms (if any)

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If you fill a kettle with water and switch it on, all the supercomputer on Earth working for the age of the universe would not be able to solve the equations that predict what all those water molecules will do - even if we could somehow determine their initial state and all that of outside influences on them, which is itself an intractable task,

— David Deutsch

Contents

List of Papers

I   **Hörberg, C.**, Sandberg, A., Arvidsson, M., O'Carroll, D.C., Johansson, F., Englund Johansson, U. (2025). Controlled Deposition of Human Neural Stem Cells on Fiber Substrates using Gel Encapsulation. (submitted).

II   **Hörberg, C.**, Englund Johansson, U., Johansson, F., O'Carroll, D. (2022). Spontaneous Cell Cluster Formation in Human iPSC-Derived Neuronal Spheroid Networks Influences Network Activity. Eneuro, 9(5).

III   **Hörberg, C.**, Beech, J.P., Englund Johansson, U., O'Carroll, D.C., Johansson, F. (2025). Population-Wide Control of Neuronal Networks *in vitro*. (manuscript).

IV   **Hörberg, C.**, Beech, J.P., Englund Johansson, U., O'Carroll, D.C., Johansson, F. (2025). Plasticity, Sparsification, and Ensembles in Designed Neuronal Circuits *in vitro*. (manuscript).

Author contributions

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To everyone I've forgotten to mention, I apologise, feel free to preclude me from your own acknowledgments as soon as the opportunity arises.

To my family, my nearest and dearest.

Popular summary in English

The human brain is a particularly interesting organ in that it's both a physiological system but also an information processing system. This dual role can be seen when you're emotionally agitated, clearly a physiological response but which also influences your thoughts. The same can be seen in the reverse case, when a thought or a particular sensory experience produces a physiological response. Physiologically, we know the brain is built up by neurons, neurotransmitters and electrical activity. On the information processing side, the brain operates by forming an internal simulation of the world, always trying to predict what is going to happen next. Both of these processes are involved in brain diseases, and in order to understand how this happens, how it can be prevented and how it can be cured, we can create little miniature versions of the brain by growing neurons in the lab.

Thanks to modern biotechnology, it's possible to create human neurons by taking a few cells (like skin cells or blood cells) from an adult individual and reprogram them into neurons, effectively reproducing the same cells which otherwise reside in their head. We can use these neurons and "listen" to their electrical activity to try to understand how they are communicating with each other. Amazingly, single neurons are capable of some rudimentary intelligent behavior, like forming memories and learning to distinguish simple patterns. However, when neurons are grown in the lab, they do not develop the intricate structure of the brain, but rather, grow chaotically in a way that isn't seen in a healthy brain, possibly making these neurons behave abnormally and undermining the potential to use them to model what happens inside the brain. In order to make neurons grow into brain-like structures, I have tested several different approaches to control their growth. In **paper I**, I describe our attempts at making a kind of "3D printer" that spits out small cell populations on a microscopic fiber substrate which functions like a scaffold that guides how the neurons can grow. We were, however, unsuccessful in "listening" to these neurons, and had to think of other approaches. In **paper II**, I looked at how the neurons instead, can be encouraged to grow into 'clusters'. These clusters were found to have a strong effect on the electrical activity of the neurons, highlighting the importance to control unwanted clustering in experiments where result consistency is important, such as when testing new medications for neurological diseases. In **paper III & IV**, I describe a new approach to get fine-control of how neurons connect with each other. We delivered artificial "sensory information" to these neurons, and saw that they may have some ability to adapt and distinguish between different patterns.

Together, this work point to several different paths ahead for improving our ability to recreate the brain's unique dual function by using cultured neurons. These techniques could prove useful both in small-scale experiments on neuronal populations ability to process information, but also in large-scale experiments where new medical treatments, toxicity of various compounds, or mechanisms for neurological conditions, can be tested.

Populärvetenskaplig sammanfattning på svenska

Den mänskliga hjärnan är ett särskilt intressant organ eftersom den är både ett fysiologiskt system och ett informationssystem. Man kan se hur hjärnan är involverad i båda dessa när man till exempel är känslomässigt uppjagad. Uppenbarligen så är det till stor del en fysiologisk respons, men det påverkar också hur man tänker. Det motsatta kan märkas när en känslomässig respons dras igång av att man tänker på någonting, eller har en särsklid upplevelse. Som ett fysiologiskt system kan man beskriva hjärnan som uppbyggd av hjärnceller, neurotransmittorer, elektrisk aktivitet och mer. Som ett informationssystem så ses hjärnan fungera genom att skapa en slags intern simulering av omvärlden, och genom att ständigt försöka förutsäga vad som kommer att inträffa. Båda dessa sidor av hjärnans funktion är inblandade i hjärnans olika sjukdomstillstånd, och för att kunna förstå varför, hur man kan förebygga det, och hur man kan behandla det, så kan man skapa miniatyrversioner av hjärnan genom att odla hjärnceller i labbet.

Dagens bioteknik gör det möjligt att ta celler från en vuxen individ (som hudceller eller blodceller) och göra om dem till hjärnceller. I princip så kan man återskapa de hjärnceller som finns i just den personens hjärna. Man kan sedan odla dessa hjärnceller i labbet och lyssna på deras elektriska aktivitet och hur de kommunicerar med varandra. Otroligt nog så kan odlade hjärnceller efterlikna vissa kognitiva förmågor som minne och inlärning. Problemet är att när man odlar nervceller så återskapar de inte de strukturer som finns i hjärnan, utan växer mer eller mindre kaotiskt, vilket skulle kunna hindra försök där man vill använda dem för att återskapa sjukdomsförlopp, exempelvis. För att få odlade hjärnceller att bättre efterlikna de strukturer och funktioner som återfinns i hjärnan så har jag under-sökt olika metoder för att kontrollera deras tillväxt. I **artikel I** så utvecklade vi ett sätt att 3D-printa små populationer av hjärnceller i ett mikroskopiskt fibersubstrat som kan styra hur hjärncellerna växer. Vi kunde dock inte använda denna metod för att undersöka deras elektriska aktivitet, och vände oss till andra alternativ. I **artikel II** så beskriver jag hur man kan få nervceller att skapa små kluster, som små 'tighta' grupper av hjärnceller. Vi kunde se ett tydligt samband mellan formationen av kluster och deras elektriska aktivitet, vilket understryker att oavsiktlig formation av kluster kan försvåra för studier där man letar efter små skillnader i elektrisk aktivitet. I **artikel III & IV** så presenterar jag om en ny metod som ger utmärkt kontroll över hur nervceller växer. Vi kunde se att om man stimulerar cellerna med elektriska signaler som efterliknar sinnesintryck så kan hjärncellerna reagera plastiskt, vilket skulle kunna betyda att de utrycker en enkel form av inlärning.

Tillsammans så pekar mina resultat på flera lovande metoder för att förbättra vår förmåga att efterlikna hjärnans funktion med odlade hjärnceller. De metoder som testats kan vara mycket användbara både i studier där man vill undersöka hur hjärnceller hanterar sensorisk information, men också i storskaliga försök där nya behandlingar, läkemedel, gifter eller sjukdomsförlopp ska undersökas.

Introduction

1 Overview

In 2006, it was shown for the first time pluripotent stem cells can be produced without the need for any embryonic material (Takahashi and Yamanaka, 2006). The following year, embryonic stem cells were used to create mature networks of neurons on microelectrode arrays (Ban et al., 2007). A few years later, in 2014, the same technique was established with induced pluripotent stem cells (Odawara et al., 2014), and by this time, the first cerebral organoids had just been developed (Lancaster et al., 2013). By 2019, the first robust network activity was demonstrated in a cortical organoid (Trujillo et al., 2019). These are just a handful of the amazing technological innovations that have occurred in the past two decades. These advancements have enabled entirely new *in vitro* representations of the human brain, allowing us to explore the physiology, development and information processing of the brain in a simplified, controlled environment. By using induced pluripotent stem cells, it is also possible to create neurons from patients who suffer various neurological conditions, giving unprecedented access to *in vitro* human disease models (Page et al., 2022).

Perhaps somewhat ironically, the spontaneous electrical activity of these neurons is essentially the same as that of cultured rat neurons, which was described observed over 40 years ago (Gross et al., 1982). Neurons *in vitro* very often develop global synchronized electrical activity, with an almost uncanny similarity between model systems (Hyvärinen et al., 2019). I will attempt to provide a comprehensive explanation of this phenomenon in later chapters, but roughly speaking, it arises from a combination of absence of neuromodulation, lack of sensory input or input from other brain regions, and the lack of ordered network structure *in vitro*.

In this thesis, I will argue that these factors have not received the attention they deserve, and that overcoming these issues is crucial in producing better models. Understanding the processes that underlies neuronal behavior *in vitro*, and how these can be improved, requires a very broad discussion of neuronal function. I will begin by discussing the brain in a "bottom-up" approach, starting with the function of single neurons, and move upwards

to networks and entire brain functions. Then, I will switch gears and discuss some specific topics in the context of *in vitro* models. Lastly, I will present my research in the form of four chapters. In the first chapter, I describe how we explored bioprinting on fiber substrates as a way of producing macroscopic organization in neuronal cultures. In the second chapter, I describe the spontaneous formation of clusters of neurons and astrocytes and how they influence network activity. In the third chapter, I describe a new kind of microfabricated device which allows control of network architecture in cultured neurons. In the last chapter, we use the microfabricated device and investigate neuronal responses to neuromodulation and simulated sensory input.

Paper I

Combination of bioprinting and 3D fiber culture substrates to manipulate neuronal network structure and macroscopic organization
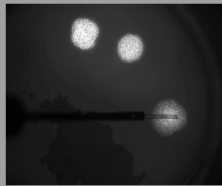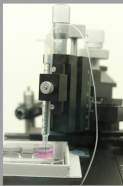
Custom made bioprinting device

Paper II

Spontaneous generation of spheroid networks creates 3D structures with modular structures, but exhibit highly syncronous activity
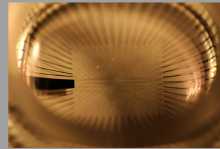
Self assembling Spheroids
What activity emerges from their interactions?

Core Problems

Neuronal Networks In Vitro
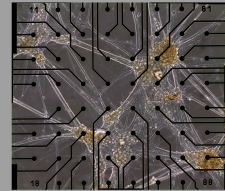
Key Questions

Cultured Neurons
lack macroscopic organization
are deprived of sensory input
lack neuromodulatory input

What spontaneous activity emerges in cultured neurons?

Can cultured neurons exhibit plasticity?

Are there structural requirements for brain function?

Paper III

Large scale control of network architecture using perforated microchannels
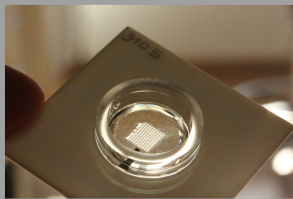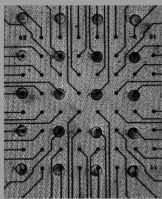
Microfabricated MEA insert for circuit design

Paper IV

Network properties, functional segregation, neuromodulation and plasticity in neurons with controlled network architecture
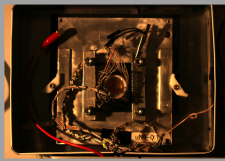
Stimulus recording setup
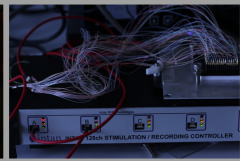
Relevance

Create more accurate models of neurological disorders - Investigate biological basis for intelligence

From neurons to networks

2   Neurons and the emergence of the mind

One of the things I find the most fascinating about the human brain and the human mind is that nobody is ever in charge of its construction. Not just the complex biological structure of the brain, but the mind itself, is entirely self assembled by individual cells. Somehow they achieve this while only being aware of their local environment, without any overarching guiding force. This includes more than just the ordinary biochemical precesses of cells, but the more ethereal notion of information processing. Take reading for example, the human brain was never explicitly designed for reading, and it can't be an inherited trait. The brain has somehow rewired itself from nothing but information to produce a productive outcome.

The *capacity* for rewiring the brain is of course, an inherited trait. Across the animal kingdom, different brains are more or less flexible in different species. In very general terms, invertebrates are much more "hard wired" and relies more on inherited circuits than vertebrates to. A fly can hatch from its pupa and fly almost immediately (Hesselberg and Lehmann, 2009), while a bird needs much more practice before being able to fly (Ruaux et al., 2020). There are different views on *where* and *how* information driven rewiring takes place, and to which extent specific brain circuits are required for these changes to be possible.

We can think of neurons in the brain a bit like ants in a colony. No ant is ever capable of understanding their role in the colony, but acts according to a simple " local program" running in its tiny brain (Bonabeau et al., 1997). A single ant will not accomplish much, but thousands of ants will be capable of building highly sophisticated colonies without any individual ant understanding what they are doing. This is an example of an emergent phenomenon; a system which arises from the collective interactions of many copies of individual components (Carroll and Parola, 2024). Other examples includes molecules in a cell, people in an economy, neurons in the brain, or gas molecules in sound waves. A fascinating aspect of emergent properties is that they can be substrate independent, meaning that

waves that emerge from the interactions of air molecules can share properties with waves emerging in the electromagnetic field, for example. Interference, reflection and diffraction are examples of properties of waves, that can be found in both the air, in electromagnetic fields or even in gravitational waves (Hou et al., 2020).

Thinking about neurons as exhibiting a local program is especially interesting if we consider the theory of universal computation. First realized by Alan Turing in the 1930's, the theory states that 'any finite realisable physical system can be perfectly simulated by a universal model computing machine operating by finite means' (Deutsch, 1985). What this means is that there exists, in theory, a machine which can simulate any physical system. These machines, called Turing Machines, are the foundation of modern computers, and the "universality" of their computational abilities underlies their diverse capabilities. Physical systems are, however, continuous, while Turing Machines are discrete. This is one of the interesting aspects of quantum computers, which display universality even in continuous systems (Deutsch, 1985). This leads us to a rather provocative conclusion. Since memory, learning, object recognition and even consciousness (assuming physicalism) are based on physical processes of the brain, then all these processes could be simulated in a computer, given the right program. Uncovering the program that is executed by single neurons, is therefore a highly attractive prospect for AI research and our understanding of cognitive processes though computational models.

If the brain is like an ant colony, with individual neurons all running a simple "program" out of which the mind emerges, what is the program that the neurons are running? Understanding the structure and function of the entire brain may be very difficult, but what if we could uncover some more simple "local" program that individual neurons are running? A simple "kernel" from which all the complexities of the brain emerges. This idea is reminiscent with that of cellular automata, in which a lattice of states are updated using a local rule, so that any combination of, for example, 3-by-3 neighbourhood, the central cell has a defined arbitrary state in the next iteration. Amazingly, using mere local and uniform update rule, cellular automata can exhibit computational universality (Von Neumann et al., 1966). The famous example of a cellular automata of "Conway's Game of Life" provides a very nice example of emergent phenomena. This cellular automata uses a simple local update rule, yet from these simple rule, a diversity of behaviors can emerge. One such example is the 'glider' which moves diagonally across the grid (Figure 1), thus a "new" rule of diagonal movement has emerged out of the local interactions of multiple cells.

If there exists such a "local" neuronal program, then we would expect it the be present even in small populations of isolated neurons. This is quite literally what we study when we grow neurons in the lab. It's analogous to taking a few ants from an ant colony to study which behavior emerges out of their collective interactions. Similarly, neurons can be "taken out" (not *always* in a literal sense) of the brain and grown in the lab. What emerges out of their interactions? Will the neurons spontaneously start to think? And if not, why?

A contrary view of the brain is that specific macroscopic structures are required for certain functions. Lesions to the hippocampus for example, will lead to an inability to form new episodic memories (Smith and Mizumori, 2006). Damage to the visual cortex will lead to blindness (Leopold, 2012), and lesions or neurodegeneration to particular areas such as the dopaminergic neurons in the substantia nigra, or cholinergic neurons in the basal forebrain will lead to very particular symptoms such as parkinsonism, or learning deficiencies (Dunnett et al., 1991), respectively. This view is however, less true if we consider that the brain is much more capable of compensating for loss or damage to large areas of the brain if they are inflicted when the brain is still developing (Kolb and Teskey, 2012). As we shall see, the adult brain establishes specialization over the course of maturation.

What are then the necessary components for a mind to emerge from neurons? Can we make a case that the mind emerges out of a neuronal "program", or are there some minimal requirements of macroscopic organization? If so, what are they? When we study the structure and the function of the intact brain, it is very difficult to untangle what is a specific realization versus a sufficient principle for a functional mind to emerge. In other words, there's not a whole lot in the experimental toolbox for testing which particulars of brain structure are necessary for the formation of a functional brain. Throughout this thesis, I will explore the use of neuronal cultures, and our ability to control neuronal circuits in vivo, as tools to investigate how neuronal circuits underlies the emergent properties of neurons.
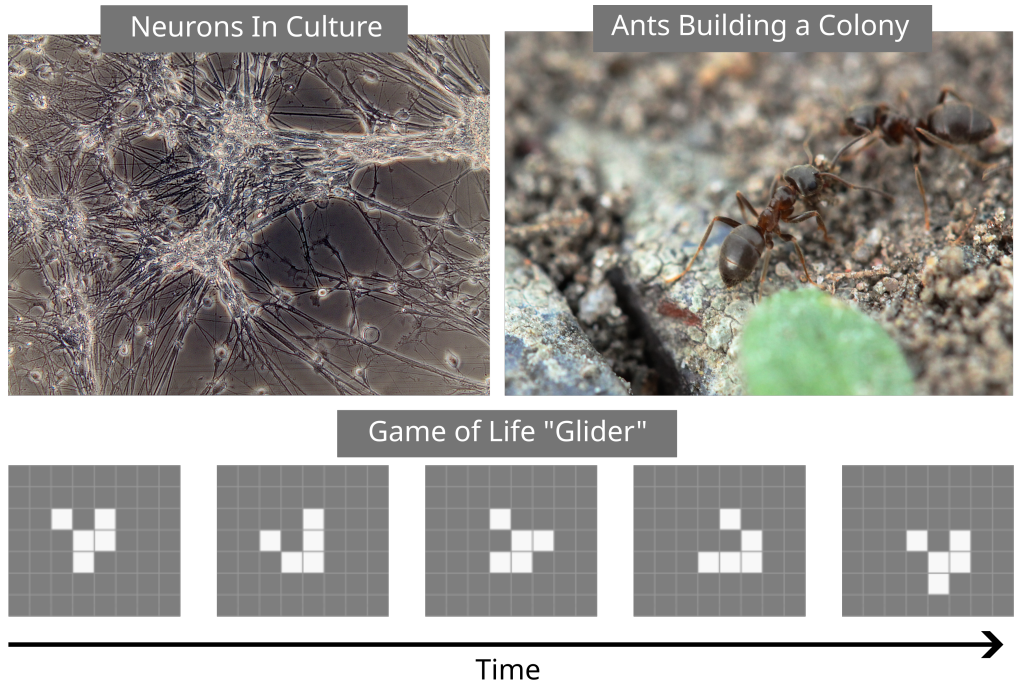
Figure 1: Examples of emergent phenomena. The neurons in culture, the ants building a colony or the grids in Conway's "Game of Life", are presumably not aware of the role they play in the construction of larger emergent phenomena.
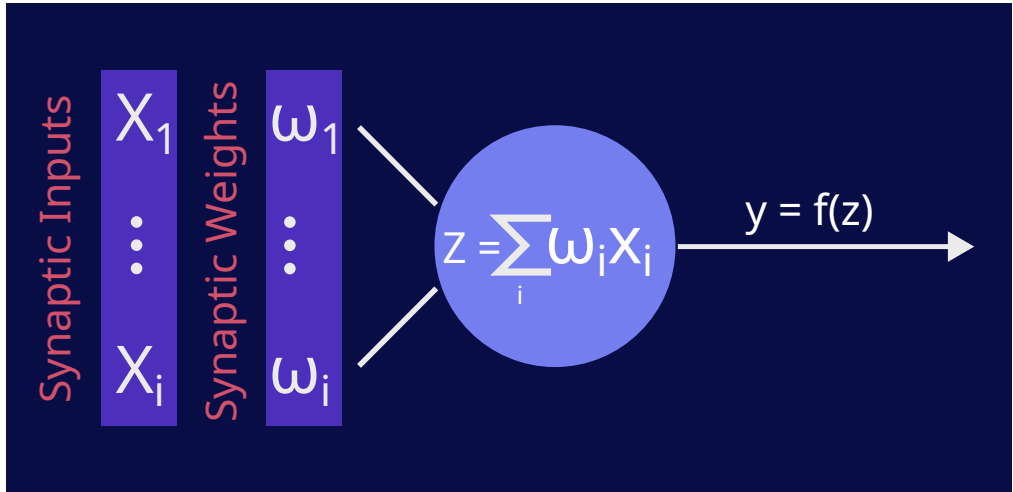
Figure 2: The McCulloch-Pitts model of a neuron. A neuron take in an input vector, x, from which a weighted sum is derived. The weighted sum is passed through an activation function to generate an output y. Learning in this model can occur by changing the synaptic weights ω and thus alter how the neuron responds to input.

3 Mathematical models Of neurons

When I studied neurobiology I was rather frustrated with how much detail about the biology of neurons we had to learn, and how little answers I was getting to the questions I had about how the brain works. There are of course, many accounts of neurons - many ways of looking at them. We can describe neurons as biophysical objects, having membrane potentials, capacitance, conductances. We can describe neurons as biological entities with cell membranes, synaptic vesicles, all kinds of proteins like receptors and ion channels. It wasn't until I learned about the various mathematical or functional descriptions of neurons that I felt like things started to make sense. That is not to say that I am a particularly mathematically oriented person, but mathematical models of neurons are just very good tools for getting a grip on what neurons are doing. These models also underlie the bulk of the AI literature. Many of these models makes some pretty bold simplifications or are otherwise not biologically plausible, but their limitations are very informative, and I will discuss these more below.

The most common mathematical formulation of a neuron has its roots in the 1940's, when the McCullough-Pitts neuron was formulated (McCulloch and Pitts, 1943). This model proposed that neurons perform a weighted sum of it's synaptic inputs (Figure 2). This was at the time based on empirical knowledge about neurons and synapses, and McCullough and Pitts used this model to show how propositional logic (AND, OR, XOR and such) could be performed by simple networks of neurons. They showed this with neurons as "all or none", either producing an action potential or not. Today, we know that neuronal networks do

not work by performing propositional logic, but the formulation of individual neurons has lived on. Their model was later modified to allow continuous outputs, by the addition of an activation function, to reflect that neurons to some extent encode information by spike firing rates (Gerstner et al., 1997). There's a large botany of different activation functions that have been proposed, but one popular activation function is the sigmoid activation function.

In the 1950's the perceptron (Rosenblatt, 1958) was developed. The perceptron was built to explore how learning and the storage of information could be achieved in the brain. In the perceptron, learning is achieved by changing the synaptic weights from experience $\Delta\omega$. The "Mark I Perceptron", a living-room filling behemoth, was built in 1958 and could be taught to recognize hand-written digits. Changing the weights was done by hand, by turning potentiometers to adjust the gain of synapses, and how much the weights had to be adjusted was calculated by measuring the difference between the perceptrons output, and a "target value", and computing how much the weights needed to be adjusted in order to minimize the error. This means that the perceptron uses a form of supervised learning, which already means that the biological feasibility of this model is rather questionable. The formalization of neurons in the McCullough-Pitts model and in the perceptron is really handy because the mathematics are rather simple, and uses some fairly straightforward linear algebra, making them highly amenable to analysis. This sets them apart from more biophysically relevant models such as compartmental models or integrate-and-fire models, which relies much more on differential equations that are not always as interpretable.

Modern artificial neuronal networks are not, in principle, very different from the Mark I perceptron (LeCun et al., 1998), and our understanding of biological neurons has diverged significantly since then. In contrast to the perceptron and most artificial neuronal networks, the brain has to rely on unsupervised learning, as there's no predetermined correct output that neurons can strive for. An example of a more biologically plausible is the "Hebbian" form of learning (Hebb, 2005). Hebbian learning is often encapsulated in the catchphrase "neurons that fire together, wire together". This expression wasn't actually coined by Donald Hebb, but it describes the intuition behind a weight update rule that is attributed to him. I think the expression is actually really bad, I must have heard it a hundred times before I realized its true significance. A better way to explain it would perhaps be, that neurons (not all neurons, but some subset of neurons to which we attribute learning) develop sensitivity to recurring input patterns, or in other words, neurons find correlations in its input information. In this view, neurons are little pattern detecting machines, which you can feed information, and which will pick out the dominant pattern in this information and become selective for this pattern.

The Hebbian weight update rule can simply be expressed like this. You can see how the catchphrase "neurons that fire together wire together" enters the picture here. When a neuron becomes activated, when y is high, then the inputs (the neurons that fired together with

this neurons) are facilitated. In other words, if a synaptic input evokes a strong response, it's facilitated.

$$\Delta \omega_i = \eta x_i y$$

A "learning rate" η is introduced which is just there to slow down the weight updates.

This rule has a big problem, and is actually a terrible update rule. If applied, the weight would start to 'runaway' since there's nothing that normalizes the weights. The Finnish mathematician Oja expanded on this idea in a very interesting way:

$$\Delta \omega_i = \eta (x_i - y \omega_i)$$

This adds a normalization term, which is proportional to the synaptic weight, and the activation of the neuron. Oja's learning rule can be used to derive principal component analysis (Oja, 1992). Principal component analysis (PCA) is a super useful mathematical tool for dimensionality reduction. In essence, PCA is a way of finding the underlying correlation structure of a dataset, and I have used expensively in my work. PCA is rather similar to Fourier transform, in that it's a way of decomposing a dataset to their principal components (or frequencies, in the case of Fourier transform). Imagine a two-dimensional linearly co-varying dataset. Rather than describing a datapoint as a 2D position, you can describe it as a magnitude, or distance along the axis of covariance. This transfers to higher dimensions, and can therefore be used for dimensionality reduction. In this way, PCA works as a kind of pattern detection, and the possibility that neurons function as little PCA algorithms is an enticing idea. Hebbian learning in this case is a candidate for the speculative "neuronal program" I discussed in the overview, as it is a *local* algorithm for how neurons change their synaptic weights.

4 The biology of plasticity

One intuitive case to consider this idea of neurons as pattern detectors is the olfactory system. When we smell coffee, for example, there's a vast number of volatile chemicals which our olfactory receptors detect. Coffee is actually one of those aromas that hasn't really been possible to create artificially, unlike some of the fruity aromas which often contain a large proportion of a single ester (although I personally despise artificial fruity aromas). Some of the chemicals present in coffee aroma are actually when isolated and presented to us in pure form, not very nice smelling (Rhoades, 1960), but the combination, or the covariance, of these chemicals evokes the sensation of the aroma of coffee. So we can think

of the olfactory system basically doing a dimensionality reduction, and represents a flavour as a projection along a high dimensional principal axis of covariance.

This is seen in the olfactory cortex, or piriform cortex, which receives direct input from the olfactory bulb, where glomeruli transduces signals from the primary olfactory sensory neurons. Neurons in the piriform cortex receives synaptic inputs from a very large area of the olfactory bulb (Haberly and Bower, 1989), that is to say, the neurons in the piriform cortex receives a very high dimensional vector which represents the relative magnitude of the activity of different odour receptor neurons.

The principle of Hebbian learning gained widespread recognition for its explanatory power. Associative memory, perception, and development of ocular dominance, to name just a few, can be quite satisfactory explained by the Hebbian framework (Lim, 2021). In the hippocampus, Hebbian synapses can be demonstrated by stimulating a presynaptic axon and a postsynaptic neuron. Stimulating the presynaptic axon will consistently yield a certain response in the postsynaptic neuron, but when the postsynaptic neuron is depolarized simultaneously as the presynaptic axon, the neuron will be facilitated and presynaptic stimulation will subsequently produce a stronger response in the postsynaptic neuron (Gustafsson et al., 1987). The same effect can be demonstrated by stimulating two presynaptic inputs to a postsynaptic neuron, where a weak presynaptic coupling may be facilitated by simultaneous stimulation of a strong presynaptic input (Kelso et al., 1986). In the framework of our artificial neuron sketched out above, this can be interpreted as the $\Delta\omega$ being 0 when the postsynaptic response y is small, such as it is when only a single presynaptic axon is stimulated. When y is large, as it is when the postsynaptic neuron is depolarized or when a strong synaptic input is stimulated, $\Delta\omega$ is big, producing facilitation.

The Hebbian facilitation first observed in the hippocampus was an example of long-term potentiation (LTP). LTP like this is driven by the action of the N-methyl-D-aspartate (NMDA) receptor. The NMDA receptor's role in Hebbian learning can easily be seen in it's molecular mechanics. NMDA receptors are ionotropic glutamate (the most common excitatory neurotransmitter throughout the brain) receptors, but their ion pore is normally blocked by a magnesium ion. If the cell is strongly depolarized, the magnesium ion is dislodged, and the NMDA receptor now becomes permeable to calcium (Nowak et al., 1984). The influx of calcium subsequently triggers signalling cascades in the postsynaptic neuron that produces the LTP effect (Willard and Koochekpour, 2013). One key player in this machinery is calmodulin dependent protein kinase II, which is stimulated by the local increase in calcium, and leads to the translocation of AMPA receptors (another ionotropic glutamate receptor), exposing them to presynaptic release of glutamate and thereby strengthening the response to presynaptic glutamate release (Rumpel et al., 2005). The role of NMDA receptors in memory formation is quite well established. Several compounds which inhibit the action of NMDA are known to induce amnesia, such as alcohol (Izumi et al., 2005) or ketamine (Newcomer et al., 1999).

The picture is of course much more complicated than the action of a single receptor. For example, presynaptic facilitation also takes place when the postsynaptic neuron is stimulated, meaning that the presynaptic axon will also produce stronger responses in neighbouring neurons that wasn't depolarized (Bonhoeffer et al., 1989), which doesn't have a straightforward Hebbian interpretation. Hebbian learning is also dependent on local excitation (Malouf et al., 1990), as inhibitory neurons and excitatory neurons form recurrent connections in the local circuit of the cortex (Douglas et al., 1989).

The calcium influx of NMDA receptor activation also regulates gene expression of genes involved in memory formation (Xia et al., 1996). These genes, called the immediate early genes (IEG), are required for the maintenance of LTP and memory formation, and can be shown by standard behavioral assays like taste aversion and novel object exploration time in gene deletion experiments (Jones et al., 2001). The genetic nature of memory formation has enabled some totally fascinating research where the expression of these genes can be tied to reporter genes, allowing the direct visualization of which cells are involved in a particular memory (Vazdarjanova and Guzowski, 2004). Furthermore, it's possible to tie the expression of IEGs with channelrhodopsin-2 (ChR2), which is a light-induced transmembrane ion channel, which becomes permeable to cations when exposed to blue light. This way (and with a few additional genetic tricks), it is possible to entrain a memory in living mice, where again, the NMDA channels leads to calcium influx, which leads to expression of IEGs but now also to the expression of ChR2, and then re-activate these memories by light stimulation (Liu et al., 2012). This way it has been possible to perform inception (yes, like the movie) of false fear memories (Ramirez et al., 2014) by re-activating a non-fear-conditioned memory with a pain stimulus, which now makes the animal associate the old memory with fear even though they were never presented concurrently. The Hebbian learning paradigm once again provides a useful way of explaining these findings, as the neurons that *fired together* with the pain stimulus, formed a lasting association -a memory.

5   Learning and neuronal circuits

In order for the brain to distinguish between external states, it requires that the different external states are associated with different brain states. This is a matter of common sense. Your computer relies on the same principle. If, for example, pressing the key 'C' produces the same binary representation in your computer as pressing the key 'J', the computer has no way of differentially responding to these. Similarly, if you see two images, and they both produce the same response in you brain, how could you possibly differentiate them? Such is the case in the developing immature visual cortex of rat, where visual stimulation evokes wide and non-specific responses in the cortex (Colonnese et al., 2010), which means that these mice have very limited representational capacity of visual states.

The observation of IEG-expression suggest that memories exists as a "sparse code" in the brain, where a small population of neurons are responsible for representing a memory. This has been seen in the human hippocampus, where identified neurons are extremely selective for what they respond to. In a famous example, people undergoing brain surgery for epilepsy were shown various images while neurons in the hippocampus were being recorded. Some neurons were discovered to respond to extremely selective images, such as the face of a famous person or a famous landmark, while being more or less silent when shown other types of images (Quiroga et al., 2005). A similar experiment showed that selective cells in the hippocampus could also be activated when the patient recalled images or image sequences (Gelbard-Sagiv et al., 2008).

The contrasting concept to sparse coding is dense coding. Going back to a computer analogy, ASCII characters in a computer are represented by unique combinations of 7 bits. This is the information theoretical maximum of how many states can be represented by 7 bits (Shannon, 1948). However, if only one of these bits are faulty, then every representation will become inaccurate. If we imagine that each bit is a neuron, which is either firing or not, then a dense coding implies that many neurons have to be active for each representation, possibly resulting in high energy consumption (Foldiak, 2003). The opposite strategy in this analogy would be that each character is represented by one bit, so 128 bits, each encoding a separate character. This is obviously much less memory efficient, but means that many bits can be faulty but leave most representational capabilities intact. If the key 'Q' is broken on your keyboard, you would still be able to write most words. This also means that several different keys, or states, can be represented concurrently. The brain uses a combination of these two modes, using both the representational capacity of dense encoding, but also minimizing the number of neurons that are active in each representation (Foldiak, 2003).

Sparse coding fits very nicely with the idea of Hebbian neurons and neurons as pattern detecting machines. Suppose we build a hierarchical feed-forward network of such neurons (Figure 3), where each neuron is a Hebbian feature extracting neuron. If we assume that each neuron is capable of feature extraction by some PCA-like mechanism, then we can imagine that layers of neurons like this can perform an increasing degree of abstraction of image features. The first layer may detect simple image features like edges and pass that on to the next layer, which can abstract the image even further by detecting combinations of lines as simple shapes, and so fourth until some neurons respond to individual faces.

Anatomically, the visual information travels from the retina via the optic nerve to the lateral geniculate nucleus (LGN) in the thalamus, which finally innervates the visual cortex (De Moraes, 2013). Throughout this pathway, the retinal layout is preserved, meaning that neighbouring retinal areas terminate in close proximity on the cortex. Interestingly, however, this retinotopic map output of the fovea is disproportionately big compared to the area of the visual field that it represents (Dougherty et al., 2003). The primary visual cor-
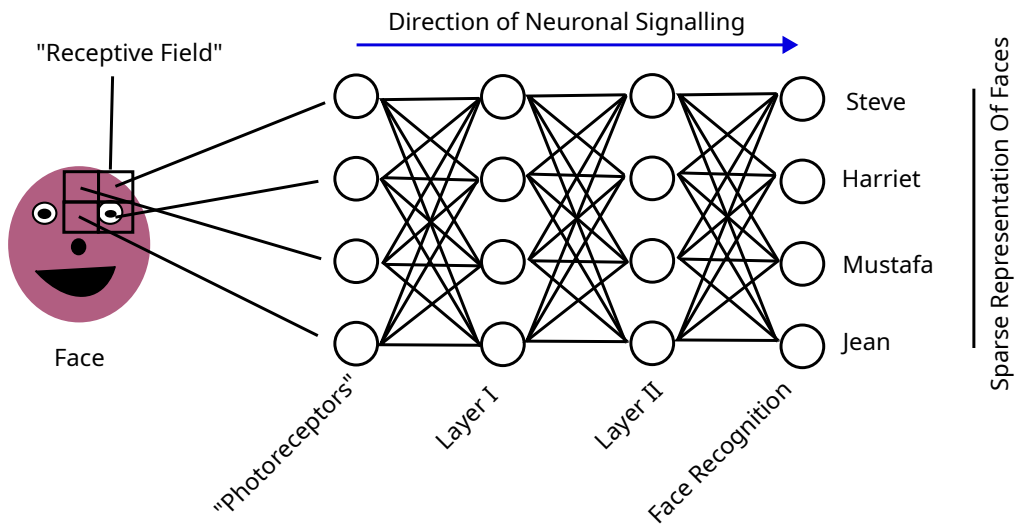
Figure 3: Illustration of sparse coding, and the incorrect view that sensory information directly drives cortical responses. Consider an image of a face being projected onto a retina. Different photoreceptors will see different parts of the image. The relative illumination of each photoreceptor will somehow be encoded and passed onto neurons in the brain. If we think of these neurons as operating by a Hebbian learning regime capable of detecting correlations in the inputs, then the first layer of neurons may pick up on basic features of the image, such as lines of different orientation. The next layer will thus be receiving a form of abstraction, representing where lines of certain orientation are found, and will perhaps -again possibly through a Hebbian mechanism- derive combinations of differently oriented lines, basically rudimentary shapes. This increasing amount of abstraction of layered feature extraction eventually lands on a few neurons representing entire faces. So the story goes, at least.

tex, or V1, is where the visual input first makes contact with the cortex. This part of the cortex is famously home to feature detection neurons, aligning with the idea of sensory processing as outlined in Figure 3. In the classical experiment by Hubel and Wiesel on the cat primary visual cortex, neurons are found which respond specifically to rudimentary shapes like rectangles of different orientation(Hubel et al., 1959). Interestingly, similar feature-detecting neurons have also been found in dragonflies(O'Carroll, 1993), but also in the machine learning models of convolutional neuronal network (LeCun et al., 1998).

These receptive fields are subject to Hebbian plasticity, and receptive fields can be manipulated by pairing an extra-receptive visual stimuli with current injections in a single neuron (Eysel et al., 1998), and thus artificially expanding the neurons original receptive field. Again, neurons that fire together... However I'd like to point out that a more informative description is that neurons in the visual cortex will facilitate synapses that are strongly correlated. In a way, by artificially depolarizing a neuron we are tricking the neuron. In the native cortex, these depolarizations would come from the summation of many synaptic inputs; the synapses that are involved in this depolarization become facilitated by the action of NMDA receptors.

This highlights another important aspect of Hebbian learning, which is that facilitation can only happen in existing synapses, and the formation of synapses isn't covered by the

Hebbian framework per se. This however, aligns well with what we know of cortical circuits. Cortical connections are typically grouped into extrinsic (via the white matter) and intrinsic (via the grey matter). Cortical neurons often have many weak and far-reaching intrinsic cortical connections (Binzegger et al., 2004). The strength of these connections follow an interesting log-normal distribution, meaning that a few connections account for much of the functional connectivity of the visual cortex (Song et al., 2005).

Similar mechanisms underlie changes in cortical circuits resulting from lesions that induce sensory deprivation in the cortex. Lesions to different parts of the retina are associated with a rapid encroachment of neighbouring receptive fields (Gilbert and Wiesel, 1992). Similar effects are seen in the somatosensory cortex. In rats and mice, the facial whiskers terminate into well-defined cortical areas which are, like the retina, topographically consistent with the whisker layout. This area is called the barrel cortex, and like the visual cortex, neurons here extend long intrinsic connections (Narayanan et al., 2015). If the whiskers are clipped, thus preventing much of their tactive activation, the barrel cortex shows strong reorganization (Diamond et al., 1993), leading to expansion or shrinkage of receprive fields, depending on which combinations of whiskers are removed (Glazewski et al., 2007).

We can begin to see and image emerging from these observations, that Hebbian mechanisms lead to a form of competition in the cortex. It's important to point out that in the scheme outlined in figure 3, this model only works if the different neurons develop a distributed selectivity for different features. Again, of all neurons become sensitive to the same face, the brain's representational capacity is reduced. This underscores the importance of inhibition in the cortex in "sharpening" cortical responses (Foeller et al., 2005). Inhibition, as we shall see later, is a crucial aspect in the maturation of the brain.

Hebbian plasticity is a great candidate for a "local neuronal program", and can be used to explain a wide range of neuronal phenomena. We will now zoom out a little bit and discuss how the brain operates on a larger scale to process information.

6   Bayesian inference and predictive processing

The idea that the brain process sensory information by a hierarchical feed-forward model is irreconcilable with the anatomical and functional data we have of the brain. Several important features of the cerebral cortex suggest a view that is almost the polar opposite to what I first described above. Much of the processing of information and learning is thought to be done by neurons in the cortex, the outer layer of the cerebrum which in humans and many other mammals is highly gyrified (wrinkled), and famously laminar in its structure, having a few different layers depending on the animal and the brain region. The cortex receives much of its sensory input from the thalamus, which is a structure that lies below
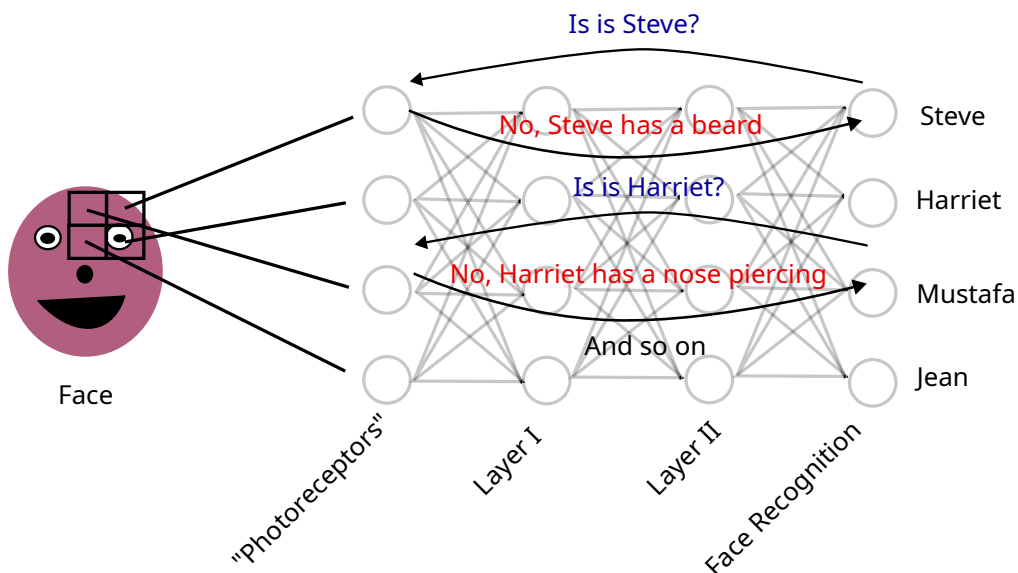
Figure 4: Predictive coding is thought to be the way our brains can make perceptual inference about the causes of a sensory stimuli. Rather than sensory information flowing from the senses and upward, a predictive model forms predictions based on a 'best guess' of sensory causes, and sends these predictions downstream to lower levels of the cortical hierarchy. The residual prediction errors are what instead flows upstream, driving changes in the brain's 'best guess' about the cause of sensory information.

the cortex and acts like a relay of sensory information. Visual information, as I have already mentioned, travels via the lateral geniculate nucleus (LGN) of the thalamus and enters the cortex in roughly the "middle" layer, layer 4. However, these thalamocortical connections only account for about 5% of the synapses in the cortex (Markov et al., 2011), and the cortex is dominated by intracortical connections which scan span several tens of mm on the cortical surface (Markov et al., 2013), in addition to long-range connections via the white matter. In addition to being laminal, the cortex is often described as being functionally grouped into "cortical columns" (Mountcastle, 1997) which operate as individual processing units and contain reciprocally connected excitatory and inhibitory neurons (Douglas et al., 1989). Sensory information reaching the cortex does not necessarily drive cortical activity, and the cortex is full of feedback and lateral connections which are also strong drivers of cortical activity (Mignard and Malpeli, 1991). These feedback connections are often laminar specific, often going from the superficial layer of one cortical column to the middle or deep layers of another (Bastos et al., 2012), and it's been suggested that the laminar organization of the cortex serves the purpose of sorting feedback and feedforward signals.

What is the reason for such pervasive and strong feedback connections in the cortex? These connections do not serve a mere modulatory role, but are thought to be essential in how the brain interprets sensory information. I think Karl Friston puts the distinction beautifully:

"In short, there is a distinction between percepts, which are the products of recognizing the causes of sensory input and sensation per se. Recognition (i.e. inferring causes from sensation) is the inverse of generating sensory data from their causes. It follows that recognition rests on models, learned through experience, of how sensations are caused"

- Friston 2005; A theory of cortical responses

What Karl is talking about here is an almost reversed view compared to that of the feed-forward notion of perception outlined in figure 2. Here we're instead talking about a top-down production of contextual expectations, rather than "generating sensory data from their causes". I have to admit, I am absolutely in love with this idea, and it's fantastic how much it can explain. Consider for example a colored object, let's say a white T-shirt. The perception of the color "white" is rather constant irrespective of ambient light levels. If you go out on a cloudy day, there's much more blue light compared to when you're having dinner in a room lit by candles, and the actual spectral components of light which reach your fovea when fixating your eyes on the white fabric will be quite different under the two conditions, yet we perceive the same color, meaning that the color white could be thought of a a recognized cause of sensory input. This phenomenon is known as color constancy (Gehler et al., 2008), and isn't believed to be simply by adaptation, or some other mundane process, but a recognized cause of sensory input (Brainard and Freeman, 1997).

A personal example that I really like is the perception of reflections. Imagine you are by a lake. You're looking down into the water and you're seeing both the brown muddy bottom of the lake and the blue light reflected on the surface. It is literally impossible to look at this scene and see what color is created by the mixture of the brown bottom and the blue reflection. Instead, what we perceive are two separate colors simultaneously, both the brown bottom and the blue reflection. This becomes very hard to explain if we think of perception as a mere feed-forward circuit.

The process of inferring causes of sensory input is thought to occur by a process of Bayesian inference. Bayesian inference is simply put a statistical model where the cause of sensory data can be inferred from sensory data if we know how a cause effects sensory data (Friston, 2003). This means the brain is basically running a complex probability model, where the posterior probability (the cause given evidence, or belief) is continuously updated based on new evidence (sensory information) weighted by the prior (background) probability. However, the probability calculation quickly becomes very difficult to compute given high dimensional sensory information, and how the brain implements Bayesian inference is not so straightforward as just a simple probability calculation. One candidate solution for this is predictive processing.

Going back to the feature detection neurons in the cat primary visual cortex. There's a very

curious phenomena known as extra-classical receptive field effects, where the response to a rectangle is eliminated if the rectangle is elongated outside of its classical receptive field. This phenomena is difficult to explain if we consider the brain as a feed-forward hierarchical system where neural spiking is interpreted as upstream signalling of sensory information. If we instead consider that the brain is doing top-down predictions of what the sensory data *should* be given the brains current prediction of the *cause* of sensory input, and that the upstream signalling instead encodes the *prediction error*, then extra-classical receptive field effects makes more sense. This is because lines in natural scenes don't suddenly stop, like the ones presented in the original work by Hubel and Wiesel, but rather continue for greater distances in the scene. The brain upon seeing the line then predicts that it ought to continue for some distance, which a truncated line doesn't, thereby making the neuron fire to signal upstream that the expectations didn't match the sensory data (Rao and Ballard, 1999).

Predictive processing matches well with my intuition for how perception works. I am able to scan the bare oak trees outside my window and quite easily spot a birds nest among the branches. You'd be hard pressed to argue that my eyes have scanned all branches and found a pattern matching a birds nest, which somehow also takes all contextual variables into account like ambient light, view angle, distance etc. Could it be that I instead detected a prediction error or a discrepancy in the statistical distribution of visual information that is consistent with a bare oak tree, and that my attention was drawn in by this discrepancy?

Human gaze has been shown to be directed by "surprise" or unpredicted stimuli, as compared to a neuronal model based on Bayesian inference (Itti and Baldi, 2005) This highlights an important aspect of the predictive processing framework, that predictions and error coding allow for the rejection of redundant information (Friston, 2018). To an animal keeping watch for predators, there's an enormous amount of redundant visual information, and if it can predict these unimportant sensory stimuli and filter them out, and instead dedicate processing of information to those sensory inputs which deviate from these predictions, then the survival benefits of such a system is obvious.

Predictive coding has been shown to be consistent with a Hebbian-style of neuronal learning (Rao and Ballard, 1999). The novelty doesn't lie in the fundamental interpretation of neurons and plasticity function, but instead in how information flows in the brain. Instead of a bottom-up feed-forward network where sensory information directly causes neural activity, we think of the brain as much more independent of sensory information, with a strong reciprocal connectivity which draws from a much wider pool of sensory context to try to infer causes of sensory information.

7 Neuronal ensembles, attractors, and predictive processing as an emergent phenomenon

Bayesian inference through predictive processing ascribes a degree of sensory independence to the brain that is consistent with cortical activity dynamics. In acute brain slice preparations with intact thalamocortical connections, the cortex shows spontaneous activity independent of thalamic stimulation. This spontaneous activity isn't random, but distinct groups of neurons, called ensembles, show strongly correlated activity. When the thalamus is stimulated, the evoked responses in the cortex are highly similar to those that occur spontaneously (MacLean et al., 2005). The ensembles also show stereotyped sequences, where one ensemble follows the activation of another in an orderly fashion (Cossart et al., 2003). Spontaneous cortical activity also includes synfire chains, neurons that fire spikes as discreet temporal sequences, possibly by an existing recurrent chain of interconnected neurons (Ikegaya et al., 2004).

Consistent with the idea that "neurons that fire together, wire together", it is possible to entrain neurons into existing ensembles by stimulation (Carrillo-Reid et al., 2016), similar to the "inception" of false fear memories discussed earlier. Neuronal ensembles have been demonstrated to behave like "attractors" which means that they act like a more or less stable state of convergence of neuronal activity. Attractors are interesting because they can instantiate such cognitive processes such as working memory, sequence generation, and pattern completion (Khona and Fiete, 2022). Partial stimulation of an ensemble may lead to the full activation of the entire ensemble, thus completing a pattern.

Neuronal ensembles and attractor dynamics are appealing concepts that matches well with the ideas of Bayesian inference and predictive processing. Ensembles have been argued to represent the building blocks of the brain's internal representations of outside causes (Yuste et al., 2024). If we consider that the brain is making predictions about sensory inputs, then the brain necessarily needs to have some way of representing sequences, and neuronal ensembles and synfire chains may be the mechanism by which the brain accomplishes this. In the context of language, words, sounds and sequences of such, can be framed as neuronal ensembles in action (Pulvermüller, 2002). As you are reading this your brain is probably making predictions about which word is going to come [****]. I bet that if we could look into your brain, the neuronal ensemble associated with the word 'Next' would have come online before seeing that bracketed frame.

Neuronal ensembles, attractors and engrams are all easily explained as Hebbian phenomena, and it follows from that that they are necessarily dynamic in nature and possibly learned traits. This is less obvious if we think about predictive processing and the hierarchical organization of the brain. The circuitry of the cortex has been studied in extensive detail for over a century, but it's really hard to pin down how much of the specifics of the cortex are

irreducible to its function. Here, I believe neuronal cultures provide a unique opportunity to examine this question, but I will cover that more in later chapters. The idea that the specifics of the brains structure is more or less redundant, and that functions are acquired by sensory experience or by some intrinsic procedural mechanism is called "neuronal constructivism" (Quartz and Sejnowski, 1997). The arguably most extreme case for neuronal constructivism is seen in hemispherectomy patients, which as a last resort treatment to severe life-deranging epilepsy, have had half their brain removed surgically (Rasmussen, 1983). This is usually done in young children, partly due to children's greater capacity for neuronal plasticity. The immediate effects of the surgery are rather obvious, like hemi-paralysis or loss of language, depending on which hemisphere is removed. However, many patients can recover to an extraordinary degree after surgery (Van Empelen et al., 2004), regaining full body control and other functions, often returning to a normal, albeit already impaired state associated with the preoperative conditions (Pulsifer et al., 2004). Hemispherectomy is perhaps the most 'macroscopic' case for neuronal constructivism, all the different senses can still reach the cortex via the thalamus, and the remaining cortex is still structured in the same laminar and hierarchical fashion. This begs the question, how 'deep' does neuronal constructivism go?

Several neural circuits have been shown to exhibit a strong dependence on neuronal activity and sensory information for development. The visual system is a particularly well-studied case where the importance of visual information is apparent. The visual system lends itself well to these experiments as it is relatively easy to deprive an animal from visual stimuli. Hubel and Wiesel, who first demonstrated the feature detection neurons in the visual cortex, also showed that development of these neurons depend to some extent on visual experience, and that there's a critical period during an animals juvenile stage where they are sensitive to visual deprivation (Hubel and Wiesel, 1970). This only demonstrated that visual experience is necessary for normal visual cortex development (of perhaps, intrinsic genetically determined circuits), but not that it is sufficient, i.e. that there's a causal link between sensory information and development of pattern detecting neurons.

A more compelling case for a causal link between sensory information and development comes from "rewiring" experiments in the visual/auditory system. If the normal visual pathway between the thalamus and the cortex is ablated in neonatal ferrets, then a new visual pathway will develop where the retinal pathway terminates onto the auditory cortex (Sur et al., 1988). In these cases, neurons which respond to primitive visual features can be found in the auditory cortex (Roe et al., 1992), strongly suggesting that the cortex learns to discriminate statistically recurring features irrespective of sensory modality.

The question whether predictive processing and Bayesian inference can in a similar way arise from a "local" neuronal mechanism, being more or less agnostic to the specific structure of the cortex is an area of active research. Some computer models have suggested that very simple homogeneous networks of neurons can utilize a 'population code' of the posterior

probability (i.e. the 'beliefs' about the cause of sensory input) (Zhang et al., 2023), (Bill et al., 2015). Other computational work has shown that predictive processing can emerge from neuronal network that are trained to minimize energy consumption (Ali et al., 2022).

An important mechanism in these models is spike-timing dependent plasticity (STDP) (Vilimelis Aceituno et al., 2020). STDP is similar to Hebbian learning, but takes into account the relative timing of synaptic excitation. Here, synapses which precede neuronal depolarization are facilitated, while those which succeeds depolarization are weakened. In this way, you can think of a neuron as finding the synaptic inputs which accurately predicts neuronal activation. STDP is known to underlie latency reduction, by facilitating synapses that predicts neuronal depolarization, one can see how latency reduction follows (Guyonneau et al., 2005).

8   Summary

In this chapter we have built neuronal network from the bottom up. We have discussed the simplified functional view of neurons as performing weighted summation of their synaptic input, and learning as an algorithm for how synaptic weights are changed, and the importance of NMDA receptors in mediating weight update. We went on to describe that neurons can be considered as little pattern-detecting devices, and the erroneous idea of a feed-forward hierarchical network of feature extraction for complex object recognition. Instead, it is believed that the brain operates under a different set of circumstances where highly contextual predictions take much more precedence. We've discussed the possibility of ensembles as the internal representations of predicted hidden causes of stimuli, and the idea of the Bayesian brain. Lastly, we've explored the reducibility of cortical structure for its function and the possibility that information is entirely sufficient for formation of predictive processing of sensory input.

Next, we will move on to how *in vitro* models can be used to model brain function. Specifically, to which extent can the functions outline in this section be explained as emergent properties of local neuronal "programs". If neurons are indeed running a local program, then we would expect that emergent phenomena of the brain could emerge from the interactions cultured neurons. As we shall see, the evidence for this is not clear-cut, and it is possible that some minimal circuit is required, or at least beneficial to the emergence of memory, learning, perception and intelligence.

I want to continue this thesis with a slightly different structure. *In vitro* models overlaps with so many areas in neuroscience and neurobiology that an exhaustive review of all relevant topics may seem disembodied from the questions at hand. Instead, I want to dedicate the rest of this introduction to a top-down view of what neuronal cultured are used for,

and how this relates to diverse phenomena of the brain, ranging from simple biophysical characteristics to complex behaviors like sleep, memory or even volition. In the end, I hope that I will have made a strong case for my position that neuronal cultures represent a unique opportunity to study the causal link between neuronal network structure and complex neurological functions.

In vitro neuronal cultures as models for brain function and biology

9 Early neuronal cultures as simple biological models

When studying the literature on cell culture, it can be surprising how long people have been culturing cells, neurons and stem cells. We think of stem cell research as this incredibly modern practice, but its roots can be traced back to over 100 years. In 1907, one of the earliest cases of culturing living tissue was performed by Ross Harrison, where the nerve growth from explanted embryonic frog tissue was observed (Harrison, 1906). In a publication from 1911, Harrison's work is mentioned as the earliest documented case of keeping living tissue alive outside an animal, but publishes results from a wide range of tissue cultures, including embryonic cells, cancer cells and cells from various organs. The procedure by then was to take very small fragments of tissue and put these on "coagulated blood plasma" upon which cells will migrate out of the tissue (Carrel and Burrows, 1911).

Many early cell culture experiments utilizes stem cells, embryonic tissue or cancer cells, in ways which may seem like they belong to a much more recent historical time period. However, the major technological advancement throughout the century has, apart from being attributed to methodological refinement, been due to a greater capability to recreate the native microenvironment of the respective tissue, and thereby enabling better recapitulation of normal cell behavior. Small molecules, proteins and more recently, mechanical factors, have been used to reduce the gap between cells in culture and cells in tissue. One great example of this is the issue of dedifferentiation, where many types of primary cells can lose their tissue-specific phenotype and function after a few weeks in culture (often measured by their enzymatic activity). In the mid 20th century, overcoming these issues by using extracellular matrix proteins allowed long-term culture without dedifferentiation (Holtzer et al., 1960).
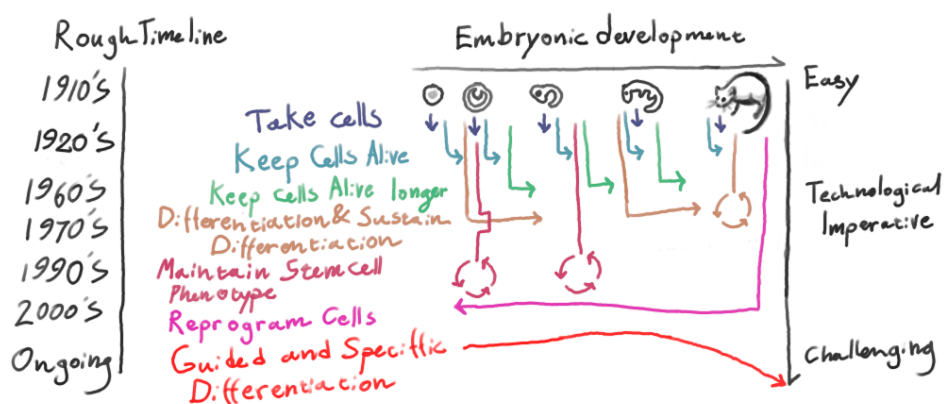
Figure 5: Rough timeline and technological difficulty of various cell culture practices. Taking cells -including stem cells- out of an organism isn't the challenging part, the challenge is to recreate the conditions of the native tissue, and to recreate the processes these cells exhibit in the organism. A major breakthrough was when adult cells could be reverted back to a stem cell state. Today, stem cells can be made to form tissue-like structures in the lab, and the process of improving these models is still ongoing.

In the 70's, researchers were developing the technique of culturing dissociated cells, and many important cornerstones of modern neuronal culture were laid down during that decade. By then, culture of neuronal tissue, known as explant culture, or ex-vivo, was already established, and it had recently become possible to perform long-term culture of tissue explants (Bunge et al., 1967). Culture of single cells was still very rare (Bray, 1973), and these early single-cell cultures were achieved by harsh mechanical dissociation of cells in tissue. The development of early single-cell cultures were driven by an outspoken re-ductionistic sentiment (Hawrot and Patterson, 1979), were the isolation of neurons from each other and from glia was seen as a new opportunity to study intrinsic neuronal mech-anisms, isolated from the rest of the tissue. Some of the questions at that time were the origin of neuronal morphology, or questions related to neuronal metabolism. It was during this time that enzymatic dissociation of cells became more common (Banker and Cowan, 1977). Enzymes such as trypsin allowed for a much greater yield in cells, which was required when studying metabolomics, for example (Yavin and Menkes, 1973). A typical problem in these early experiment was that neurons tend to survive poorly in the absence of glia, while culture overpopulation from glial contaminants was equally troublesome. In a report from a 1975 workshop on neuronal cell culture, emphasizing the critical need for technical improvements in the field, it was noted that culture technique and cell source can have a significant impact on the relative proliferation rates of different cell types (Varon and Saier, 1975).

In 1980, the first culture of dissociated neurons on micro electrode arrays was published (Pine, 1980), and it was only a few years before some of the first observations of "network

bursts" were reported (Gross et al., 1982) in dissociated mammalian CNS neurons. It's not surprising that network bursts were found so soon after the utilization of micro electrode arrays, given what we now know about how common these phenomena are. The digital data processing tools required for making the most out of the technology was not ripe by the time of these early experiments, but the practice of using dissociated neural cells from animal tissue is still used extensively today. Some of the most groundbreaking work on *in vitro* models utilizes animal-derived neurons (Kagan et al., 2022; Isomura and Friston, 2018). I will discuss the various underlying processes of network bursts throughout later sections, but in most simple sense, they arise from recurrent excitation as synapses form after seeding (Brewer et al., 2008).

Somewhat confusingly, the term "*in vitro*" is used for both dissociated cultures as described above, and the use of sliced brain tissue. The use of brain slices has been extremely important in neuroscience as it provides access to brain tissue without the interference of other brain regions or afferent stimulation. Brain slices can be used to create organotypic cell cultures (Hild, 1957), preserving some of the original cell type distributions and circuitry for prolonged periods of time (Gähwiler, 1981). I will discuss experiments from brain slices throughout later sections.

When neurons are dissociated and cultured *in vitro*, all dendritic and axonal projections are destroyed, meaning that any synchronized activity seen in such cultured neurons is a product of the formation of new functional connections between neurons. Given the right culture conditions, plated neurons will spontaneously grow new axons and dendrites, resembling that of the brain (Dotti et al., 1988), (Bartlett and Banker, 1984), and form synapses (Burry et al., 1986). The benefit of using culture systems to study synaptic formation is rather clear, we're able to continuously track the outgrowth of neurites, away from the clutter of the brain, as well as apply environmental and genetic perturbations that would be more challenging in intact tissue. Synapse formation is a highly complex, multi-stage process, and the process of synapse formation has been elucidated by utilizing cultures of neurons (Fletcher et al., 1994), where the days-long process from initial contact, to mature synapse, can be studied by fixation and histological examination at various time-points.

In vitro models also allow for control of cell composition. It is know, for example, that the appearance of astrocytes in the developing brain coincides with a great increase in synapse density. Is this a coincidence or are astrocytes actively causing synapse formation? These are the types of questions that lend themselves well to be investigated by culture models. Isolated astrocytic populations can be acquired from primary mouse tissue as astrocytes are proliferative unlike most primary neurons, meaning that the biochemical characteristics of astrocytes can be studied (Furukawa et al., 1986). Astrocyte cultures were well established already in the early 80's, and is a great example for how cell cultures gives control of environmental properties which isn't otherwise possible in living organisms (Kimelberg, 1983). Cell cultures have also elucidated astrocytes role in regulation of synaptic formation

(Meshul et al., 1987). In culture, astrocytes have been shown to support development of synapses in neurons (Nakanishi et al., 1994).

10 Stem cell derived cultures and induced pluripotency

As I mentioned earlier, many varieties of cells, including stem cells and progenitor cells, have been cultured for a very long time. The ability to maintain stem cell in a proliferative state, however, required the discovery of several key growth factors. In absence of these growth factors, neural stem- or progenitor cells may only proliferate for a limited time. Before the relevant growth factors were isolated, it was known that extracts of various embryonic brain regions could support extended proliferation of such cell (Barakat and Sensenbrenner, 1981), suggesting that some molecular component was underlying stem cell maintenance.

The identification of important growth factors took off in the late 80's. Basic Fibroblast Growth Factor (bFGF) was established as stimulating proliferation in embryonic rat neurons in 1987 (Gensburger et al., 1987), but true stem cells need to divide symmetrically, giving rise to new stem cells which are in turn capable of further symmetric division. This was demonstrated in 1994, where colonies derived from embryonic rat cerebral cortex cells could be dissociated, where these cells in turn could form new colonies (Davis and Temple, 1994). These cells formed colonies which were a mixture of both stem cells, neurons and glia, indicating that spontaneous differentiation was still occurring. The identification of epidermal growth factor (EGF) was later found to allow for sustained self-renewal of neuronal stem cells (Reynolds and Weiss, 1996).

The history of pluripotent stems cells distinguishes itself from that of neural stem cells, but share a similar historical timeline. Plurpipotent stem cells are self-renewing stem cells, just like neuronal stem cells are, but which have not committed to a particular lineage, and can differentiate into any tissue type. These cells were isolated and cultured in 1981, but relied on undefined factors, secreted by a population of teratoma cells, in order to maintain self-renewal potential *in vitro* (Martin, 1981). It wasn't before 1988 that the factors needed to sustain a pure population of self-renewing pluripotent stem cells was identified (Smith et al., 1988), which included the leukaemia inhibitory factor (LIF). This was done in mice, but it took until 1998 before the same could be achieved with human embryonic stem cells (Thomson et al., 1998), which required yet another growth factor, FGF2.

The late 20th century saw a rapidly growing capacity to recreate the biological conditions that influences cell behavior, such as we've seen with maintenance of stem cell phenotypes *in vitro*. In 2006, a major breakthrough came when induction of pluripotency was obtained by viral forced expression of transcription factors which could generate pluripotent stem cells (Takahashi and Yamanaka, 2006). This opened up completely new possibilities for *in vitro*

models, and researchers rushed to create human disease models by generating pluripotent stem cells from patients (Park et al., 2008).

It's difficult to overstate the importance of these discoveries. Stem cells, in its ideal case, have the potential to provide an endless source of any cell type (stem cell, progenitor, or mature cell) of the human body. The medical and scientific implications of stem cell research are therefore profound. However, generation of stem cells is only the first part of this story, as one then has to figure out how to derive the various cell types of interest from the stem cell source, a process of technological development which continues to this day. Thankfully, the differentiation protocols from one stem cell source often transfers directly to the same stem cell type derived from a different source (Chin et al., 2009), and when induced pluripotency entered the scene, there was already a large body of differentiation protocols available to generate neuronal progenitors (Okabe et al., 1996), and subsequently mature neurons (Temple, 1989) capable of exhibiting electrical activity (Bain et al., 1995), where retinoic acid was used for induction of embryonic stem cells into a neural lineage.

One potential milestone for the field is the replacement of dopaminergic cells in patients with Parkinson's disease (PD). In PD, dopaminergic neurons situated in the substantia nigra which normally innervates the striatum (among other targets) are lost. Potentially, dopaminergic neurons could be transplanted directly into the striatum, thereby circumventing the challenge of guiding these neurons to innervate the striatum (Björklund and Lindvall, 2000). Early attempts dates back to animal trials in the 70's (Bjo et al., 1979), but challenges in suitable cell sources, sufficient cell survival (Lindvall and Hagell, 2000), and adverse reactions such as dyskinesia (Greene, 1999), possibly due to impurities in cell grafts (Hagell and Cenci, 2005), meant that clinical implementation was stalled. The field has since developed and today, preclinical trials for cell treatment of PD are undergoing (Kirkeby et al., 2023).

Replacing cells in damaged tissue isn't always as straight forward as one might naively think. Attempts at regenerating lost tissue in for example, spinal cord injury, hasn't been very successful. Simplified, this is due to structural changes in damaged tissue, such as severed neuronal fibers and glial scarring that occurs in spinal cord injury (Clifford et al., 2023). The field of tissue engineering is the response to such challenges, which incorporates stem cell research with material science in order to create artificial grafts to repair damaged tissue (Madigan et al., 2009). Spinal cord injury is of course, symbolically a significant milestone in this field, which has yet to be reached, but important advances in other tissue types have been made

With the arrival of induced pluripotent stem cells, and the developments in maintaining and differentiating embryonic stem cells, human cell cultures became widely available. Studies deriving spontaneously active mature neurons from human stem cells began to emerge in the late 00's (Ban et al., 2007), and since then, there's been an explosion of

various model systems which utilize human stem-cell derived neurons. The use of micro-electrode arrays in combination with human neural cell cultures is a particularly attractive concept for a couple of reasons. Primarily, it enables the investigation and perturbation of the electrophysiology of human neural cells and networks, which are otherwise very rare (Howard et al., 2022). It also allows the continuous monitoring of electrical activity as the network develops over time, or as a compound or some treatment is being applied (Nimbalkar et al., 2023), unlike many other techniques where analysis constitutes an end-point. As mentioned, iPSC technology also enables the production of neurons carrying mutations which makes them susceptible to various neurological conditions, including Alzheimer's Disease or Parkinson's Disease (Caneus et al., 2024). Lastly, the use of stem-cell derived neurons and microelectrode array analysis is highly scalable and allows for deployment of high-throughput experiments and for screening of drugs or hazardous compounds (Garcia-Leon et al., 2020), (Tukker et al., 2018). In these cultures, like that of dissociated neuronal cultures from animal tissue, the spontaneous activity is characterized by highly synchronized bursts (Hyvärinen et al., 2019). The functional readout is largely based on the dynamics of these synchronous bursts (Lv et al., 2023; Obien et al., 2015; Hu et al., 2022). Sometimes connectivity metrics are also used, but it's possible that connectivity is obscured by strong synchronized activity (Lonardoni et al., 2015). I will discuss this more in the chapter related to paper IV.

Here we have looked at some of the important historical landmarks of stem cell research. The development of stem cell technology and cell reprogramming has laid the foundation of modern *in vitro* methods. Microelectrode arrays and calcium imaging has emerged as vital tools for assessing the functional properties of these models, which develop spontaneous network synchrony similar to that seen in dissociated neuronal cultures of earlier work. Given its ubiquity, synchronous network bursts have been loosely used as an indicator of a functional and "normal" network, but does this behavior have an *in vivo* counterpart? Why are synchronous bursts so widespread? I began this thesis by proposing that the functions of the brain could be thought of as emergent properties of neurons acting on a 'local' program. As we saw previously, many complex behaviors can emerge out of Hebbian and STDP plasticity rules, and presumably these should also be present in cortical cell culture models. How does this image fit with the spontaneous activity of neurons?

11 Cultured neurons as models for epileptic network activity

As mentioned, when neurons are cultured *in vitro*, they very often develop synchronized spontaneous bursts. These bursts superficially resemble epileptiform activity otherwise seen in vivo or in brain slices (Swartzwelder et al., 1987), but these similarities does not include their respective mechanisms to any large degree. Epilepsy is characterized by spontaneous seizures, and is generally thought to result from an excitatory-inhibitory imbalance,

which can become self-propagating and disrupt the normal electrical activity of the brain (Margineanu, 2010). Epileptic seizures often result in large areas of the brain becoming abnormally synchronized, showing large and often stereotyped EEG deflections (Cho et al., 2012). Similarly, many cultured neurons develop spontaneously occurring bursts, which propagate across the network to recruit a large population of neurons (Maeda et al., 1995).

Network bursts shares several pharmacological features of epilepsy. The application of GABA receptor antagonists increases the prevalence of network bursts (Gross et al., 1992), while increasing inhibition decreases their occurrence (Puia et al., 2012). This has been used to investigate a large number of neuroactive compounds, and analysis of network activity of cultured neurons has been suggested as a way to screen for potentially seizurogenic side-effects of novel drugs (Yokoi et al., 2021). However, many important features of epilepsy are not reflected in network bursts, meaning that pharmacological similarities may simply reflect that network bursts are a general indicator of network excitability. Epileptic seizures are known to cause excitotoxicity, for example, where excessive excitation leads to elevated intracellular calcium levels, triggering cell death. This is obviously not seen in spontaneous network bursts as such cultures can continuously exhibit network bursts for many months (Deshpande et al., 2007). Excitotoxicity however, can readily be induced in cultured neurons by modulating their excitability (Furshpan and Potter, 1989), resembling that of a prolonged seizure. Epilepsy has diverse causes, which can sometimes be traced to a very particular cellular mechanism (Koyama, 2013). The mechanistic resemblance of cultured neurons therefore need to be addressed on a case-by-case basis.

Another important difference is that an epileptic seizure can cause a period of transient cognitive impairment and memory loss. This can be due to indiscriminate NMDA-driven long-term potentiation which saturates the brain's ability to form new memories (Reid and Stewart, 1997). Neuronal cultures which exhibit spontaneous bursts can exhibit long-term potentiation (Shahaf and Marom, 2001; Odawara et al., 2016; Molnár, 2011), which is not expected if network bursts were mechanistically similar to seizure events.

Interestingly, if you take a slice of the hippocampus and culture it, it can develop spontaneous "interictal" (between seizures) and "ictal" (seizures) events in a time course similar to that of cultured neurons (McBain et al., 1989), showing pronounced synchronized activity in less than 30 days. Interictal spikes are interesting because they can be "silent", meaning that they are seen in EEG but show no obvious symptoms in the patient. In some animal models of acquired epilepsy, interictal spikes precedes the onset of ictal events (Staley et al., 2005). It is believed that the emergence of epilepsy-like activity in slice cultures is due to sprouting of new axons, and formation of new synapses, and subsequent reinforcement by plastic mechanisms that strengthens recurrent connections (Dyhrfjeld-Johnsen et al., 2010). Sprouting of axons is believed to be driven by activity-dependent mechanisms that are present in neurons throughout the life of an animal, having roles in development and normal plastic mechanisms of the adult brain (Sutula and Dudek, 2007).

The activity-dependent regulation of axon growth and synapse formation, and it's role in normal development, and possible involvement in the behavior of cultured neurons, will be discussed more in the next section.

12 Neuronal cultures and synaptic homeostasis

Epilepsy is a common complication following traumatic brain injury, with onset of seizures occurring weeks- to years after initial injury (Salazar et al., 1985). In animal models, it's known that deafferentation of a cortical area, by severing the white matter, quickly (hours) leads to abnormal self-excitation, and continues to develop hyperexcitability over several weeks (Topolnik et al., 2003). One popular explanation for this is that the deafferented neurons exhibit a phenomenon called homeostatic synaptic plasticity, and explains the increased excitability as the formation of new synapses in response, not to the injury, but to the decrease in synaptic transmission that arises from severing of white matter (Avramescu and Timofeev, 2008). Homeostatic plasticity is a theory that states that neurons have a set-point in excitability that they will actively maintain. Too little excitation will lead to formation of new synapses, and vice versa.

Synaptic homeostasis solves a more fundamental issue about brain plasticity. As we discussed in the earlier chapters, many neurons have the capacity to enhance synaptic connections from the timing and patterns of incoming synaptic transmission. If synapses are dynamic in strength, how do they remain stable and prevent runaway plasticity? Synaptic homeostasis offers a potential solution to this problem by balancing neuronal activity in the face of plastic changes (Desai et al., 1999).

Synaptic homeostasis is easily observed in cultured neurons, and stability of excitability likely occurs by several different mechanisms. If neuronal signalling is blocked by application of tetrodotoxin (TTX) for prolonged periods of time, then neuronal networks will become more excitable following removal of TTX (Yuan et al., 2023). This has been seen reflected in the intrinsic excitability of single neurons, that is, neurons will produce more action potentials following equivalent depolarization than before TTX application (Desai et al., 1999). Similarly, the amplitude of single EPSP events are scaled in an activity dependent manner, such that prolonged inhibition of a network will increase EPSP amplitude, while prolonged disinhibition by GABA receptor blockers will decrease EPSP amplitude (Turrigiano et al., 1998). NMDA receptor-driven dendritic protein synthesis and AMPA receptor expression has been implicated in regulating synaptic scaling (Sutton et al., 2006), as well as formation of dendritic spines (Hamilton et al., 2012).

Considering neuronal cultures as sharing some mechanisms with deafferented cortex is a popular idea and seems to align well with our observations and knowledge of neuronal be-

havior. It's interesting to speculate that this is what leads to the stereotyped developmental time-course of many neuronal culture models. Many neuronal models develop synchronized bursts around the same time, after about two weeks (Hyvärinen et al., 2019), after which they enter a more or less steady state of network activity(Maeda et al., 1995; Van Pelt et al., 2004). Could it be that the neuron's form synapses until they reach a set-point in activity?

The interpretation of neuronal cultures as exhibiting abnormal synaptic development, similar to a cortex which has been deafferented following head trauma, provokes several interesting questions. Firstly, how does this influence the translational value of such neuronal cultures? Ideally, if we want to use such neuronal cultures to model diseases or toxicological response, we would want the culture to resemble the target tissue as much as possible, and having an abnormal synaptic profile could lead to unfavourable discrepancies in the model. Secondly, the developing brain does not receive a wealth of sensory information during embryonic development (although spontaneous sensory information is a vital part of development), and evidently, the developing brain does not show aberrant hyperexcitation, so how is synaptic homeostasis kept in check during development? And lastly, are there techniques which let us overcome these issues and create neuronal models which do not form hypersynchronous activity but rather resemble the structure and functional behavior of a real brain? I will attempt to answer these questions in the following sections. First, we should briefly look at another interesting candidate explanation for synchronous network bursts in cultured neurons - sleep.

13   Sleep in cultured neurons

Sleep - is a surprisingly interesting phenomena - one could say somewhat humorously. From our personal subjective experience, sleep seems to be where things stops happening. A bit like when a computer is powered off, the hardware is still the same but most of the doing, the action, is gone. Surely this state of mere vacancy wouldn't be a very useful and interesting subject to dedicate one's academic career to? Today, sleep isn't believed to be simply the eclipse of wakefulness, but an active and highly derived system involved in processing of information and homeostatic control.

Our dreams are perhaps a glimpse into the world of sleep. Dreams are subjective phenomena, but their structure shares some similarity to what is known to happen in the brain during various stages of sleep. Dreams, at least for me, seem to whizz past at unrelenting speed. The ordinary pace and rhythm of life, the moments of reflection, the monotony, anticipation, all seems to be missing in dreams. When was the last time you had a dream where not much happened for a long time?

In the hippocampus, sequences of experiences that have been acquired during wakefulness, are replayed at many times their ordinary speed during sleep (Skaggs and McNaughton, 1996). The rat hippocampus is known for its accommodation of place cells, which are active when freely moving rats perceive themselves to be in a certain place (O'Keefe, 1976) - a neurological basis for a cognitive map perhaps? (Shapiro, 2001) If a mouse is left to walk around in a maze, the sequence of places it visited during the awake state, will be replayed in the hippocampus when its sleeping. This "replay" of memory sequences, is believed to be a way for the hippocampus to consolidate memory. Memory consolidation is generally thought of as a process of storing memory in a more lasting, and more distributed format. The hippocampus can be interpreted as temporary memory buffer for volatile memory, which is transferred to the cortex during sleep, and where replay of memories could be an important mechanism by which this process occurs (Ji and Wilson, 2007). Damage to the hippocampus, consequently, can result in the ability to acquire new memories, but not the loss of past memories (Smith and Mizumori, 2006).

Sleep, or perhaps more accurately, wakefulness, is regulated by several important neuro-modulatory agents that are secreted by subcortical brain structures. Most cultured neurons are grown in the absence of neuromodulatory cells. It is, however, fairly straightforward to mimic the effect of neuromodulatory cells by applying neuromodulatory agents to the culture medium. When adding wakefulness-stimulating neuromodulators such as serotonin, histamine, dopamine, acetylcholine or norepinephrine (or often, analogous compounds such as carbachol rather than acetylcholine), cultured neurons will transition from exhibiting spontaneous synchronized bursting, to a desynchronized pattern of activity (Yokoi et al., 2019).

This is akin to the characteristics of network behavior in the cortex during sleep and wakefulness, and prolonged exposure of cortical neurons can induce expression of genetic markers of wakefulness (Colombi et al., 2016). Making cultured neurons "wake up" can trigger sleep rebound (Yokoi et al., 2019; Hinard et al., 2012), similar to how parts of the cortex that have been persistently activated during wakefulness, will show a stronger sleep signature during subsequent sleep (Vyazovskiy et al., 2000). A possible interpretation of this is that sleep and wakefulness is a bit like a playground swing, where neuromodulatory circuits and sensory stimulation pushes the rest of the brain into wakefulness, after which is will on its own swing back into a sleep state (Krueger et al., 2019). In cultured neurons, no one is pushing the swing, and perhaps the cultured neurons are stuck in the penumbra between sleep and wakefulness.

Normal Brain   In Vitro Culture Models

Figure 6: Neuromodulators pushes the brain into an awake state, and prolonged wakefulness makes the brain swing back into sleep. In many neuronal cultures, this process is absent, leaving the cells potentially as neither quite awake nor sleeping.

14   Developmental processes in neuronal cultures and organoids

In the previous chapters, I may have alluded to a false dichotomy that the development of the brain, and the subsequent plasticity in response to sensory information are two entirely separate events. As we've discussed in the previous chapters, neurons are plastic in hebbian and homeostatic ways, and it's not like these functions suddenly come online at birth, rather, these processes are intimately linked to the development of the brain.

The development of the brain begins with neurulation at 2-3 weeks of gestational age, where the neural plate folds to form the neural tube. This establishes the fundamental radial morphology of the central nervous system, which acts as the scaffold for many subsequent developmental events, including the formation of cortical layers. The neural tube subsequently forms a rostral-caudal organization, where the precursors of forebrain, midbrain, hindbrain and spinal cord form along the length of the neural tube (Rhinn et al., 2006).

The lumen of the neural tube, which later develops into the ventricles of the central nervous system, are lined with the proliferative ventricular- and subventricular zones. Neurons are proliferating in the ventricular zones from gestational week 5 and onward (Bystron et al., 2008), and migrate outward to form the characteristic layers of the cerebral cortex. Radial glia, which as the name suggest, radiate outward from the ventricular zones, and serves as guides for neurons on their migratory path towards superficial laminae (Rakic et al., 1994). The deeper layers of the cortex are formed earlier than the superficial ones (Hatten, 1993), and the characteristic gyrification of the human brain is due to the "lateral" proliferation of intermediate radial glia cells, which are not connected to the ventricular zone (Borrell and Götz, 2014).

It's these developmental processes which are recapitulated in cerebral organoids. Organoid technology, which took the world by storm about 15 years ago, are made from clusters of pluripotent stem cells. I said earlier that the developmental trajectory of *in vitro* research can be summarized as gradual increments of recreating the tissue micro environment *in vitro*. Organoids represent the pinnacle of this developmental trajectory, where much of the process from pluripotent stem cell to formation of tissue structures and mature cells is reproduced. The first cerebral organoids, described in 2013, utilized a complex multi-step induction protocol, drawing from the knowledge of *in vitro* differentiation that had accumulated over several decades, but with the important addition of embedding cell clumps in a gelatinous extracellular-matrix derivative (Lancaster and Knoblich, 2014). These early cerebral organoids showed a somewhat chaotic, but nevertheless clear organotypic laminar and radial features that we otherwise see in the brain (Lancaster et al., 2013).

These early cerebral organoids generated highly diverse cell populations (Camp et al., 2015), showing markers for cortex, forebrain, midbrain and hippocampus (Lancaster et al., 2013). Thanks to another piece of modern technology, single-cell RNA sequencing enabled large scale analysis of cell populations in cerebral organoids, showing that many developmental signatures are accurately recapitulated in cerebral organoids (Camp et al., 2015). Another important issue of early organoid technology was that the cells did not reach full maturity, and retained a large population of stem cells, progenitors and immature neurons, even after many months of differentiation (Quadrato et al., 2017). These challenges persist to this day (Andrews and Kriegstein, 2022), and the field can still be considered to be in a quite young stage.

There's been extensive research in developing region-specific organoids. These include cortical organoids (Magni et al., 2022), striatal organoids (Chen et al., 2022) and midbrain organoids (Smits and Schwamborn, 2020) to name a few. Another more recent innovation is to differentiate organoids separately to induce organoids of different identity, and later fuse them to create an "assembloid" - a structure which recapitulates the region-specific interconnections of separate brain regions. This has been done to recreate the thalamo-cortical innervations (Xiang et al., 2019), cortico-striatal (Miura et al., 2020), and the specific developmental events underlying the migration of interneurons to the cortex (Xiang et al., 2017).

Spontaneous electrical activity of cerebral organoids was, up until a few years ago, characterized by weak and asynchronous electrical activity (Sakaguchi et al., 2019). Only as recent as 2019 was robust electrical activity described in brain organoids (Trujillo et al., 2019). This experiment not only showed that organoids could develop spontaneous activity, but that it shares a developmental timeline with that of human fetuses. Further studies have revealed the expression profile of organoids that underlie the development of spontaneous activity (Fair et al., 2020). Only last year, synaptic plasticity in thalamocortical assembloids was demonstrated (Patton et al., 2024), really demonstrating how far we have gotten, and
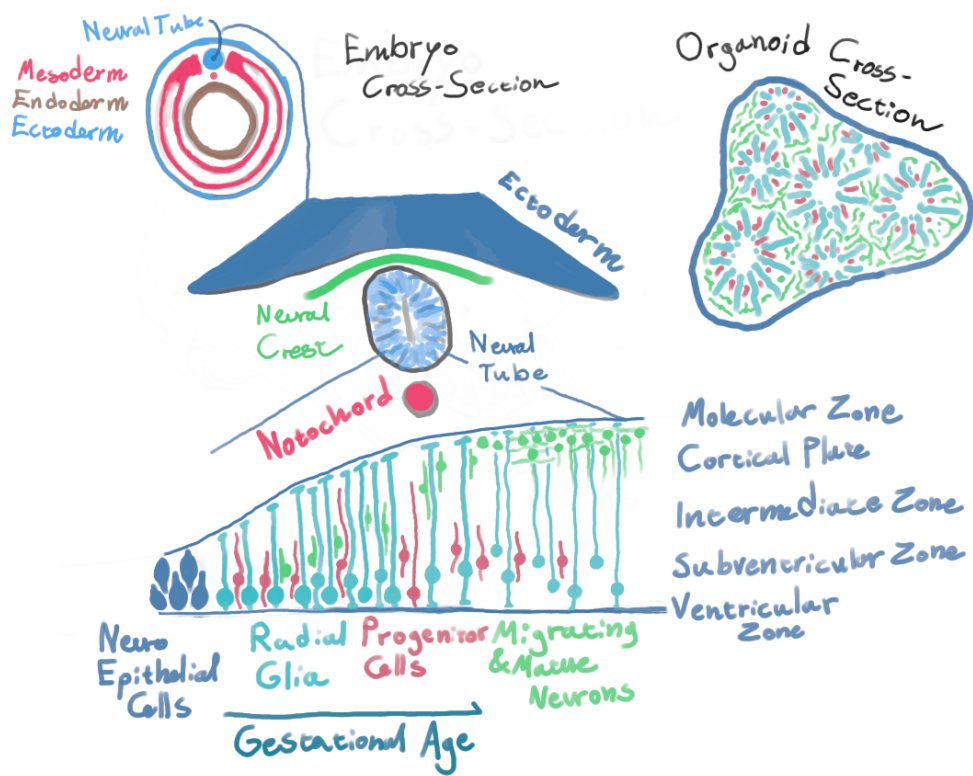
Figure 7: The development of the central nervous system starts early in embryonic development by the formation of the neural tube. Throughout development, the brain retains this fundamental organization of having an inner lumen, what eventually becomes the ventricles, and an outer surface which develops into the cortex. Cells are proliferating close to the ventricular side of the tube and migrate along radial glia to the outer regions of the tube. This is the process that organoids attempts to reproduce.

perhaps even instilling some trepidation as to what are ahead of us.

Ironically, or perhaps absurdly so, the spontaneous electrical activity of cerebral organoids come in the form of synchronized network bursts, highly reminiscent to that of dissociated neuronal cultures. It's quite remarkable that throughout the almost 50 years of growing neurons *in vitro* and analysing their electrical activity, the formation of synchronized network bursts has been constantly looming over us.

15   Electrical activity in developing neuronal networks

The developing nervous system shows a diverse form of spontaneous electrical activity. These spontaneous activity patterns have been studied extensively, and are driven by vari-

ous mechanisms depending on developmental age and region. Generally speaking, activity in the immature brain is characterized by synchronized activity, perhaps pointing to some overlap with the spontaneous activity exhibited by neuronal cultures. Spontaneous activity in the cortex and hippocampus is involved in many basic features of neural development, such as differentiation, apoptosis and migration (Kirischuk et al., 2017). In rodents, spontaneous correlated neuronal activity emerge around the time of birth (Garaschuk et al., 2000). These spontaneous events include intrinsically generated synchronized bursts, which interestingly, are at least partially driven by excitatory GABA activity (Ben-Ari et al., 1989). This is made possible by a reversal in chloride potential, making chlroide conductance excitatory rather than inhibitory (Lombardi et al., 2018). Spontaneous activity is eventually replaced by less correlated spontaneous activity, and is correlated with the transition of GABA from being excitatory to being inhibitory (Ben-Ari, 2001). A similar mechanism of a "GABA switch" has been observed in some organoid models (Zafeiriou et al., 2020).

Spontaneously activity is also present in sensory organs, such as in the retina. Dark-reared animals, which have no visual experience, still develop rudimentary orientation-selective neurons in the visual cortex, but visual experience is required for full development of mature orientation-selectivity (White et al., 2001). Experiment with sensory deprivation has elucidated a "critical period" for which orientation-selectivity can develop (Hubel and Wiesel, 1970), although some experience-dependent recovery can be exhibited outside this critical period (Hooks and Chen, 2007). This image is however complicated because sensory experience also influence how plastic the given circuit is (thus a form of meta-plasticity) and sensory deprivation can increase plasticity in the visual cortex (Lee and Nedivi, 2002).

Experience-independent formation of circuits in the brain are strongly driven by patterned spontaneous activity in sensory organs. In the retina, spontaneus patterned activity, "retinal waves" drives several important circuits to form in both the cortex and in subcortical regions. Ocular dominance in the lateral geniculate nucleus (LGN) is dependent on retinal waves which drives segregation of synaptic fields from the retina (Penn et al., 1998). Disrupting retinal waves inhibits the formation of ocular dominance, possibly by a form of hebbian competition (Burbridge et al., 2014). Artificially synchronization of spontaneous retinal activity can also inhibit formation of ocular dominance, indicating a form of Hebbian source-separating mechanism (Zhang et al., 2012). Interestingly, retinal waves play a role in developing optic flow sensitivity, and act to simulate how visual features propagate over the retina as a mouse is moving forward (Ge et al., 2021).

The formation of orientation-selective neurons in the visual cortex is believed to be driven by a similar Hebbian mechanism. Again, by inducing artificial correalted activity disrupts formation of orientation-selective neurons in the visual cortex (Weliky and Katz, 1997). Similarly, the barrel cortex in rats, which is distinguished by a clear topographical map of the rat's facial whiskers, does not develop properly in absence of sensory stimuli (Erzurumlu and Gaspar, 2012).

In the context of modelling various forms of plasticity and circuit formation *in vitro*, these findings raises several important questions. The presence of a "critical period" for establishment of orientation selectivity begs the question if a given neuronal culture system exhibits the neccessary phenotype for supporting plasticity. Another interesting question is if there are structural prerequisites that enables these plastic changes. Investigating an 'enabling' role of brain structure is difficult due to the multi-faceted spontaneous activity in the cortex. The thalamus also show spontaneous activity, which influences circuit formation in the cortex as well (Martini et al., 2021). However, as these phenomena are likely driven by a Hebbian learning mechanism, we can expect that at least *some* cortical organization is required, or at least can facilitate, the source-separation by cortical neurons. As I have mentioned, Hebbian neurons utilizes input correlation structure in order to develop selectivity for input sources. If different input stimulation (such as from different visual patterns on the retina) all lead to strong depolarization of a cortical neuron, it will have a hard time developing selectivity to any of these inputs. It's possible that neurons *in vitro* show exessive recurrent connections, leading to large and unspecific responses to different stimuli (Ruaro et al. (2005)). This is contrasted by the sparse activity which develop as a brain matures (Colonnese et al., 2010), which is hyperexcitable before eye opening in mice (Shen and Colonnese, 2016). An interesting interpretation here is that increased excitation by GABA acts to prevent formation of excessive recurrent synapses, thus allowing more selective responses as eyes open and sensory experience begins (Colonnese et al., 2010).

Recently, implanting organoids into the cortex of animals have shown that organoids can functionally integrate with the host network and participate in sensory processing, even exhibiting orientation-sensitive cells when transplanted to a cavity in the visual cortex (Jgamadze et al., 2023). Transplanted organoids develop mature network activity (Wilson et al., 2022), unlike the synchronous activity of organoids grown *in vitro* in absence of sensory stimuli. I mentioned the concept of neuronal constructivism, which argues that neuronal circuits are highly flexible and that sensory processing is an emergent phenomena of underlying principles of plasticity, rather than deriving from a genetically encoded circuit. An organoid integrating into a host brain and participating in sensory processing quite strongly supports this idea. However, it could be that the organoid forms some important microcircuit which facilitates this capacity to effectively process information. Therefore it would be very interesting to see what would happen if a dissociated organoid, so a similar cell population but without any tissue-like structure, could also functionally integrate. Long before organoids were popular, it was known that neuronal precursors could be transplanted into a living host, and integrate and form long-range connections (Shin et al., 2000). However these neuronal transplants relies on existing cortical structures (laminae and white matter fibers) which likely serves as guides for transplanted neurons. Transplanting spheroids, which are cell clumps but without the tissue organisation of an organoid, shows ability to integrate and replace a cavity in the brain (Kim et al., 2023b). Although some recovery of cognitive function following a stimulated injury, direct evidence of functional integration

hasn't been demonstrated to my knowledge. So far, the question remains unanswered, if there are some minimal circuit architecture that is required in order for information processing circuits to develop from information input.

The ability for organoids and spheroids to functionally integrate into cortical networks suggests that these cells have the potential to self-assemble into information-processing circuits. Perhaps they could even develop, through an experience-dependent mechanism, the ability to perform Bayesian inference through predictive processing. Again, the reason why we should be interested in the potential for neurons to develop these functions from mere sensory input, with little or no structural specificity, are the following: Understanding the underlying "rules" governing sensory-dependent plasticity could lead to a more fundamental appreciation of neuronal function and inspire the future generations of AI. Also, the understanding of how these processes underlie normal brain development shifts the focus from circuits to the dynamics underlying the formation of circuits, and could help us understand neurodevelopmental or psychiatric disorders.

16 Plasticity in neuronal cultures

Micro electrode arrays often allow for both recording and stimulation of neurons *in vitro*. In the 90's, digital development made recording, data processing, and complex automated stimulation regimes possible. In the 90's, extensive attempts at observing learning and memory in cultured neurons was performed. There was an explicit sentiment to uncover a "universal learning rule" that could be independent of a specific circuitry (Marom and Shahaf, 2002). Early attempts showed that cultured neurons spontaneously change their response if electrical pulses were repeatedly delivered at single electrodes (Shahaf and Marom, 2001). Because cultured neurons often exhibit strong spontaneous activity, it's possible to observe how the spontaneous activity is changed by stimulation (Jimbo et al., 1999). By stimulating repeatedly on a single electrode, it's possible to observe how changes in spontaneous activity increases over time, and eventually reaches a plateau. When another electrode is stimulated, a similar curve appears, and when the first electrode is stimulated again, changes in connectivity is much smaller than initial stimulation (le Feber et al., 2015). Potentiation by tetanic stimulation can also change stimulus-evoked responses (Maeda et al., 1998), and simple pattern-recognition has been demonstrated (Ruaro et al., 2005).

Even early on, attempts to create embodied closed-loop systems were made, where simulated sensory information from a robot (Bakkum et al., 2007; Novellino et al., 2007) or a computer-simulated environment (DeMarse et al., 2001) was fed to a culture of neurons while neuronal responses were interpreted in real time as motor actions. Some work has been able to show that an embodied system can show experience-dependent improvement in simple object-avoidance tasks (Tessadori et al., 2012). These experiments showed that

neuronal cultures, even though they lack the structure of the cortex, are capable of exhibiting very simple intelligent behavior (Bakkum et al., 2004). However, an important question here is if the process by which these neurons learn is mechanistically similar to how neurons learn tasks in the brain.

Since these early attempts at using cultured neurons to model intelligent behavior, some notable advancements have been made. In 2015, a rather clear case for source separation by cultured neurons was made, where neurons were fed complex noisy composite stimuli of two sources, analogous to hearing two voices with different characteristics (Isomura et al., 2015). The researchers went on to elaborate their analyses of these results in two follow up papers, indicating that the neurons may perform source separation by a process of Bayesian inference (Isomura et al., 2023, 2015). In 2022 it was shown that neurons were able to learn to play a simple video game with some non-zero measure of proficiency (Kagan et al., 2022). This publication became very noticed, and was strongly criticised for being sensationalistic in their communication of their results (Balci et al., 2023). This was publicly addressed by the original authors (Kagan et al., 2023) on the account that the particular terms they used to describe their results was in fact in line with their definitions. Nevertheless, it's worth pointing out that while the authors felt it was warranted to describe their neurons as "exhibiting sentience", the success-rate of the neuronal network where just barely better than chance (still impressive, in my opinion).

It's surprising to me, that with the surge of interest in organoids and human cell culture, and the wide adoption of microelectrode arrays for investigating their spontaneous electrical activity, that the study of neuronal response to input stimulation hasn't been more widespread. By feeding cultured with simulated sensory input, it could be possible to create models of human cognition *in vitro*. It's not clear to me why this remains a rare topic of study, but I am willing to make a few informed guesses: 1) Technical challenges and scalability. Each system for recording and stimulating a neuronal culture on a micro electrode array is expensive, meaning that most people will only be able to stimulate one culture at a time. Providing complex stimulation patterns requires specialised equipment, and often uses in-house manufactured equipment and software. 2) Interface limitations. Electrodes are rather crude tools for stimulating neurons, probably stimulating rather big populations from a single pulse. Recording the neuronal responses with standard passive MEAs will at best record from around 100 neurons, which is often less than 0.1% of the total population. It could also be that we don't know how to properly encode sensory information to stimulate the neurons effectively, and likewise may be true for how we interpret their responses. 3) Biological limitations. It is possible that neurons in culture are somehow biologically not representing real tissue in terms of micro-environmental factors, or necessary modulatory input from other brain areas. 4) Lack of circuit structure. It is possible that some degree of network organization is required for neuronal cultures to effectively learn and respond to stimuli.

There are some promising results which indicate that cultured neurons are competent to replicate plasticity and learning *in vitro*, but several questions remain. These experiments I have mentioned above don't elaborate much on which mechanisms are involved in plasticity. (Isomura et al., 2023) saw that blocking NMDA receptors reduces the ability to distinguish between sensory inputs, but the effects size was not very big. One interesting way of examining the mechanisms involved in these experiments would be to utilize reporter constructs of immediate early genes to observe which neurons are expressing long-term potentiation (Xie et al., 2014). Learning, as I've mentioned before, is expected to involve a sparse representation in the neurons. In the experiments from isomura et. al. responses from stimuli are very large, and recruits most of the neurons in the culture, even after training (Isomura et al., 2023, 2015).

Very few experiments look at long-term changes in spontaneous neuronal activity following training. In one experiment, micropipette injections of GABA receptor blockers could stimulate formation of memories (Baruchi and Ben-Jacob, 2007), giving rise to new states of spontaneous neuronal activity patterns. However, the "memories" that were created showed a strong statistical inter-dependence, as evident by the formation of discrete clusters in PCA space. Clearly, new states could be exhibited by the cultured network, but it's not clear if this resembles how memories are formed in the brain, and further studies on using micropipette injections to entrain memories have not been done, to my knowledge. The formation of new states in cultures of neurons by delivery of sensory stimuli would be an important finding on the path toward *in vitro* models of cognition. We know that cortical neurons organize themselves into ensembles, which become activated both spontaneously and by thalamic stimulation (MacLean et al., 2005), and that neurons can be recruited to ensembles by concurrent stimulation (Carrillo-Reid and Yuste, 2020), similar to how receptive fields can be modified by Hebbian mechanisms (Eysel et al., 1998). At least one experiment has demonstrated that it's possible to evoke several different activity patterns in cultured neurons, patterns which occur spontaneously as well (Pasquale et al., 2017), possibly connecting cultured neurons to the behavior of spontaneous activity in the cortex. These events are however, still encompassing much of the neuronal population, unlike ensembles, which are sparse.

In one recent publication, organoids were argued to exhibit spontaneous arealization and formation of clusters of activity (Sharf et al., 2022). However, this was based on the observation that spikes across different areas of the organoid were phase-locked to different angles of a theta-oscillation. It strikes me that given the large size of the organoid (several mm in diameter), such phase locking could occur simply by slow spatial propagation of neuronal activity, so I am personally a bit sceptical. These organoids, of course, also exhibited spontaneous synchronized bursts.

The results that I have outlined in the previous sections leads me to believe that synchronized activity in neuronal cultures results from excessive recurrent synapse formation, as a

result of homeostatic plasticity. The lack of afferent input leads these neurons to become self-excitatory, and unable to develop something analogous to "receptive fields" in the visual cortex. The formation of highly recurrent connections means that stimulation will evoke large portions of the network (Ruaro et al., 2005). Remember that, in order for neurons to facilitate synapses, NMDA receptors acts to strengthen synapses which are involved in strong neuronal depolarization. If several different stimuli all provokes most of the neurons to become depolarized, then the neurons will have a very hard time distinguishing between which stimuli was presented. In other words, the representational ability of neurons is severely diminished by excessive recurrent synapses. In paper IV, I show that stimulating various patterns in cultured neurons when cultured in microchannels that control their circuitry, the different stimuli provokes separate responses, but I will discuss this in more depth later.

17 Summary and justification for my experiments

We've now looked at the field of *in vitro* neuronal culture research and the important limitations of standard neuronal cultures and organoid research. In summary, we can describe the field and the historical development as having been largely successful in generating biologically and developmentally relevant model systems. We've gone from the use of dissociated neuronal cultures to model simple processes of axonal growth and synaptic formation, to complex stem-cell derived cultures recapitulating developmental processes and human pathological states. The ability to model cognitive functions has on the other hand not been realized to the same extent as the biological functions, and several findings point to the possibility that the lack of neuronal network structure and excessive recurrent connections could be an underlying factor. My work has been centred around overcoming this obstacle by utilizing various techniques in order to guide neuronal network formation *in vitro*.

Bioprinting neurons on electrospun fiber substrates (Paper I)

18 Background - The predicament of *in vitro* models

An important limitation for many *in vitro* neuronal models is the lack of macroscopic control of neuronal circuitry and cellular localization. Dissociated neuronal cell cultures do not form structured networks resembling those found in the brain, which are structured and according to several different principles, such as hierarchy (Markov et al., 2014), modularity (Narayanan et al., 2015), (Mountcastle, 1997), small-worldness (Bassett and Bullmore, 2006), integration and segregation (Friston, 2002). Additionally, ordinary culture protocols do not allow the creation of multiple populations of cells with different identity, which in the brain underlies several important circuits such as cortico-thalamic (Steriade, 2001) or cotico-striatal (Peters et al., 2016) circuits. There's also growing concern that the mechanical properties of flat and hard culture substrates are directly altering the cellular phenotype of neurons (Ko and Frampton, 2016), possibly producing abnormal properties even on the cellular level.

Organoids are heralded as the champions of these predicaments, allowing neuronal stem cells and precursors to self-assemble according to their intrinsic genetic instructions (Lancaster et al., 2013). Organoids are, however, not the be-all and end-all for *in vitro* models, and apart from simply not being technologically fully mature, organoids are expensive and take a long time to produce (Andrews and Kriegstein, 2022). More light-weight alternatives to organoids are therefore in high demand. If there were an approach which could generate the native structure, 3-dimensionality and cellular localization which organoids bring to the table, yet increase throughput and reduce culture times, then this approach could fill an important vacant niche in the *in vitro* repertoire.

This is the prospect that drives the field of 3D culture, and a diverse set of different materials and fabrication techniques have emerged for creating culture substrates that promote

formation of cultures with various tissue-like characteristics (Ravi et al., 2015). In the simplest case, cells can be cultured in a gel to recreate suspension in soft tissue. The gel can either be a derivative of extracellular matrix, or defined and functionalized hydrogels such as agarose or alginate gels (Caliari and Burdick, 2016). This was the technology which enabled organoid research, but also other interesting findings, such as *in vitro* Alzheimer's pathology showing formation of amyloidβ plaques (Choi et al., 2014).

Hydrogels only solve the issue of substrate mechanical and biochemical discrepancies of traditional cell cultures. Using fibers, instead of uniform gels, is an interesting alternative as fibers of the right diameter have the ability to guide neurite growth (Schnell et al., 2007). This has been explored as a way of creating structured human neural stem cell cultures (Englund Johansson et al., 2017). fibers can be mass-produced by a low-tech process called electrospinning, where a high voltage source is used to charge a polymer solution such that the solution self-repels into a thin fiber. Electrospinning is a mature technology that has been around for a long time, and there's a great variety of protocols for spinning different material with different morphologies (Kim et al., 2023a). Electrospinning yields a thin "sheet" of fibers, and by using multiple sheets and assembling them in various ways, it is possible to create laminar and aligned morphologies which underlies many tissue morphologies. This principle has been used to create artificial tissues such as myocardium (Eom et al., 2021), blood vessels (Ercolani et al., 2015), tendons (Brennan et al., 2018), skin (Sundaramurthi et al., 2014) and of course nervous tissue (Panseri et al., 2008; Bourke et al., 2014; D'Amato et al., 2019). Small physical structures are known to influence the orientation of growing neurites (Johansson et al., 2006), which likely underlies the guidance of neurites by fibers. By using fiber constructs, it is possible to create porous nerve grafts which allow perfusion and infiltration of host neurites (Frost et al., 2018).

18.1   Our approach and key findings

The use of fibers to control neuronal circuitry *in vitro* remains an understudied topic. Most approaches for controlling neuronal circuits *in vitro* have been through the use of soft lithography (paper III), with only a few reports demonstrating multi-unit recordings from neurons grown on electrospun fibers (Han et al., 2022). When I started working in the lab, we were in a collaboration that was trying to develop a system which could facilitate electrophysiological access of neurons grown on fibers. Initial attempts were unsuccessful in obtaining reliable electrophysiological recordings. Several techniques were attempted, which either involved placing the fiber mesh, with cells and all, on a micro electrode array, or using a variety of extracellular electrode probes. We reasoned that the relatively low cell densities our seeding technique produced simply made a successful recording unlikely. Live cells in electrospun fibers are almost impossible to discern by microscopy, so probing with an electrode always has to be done blindly. If using a micro electrode array, there's of

course very limited ability to control the position of the electrodes relative to the fibers.
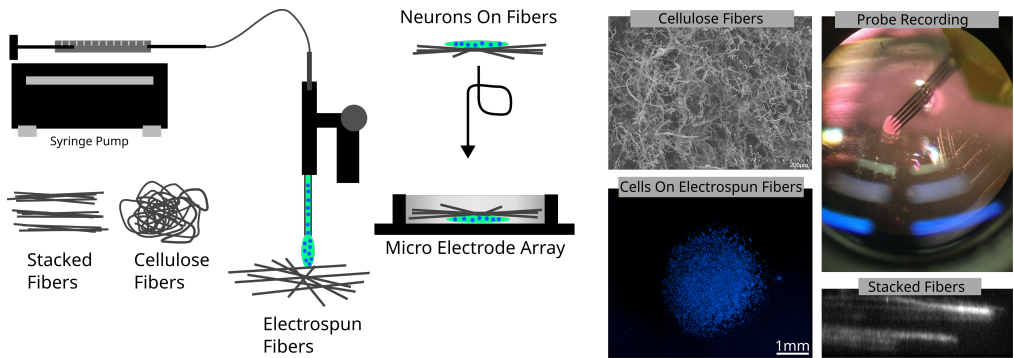
Figure 8: Conceptual outline. A syringe pump extrudes a alginate/cell suspension onto or into a fiber substrate of choice. The fibers are submerged in calcium-containing medium which causes gelation of the cell suspension. The high density gel-fiber hybrid cultures are then placed on microelectrode arrays for elecrophysiological recording, or recorded using extracellular probes.

To overcome these challenges, we decided to develop a new seeding technique to control cell distribution on electrospun fibers (Figure 8). In traditional flat solid culture substrates, it's easy to create high cell density by simply placing a small droplet of cell suspension on a dry substrate, wait for the cells to attach, and then fill up with culture medium. This isn't possible in electrospun fibers, as the fibers are hydrophilic (they have to be in order for cells to attach and for the fibers to be wettable) and have a very high surface area, so a small droplet of cell suspension will immediately become dispersed throughout the fiber mesh. We therefore took inspiration from the field of bioprinting, and developed a simple device that could extrude alginate-suspended cells through a small diameter syringe onto a fiber substrate. Alginate was used as it has the property of a viscous fluid in the absence of calcium, but becomes a solid gel when calcium is added. This way, the cell suspension can be fluid inside the syringe, but extruded out into a calcium-containing medium, leading to gelation on the fiber substrate (Axpe and Oyen, 2016). Alginate gelation is also reversible either by chelation of calcium by citric acid, or by enzymatic degradation, meaning that we could also release the cells from the gel to facilitate fiber entry. This approach would also open up new possibilities for *in vitro* models. In theory, one could seed several small populations of different cell types in controlled locations. Combined with the ability to control neurite growth by fiber morphology, this could allow reconstruction of circuits of the nervous system.

Developing this method implied several challenges. Firstly, the extruded gel needs to successfully attach to the fibers. If gelation occurs too quickly, then it will not 'grip' onto the fibers. If gelation occurs too slowly, then the cells will become too dispersed. Further challenges included the gel attaching to the syringe nozzle, and gelation causing clogging of the nozzle. We eventually came up with a protocol that could create neat cell deposits of

a few mm in diameter, both retained in alginate but also released by enzymatic degradation of gel. We then had to test if the procedure was compatible with living cells. The primary concern was that shear forces, caused by the movement of fluid through the nozzle, could kill the cells (Axpe and Oyen, 2016). Shear forces are also stronger in viscous fluids, and with small diameter nozzles. We tested the approach using a neural stem cell line, and could confirm normal cell development, cell viability, and quantify the reliability of cell deposition area, and increased cell density. We did some preliminary testing on using this approach to seed on other fiber substrates, including stacked fibers and cellulose fibers.

The human neural stem cell line that we used sporadically develops functional synapses and spontaneous network activity (Jakobsson et al., 2017). In order to see if our new method for creating high density cell cultures on fibers could create electrophysiologically accessible cultures, we seeded commercial iPSC-derived neurons using this approach. We attempted recordings with micro electrode arrays, 3D micro electrode arrays, silicon probes, tetrodes, and even single sharp electrodes, without ever managing to record spikes. When the same iPSC-derived cells were seeded directly on micro electrode arrays, electrical activity could be observed very reliably, so what was the cause of this discrepancy? On several occasions, we used the same extracellular probes to successfully record from spheroids (Paper II). My guess is that the fibers were obstructing the electrodes to come into sufficiently close contact with the neurons. This could be further hampered by passive electrical properties of the fibers. It's known that neurons can form a 'seal' on extracellular electrodes, creating something akin to a patch recording (Obien et al., 2015). When the iPSC-derived neurons (Paper II) were seeded on micro electrode arrays, we noticed that visible neurons close to the electrode was no guarantee to seeing electrical activity. Electrical activity was much more reliable if cells formed a cluster immediately on top of the electrode. Could it be that the mass of cells created electrical insulation that prevented the current from dissipating? This would not be present in the cultures grown on fibers, as they do not form clusters like cells grown on micro electrode arrays do.

19 Conclusions

The combination of electrospinning and bioprinting is an interesting approach to controlling circuit formation and macroscopic cell localization *in vitro*. We developed a working approach that could be used for low-cost and scalable *in vitro* models, opening doors to new *in vitro* models that could circumvent some of the challenges facing organoid technology. Unsolved persistent problems with electrophysiological access are still open challenges that need to be resolved before this approach can be widely adopted.

An unexpected 3D culture (Paper II)

20   A little background story

The cells that we used to unsuccessfully test the various fiber substrates and seeding techniques, were in parallel plated on microelectrode arrays to give us an idea of when the cells were electrically active and ready for probing. We wanted to use a traditional 2D culture as a reference to compare to the electrical activity of neurons grown in fibers. We seeded similar numbers of human iPSC-derived astrocytes and neurons on MEAs as on fibers, and continuously monitored their electrical activity. Time after time, these MEA cultures yielded viable data while fiber experiments kept falling short. During this time, I was continuously capturing complete microscopy images of every individual MEA culture, driven only by a vague sense that "this may be useful data". I noticed that the cells were surprisingly motile, and slowly migrated to form clusters of cells, interconnected by long bundles of what were presumably projections of neurons and astrocytes (Figure 9).

Using fluorescent labels and confocal imaging, we could confirm that these cultures had spontaneously aggregated to form a kind of 3D culture of its own, highly ironic as these were intended to be our "2D control" to compare with our 3D fiber substrates. Confocal images revealed that astrocytes and neurons were intimately associated with each other, and that the bundles of projections that interconnected the clusters were largely free of cells. Imaging the interior of the clusters showed no signs of distinct tissue-like structures, or organized cell localization as seen in organoids, rather, these clusters resembled spheroids.
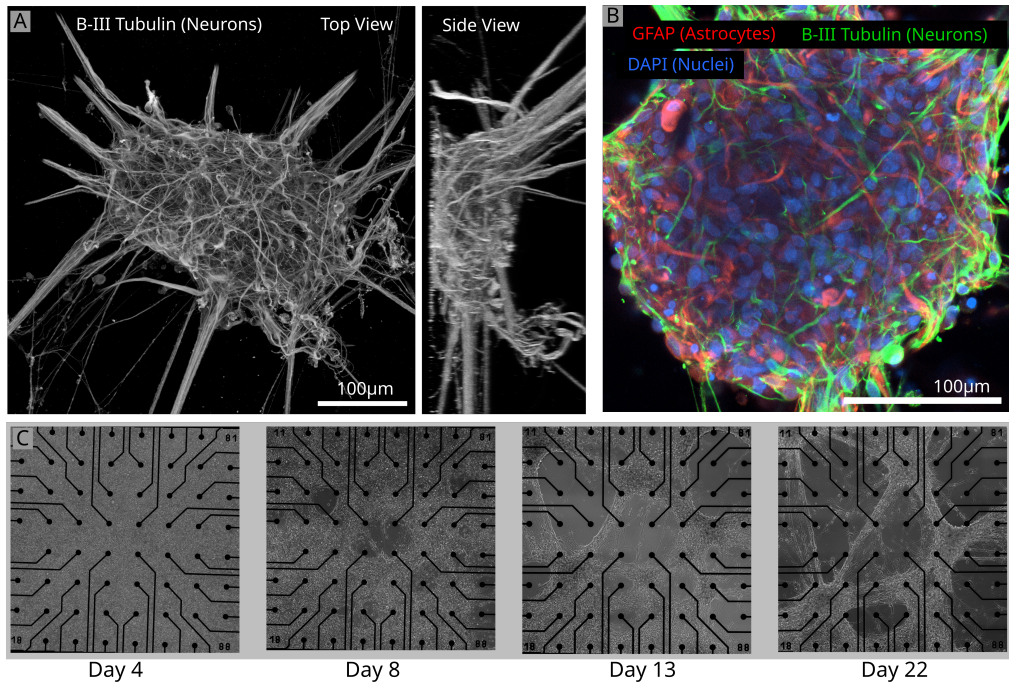
Figure 9: A) A typical spheroid imaged by confocal microscopy. This shows neurons aggregated as a large cluster, with several long connections which reach out to other clusters outside the frame. B) Confocal cross-section of a spheroid, showing that the inside is densely packed with both neurons (green) and astrocytes (red). C) Spheroids form spontaneously on microelectrode arrays, from homogeneous cell fields to distinct clusters over the course of a few weeks.

21 Spheroids as 3D cultures

Spheroids are popular 3D culture alternatives as they are self-assembled, form a tissue-like micro-environment that resembles native tissue (Fennema et al., 2013). Unlike some scaffold-based 3D culture (Pautot et al., 2008), spheroids can achieve high cell density, and mechanical properties which resembles that of the brain (Dingle et al., 2015). Unlike organoids, which aim to recreate the developing brain, including many of the macroscopic structures present throughout the developmental timeline, spheroids aim to recreate the a particular tissue microenvironment, such as the grey matter (Choi et al., 2013). Spheroids can be derived from differentiated induced pluripotent stem cells, allowing the *in vitro* recreation of a patients tissue pathophysiology (Lee et al., 2016). Spheroids of various shapes can also be created by casting, and later fused, to recreate modular and even hierarchical network architecture (Kato-Negishi et al., 2013). Using such an approach, it has been shown that spheroids assemblies can exhibit more diverse network activity patterns (Rabadan et al., 2022).

The self-assembling spheroids we found on microelectrode array seemed to create modules

of presumably highly interconnected neurons, interspaced by sparse connections. Could they spontaneously exhibit a modular network architecture?

22   Key findings

Unlike what we have hoped, formation of spheroids didn't produce any obvious modular network activity. On the contrary, spheroid formation led to an increase in network synchrony. It's worth pointing out, however, that spheroid formation comes at a cost. As cells become more clustered, less of the area of the microelectrode array becomes inhabited, reducing number of active channels drastically, limiting our ability to assess if the network activity is indeed modular.

As we purchased new MEAs, and used them together with the old ones, we noticed that the clustering was much more extensive on the new MEAs. In some particularly extreme cases, we saw dramatic cluster formation already after a few days in culture. We began noticing that the cultures that were clustering the most would also develop synchronized bursts of activity much earlier than those that did not cluster. As I had been collecting microscopy images of these cultures, I was able to correlate cluster formation with onset of synchronized activity. Using the fact that clusters show a slightly more brown color in light microscopy, I could use the pixel color and an arsenal of image filtering and eventually threshold the images to get a rough, but decent estimate of cluster formation. We were able to show that cluster formation had an influence on the development of synchronized activity. By using different cell densities, different ratios of astrocytes, and new-vs-old MEAs, we could see that all these factors contributed to cluster formation, and thereby indirectly to network activity.

By using tissue clearing, together with very long antibody incubation techniques, it was possible to perform confocal imaging of intact spheroids. This showed that spheroids were uniformly populated by a dense packing of cells, and that astrocytes and neurons were in close contact with each other. Interestingly, although higher astrocyte concentration was involved in greater cluster formation, which in turn decreased burst durations, higher astrocyte concentration also prolonged bursts, potentially highlighting astrocytes role in regulating network excitability.

23   Conclusions and comments

Culture systems like these are of great interest as *in vitro* models for diseases, toxicological assays, or for drug screening. Neuronal cultures can be sensitive to a broad range of environmental toxins, for example (Nicolas et al., 2014). As electrophysiological record-

ing though microelectrode arrays are non-invasive, this allows the readout of continuous dose-dependent responses, long-term effects, and recovery (Johnstone et al., 2010). Cultured neurons require little maintenance once cells are properly seeded, allowing cost-effective high-throughput screening, especially when applying multi-well microelectrode arrays (Tukker et al., 2016). Our results showed that cluster formation, even driven by something as seemingly innocuous as history of MEA use, can be a source of systematic errors. This could be highly problematic if these systems are to be used as screening platforms for medical research, where synchronous network bursts dynamics are often used as an indicator for neuronal function.

Spheroid assembly on microelectrode arrays has interesting applications for creating accessible 3D cultures, but can be difficult to work with as cluster formation decreases electrode coverage, and could also increase the risk of cell detachment, which was usually the inevitable fate for our cultures. Different types of microelectrode arrays which are more compatible with spheroids have been developed (Shim et al., 2020).

Controlling neurite outgrowth with microstructures (Paper III)

24 Soft lithography for axon guidance

There's been extensive research on controlling axon growth using various microstructures (Habibey et al., 2022). By using microfabrication, it's possible to produce organized compartmentalization of different cell types and to control their connectivity (Neto et al., 2016). Most of these approaches achieve guidance in a small subpopulation of the neurons in culture. Often a small strip of microscopic channels are used to create an asymmetric connection between two adjacent populations, which otherwise grows randomly like traditional neuronal cultures (Pigareva et al., 2021). We wanted instead to provide network-wide control of network architecture. Why this is challenging can be seen if we look at how these microstructures are manufactured.

The most utilized approach is the photopolymer-PDMS approach, a form of soft lithography. This approach involves taking a dissolved photosensitive polymer and spin-coating it onto a crystalline silicon wafer. Spin coating lets you control the thickness of the photopolymer. When the dissolved photopolymer spreads out on the silicon wafer, the solvent evaporates and leaves a solid photopolymer on the wafer. Through various techniques, the photopolymer can now be exposed with patterned light, which depending on the polymer, either polymerizes or weakens the polyer, and the exposed/unexposed polymer can subsequently be etched away, leaving a patterned structure on the silicon wafer, often called a 'master' (Jenkins, 2012).

The master can now be used as a mold for casting various materials. For biological applications, polydimethylsiloxane (PDMS) is often used for its biocompatibility (Habibey et al., 2022). PDMS is mixed with a curing agent, and poured onto the master. The mixture then cross-links and forms an elastomere, a tough rubbery material that now has the structure of the inverse pattern of the master (Zaouk et al., 2006). This gives you something resembling

a pancake with a patterned surface on one side, which can be applied to a glass surface, or a microelectrode array, creating microscopic channels between the glass and the PDMS "pancake". The problem is now, how do you get the cells into the channels?

There's been extensive research different channel designs and a seemingly endless variety of different patterns which can more or less effectively control neurite outgrowth. The most common approach to get cells into the channels is to punch a big hole, or cut a small section of the pattern out, so that cells can enter the channels (Pan et al., 2015). This however, creates large clearings where the cells are left to grow without the influence of the pattern, again, forming an unstructured network. There are only a few cases where researchers have successfully created small perforations that allow cell entry into the channels (Yamamoto et al., 2018).

The structure of neuronal networks in a brain can be thought of as having varying degrees of integration and segregation. Sensory input needs to be segregated in order to effectively transmit information, while integration is required in order to make associations between different information streams (Sporns et al., 2000), (Narayanan et al., 2015). In this light, cultured neurons show little segregation, and very high integration (Bettencourt et al., 2007). Most microstructures that have been developed create highly segregated networks, where a few fully integrated networks are sparsely interconnected, often entirely asymmetric (with no feedback) forming a very segregated network (Monma et al., 2025).

25   Creation of perforated microchannels

We instead wanted to create a network with asymmetric control of both integration and segregation. We chose to create perforated PDMS membranes (Figure 10), with structured microchannels and numerous small perforations that would serve as entry points for cells. Although creating normal microchannels is relatively easy, creating perforations proved to be extremely challenging. After over a year of failed attempts, we finally figured out a technique that worked reliably. The trick was to first create perforated thin PDMS films, and subsequently add the microchannels in a separate step.
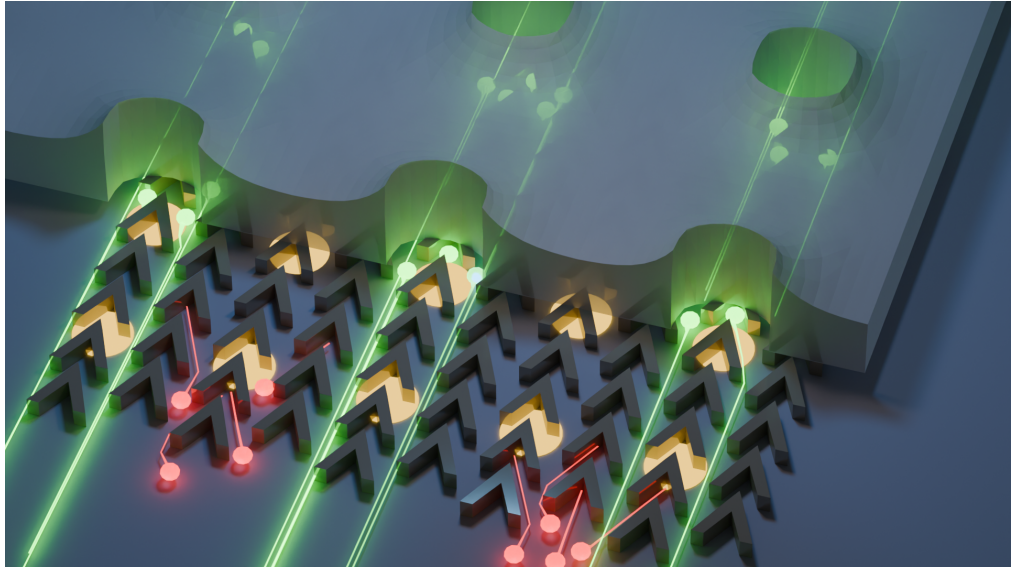
Figure 10: Schematic of a perforated membrane, showing the underlying V-shaped microchannels. Neurons in green show the hypothetical 'forward' growth of neurons, while neurons in red shows how 'backwards' growth is inhibited.

Because spin coating typically only gives thickness less than 50 microns, we used thicker films of 100 micron photoresist and laminated them onto silicon wafers. These had to be exposed through a chrome mask, instead of the maskless lithography (MLA) machine used for exposure of ordinary spin-coated photoresists (the MLA was actually damaged and had to be repaired when we first attempted exposure of a laminated photoresist). By using a chrome mask with an array of holes in it, we could create a master with 100 micron tall pillars of different radii and spacings.

The pillar array could then be used to create perforated membranes of various kinds (Figure 11). The simplest approach involved just pouring a very small amount of PDMS mixture onto the pillar array, such that it didn't cover the top of the pillars. Then, when the PDMS cured, it could be peeled off, giving us a thin perforated membrane. Then, we could add the microchannels to the perforated membrane by taking the other master (the spin coated one) and once again, add just a tiny amount of PDMS, and manually spread it across the surface so that it resided only in the grooves of the master. Then, we could simply apply the perforated membrane of top of it, leave it to cure, and voilà, a perforated membrane with microchannels.
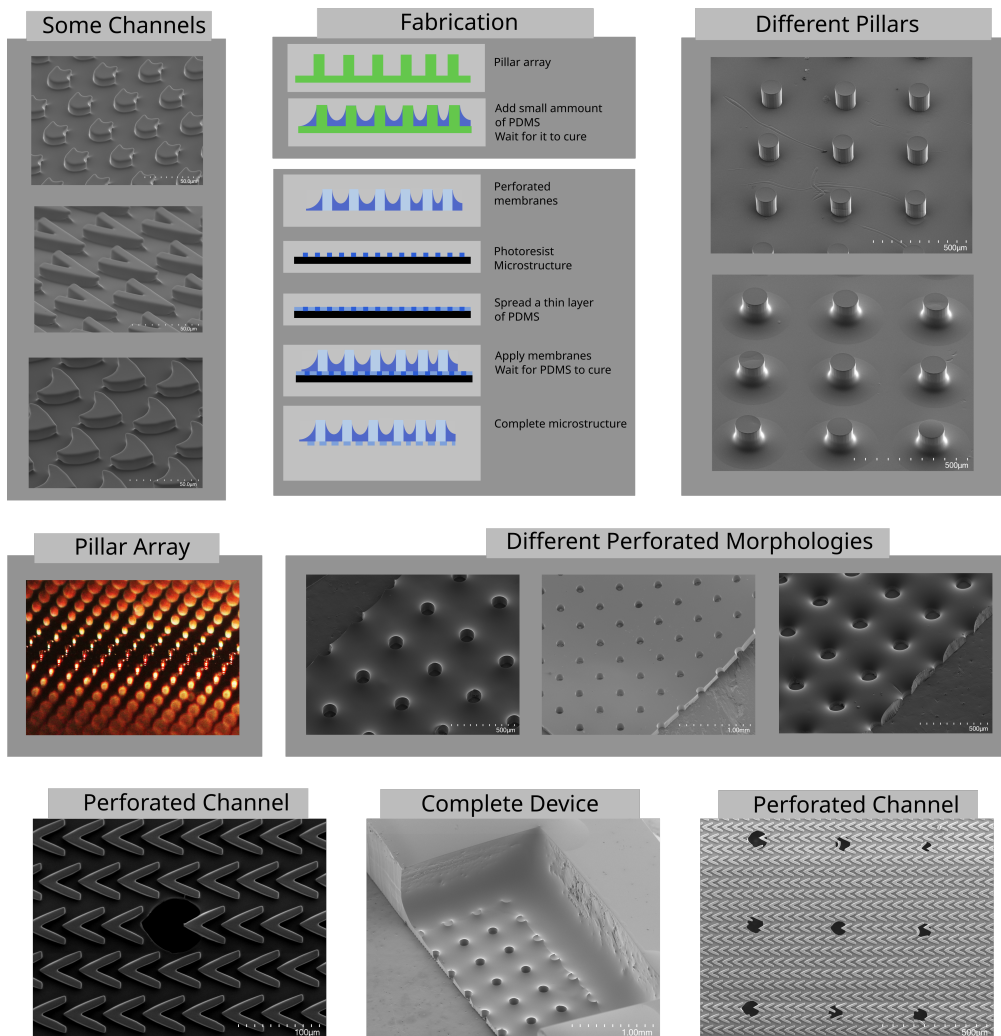
Figure 11: Fabrication of perforated microchannels. Some different microchannel designs that were tested (top left). Fabrication involved using an array of pillars and applying a small amount of PDMS such that the top of the pillars were not covered. When this cured, perforated membranes were formed. We could subsequently add microchannels to these perforated membranes by taking a micropatterned surface and spreading a small amount of PDMS over the surface, and placing the perforated membranes on top of these. When this cured, the complete perforated microchannels were completed (top middle). Pillars could be "smoothed out" by spreading a very small amount of PDMS on them (top left). Pillar arrays were created by using films of 100μm thick photoresist and exposing them through a chrome mask (middle left). Different morphologies of perforations could be fabricated using these techniques (middle right). (Bottom left) A perforation seen from "below" together with the microchannels. (Bottom middle) The complete device involved a larger hand-cut frame which created a basin to simplify cell seeding. This is showing a cross-section of the complete device. (Bottom right) Several perforations seen from below.

26 Microchannel control of neurite growth

We developed various microchannel designs to test their efficacy in controlling neurite outgrowth. Because of our two-stage approach, any existing master could be repurposed to create perforated membranes, meaning we could rapidly generate prototype patterns and test them before settling on a pattern to use for further evaluation. We used the same neural stem cells as used in paper I, and cut small pieces of PDMS non-perforated microchannels and attached them to glass coverslips to see if their neurites were successfully guided by the various patterns. These neural stem cells are highly migratory before they differentiate, so quite a few cells spontaneously migrated and settled inside the channels, allowing us to perform fluorescence immunocytochemistry to image and quantify their growth patterns (Figure 12).

This way, we could confirm that the patterns were effective instruments for guiding neurites, and that different patterns yielded highly diverse growth patterns. We were quite surprised by the growth patterns in some cases, with neurites often defying our expectations of how neurites ought to grow. We had set up a simple time-lapse microscope that used a low-intensity red LED light source to monitor neurite growth and migration, and from many hours of time-lapse videos, together with the morphological data obtained from stained samples, I was able to piece together an ad-hoc explanatory model for neurite growth, and replicate the experimental data in a simple simulation (see paper III for details).
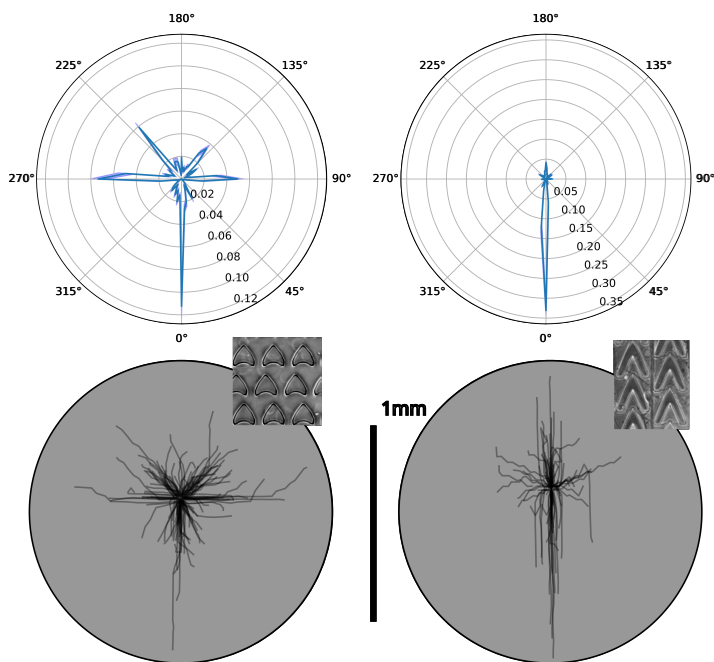
Figure 12: Two example microchannel designs and the corresponding growth of neurites they produced. The microchannel to the right was utilized for further experiments.

27 Functional confirmation of microchannel guidance

Once we had a reliable protocol for creating perforated membranes with microchannels, we applied them to micro electrode arrays, and seeded commercial iPSC-derived neurons and astrocytes (as in paper II). With this approach, it's possible to seed cells by pipetting cell suspensions onto the perforations, with no need for further protocol adjustments. Cells simply fall into the perforations, creating small populations of neurons which can only reach each other via the microchannels (Figure 13). This allowed us to confirm the morphological data by measuring how action potentials propagated throughout the network (Figure 14). I had some initial worry that the PDMS would not live up to its reputation as being biocompatible, but these worries were met with staggering evidence of the contrary. We noted that not only do neurons survive well in these microchannels, but we saw a dramatic increase in number of active channels and signal-to-noise ratio. Interestingly, the improved signal-to-noise ratio was only apparent on electrodes that were 'inside' the channels, and not the ones that were situated under a perforation. The best explanation for this, I believe, is that the PDMS acts as a strong resistor, and that the constrained volume inside the microchannels leaves less space for the current produced by the neurons to dissipate through.
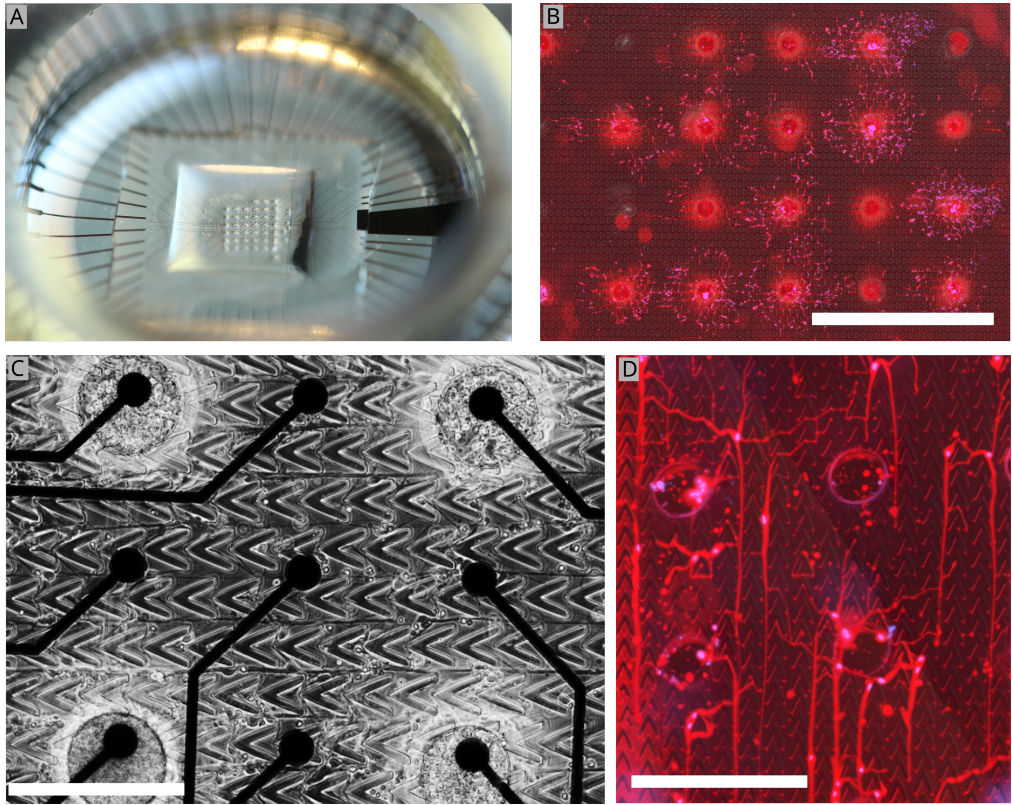
Figure 13: A) The complete device assembled in a microelectrode array. B) Three days after seeding, cells are seen infiltrating the microchannels through the perforations. Here, neurons are stained in red, and cell nuclei in blue. C) Neurons growing in a perforated microchannel on a microelectrode array. D) Two months after seeding, neural stem cells exhibit long projections, showing the influence of the microchannels. Scale bars: B: 400μm; C: 200μm; D: 200μm.

28 Discussion

This technique is an improvement of current microfluidic practices in several important respects. The current standard design for microfluidic control of circuits is the "two compartment" model where two neuronal populations are separated by a strip of microchannels (Pan et al., 2015). As mentioned before, this is a consequence of the casting process, which generates thick slabs of PDMS with a micropattern on one side, making cell entry quite challenging. In general terms, creating large arrays of very small features isn't especially challenging, but the seamless integration of a range of different scale of features is more challenging. Our approach of sequential fabrication is an example of this type of challenge, where larger features of perforations has to be integrated with small microchannels.

Neuronal networks in the brain are interesting, because they both show clear modularity
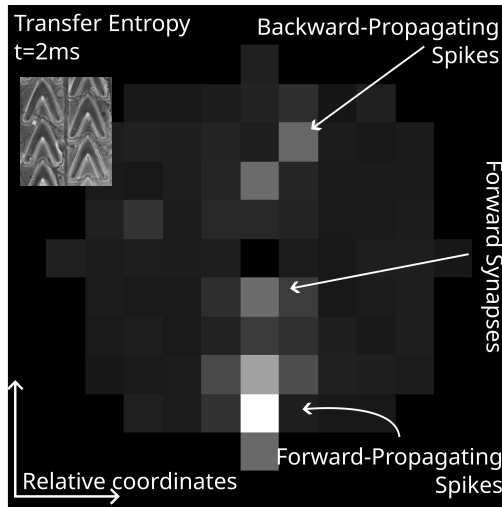
Figure 14: Propagation of action potentials as measured by transfer entropy. 2ms after a spike is seen on a channel, action potentials propagate with a strong preference in the 'forward' direction. The two hot-spots of forward activity could be due to fast-moving action potentials and slower synaptic transmission.

and segregation, but there's also a significant degree of integration between distant and seemingly unrelated areas. It's believed that the ability to recover from hemispherectomy is attributed to the prior existence of latent synapses innervating ipsilateral appendages (normally, the each brain hemisphere controls the contralateral side of the body), and that these existing connections only need to be strengthened in order for recovery to occur, rather than requiring completely new synapses (Sebastianelli et al., 2017).

Anatomical studies of the murine barrel cortex corroborate this view. The barrel cortex has several clearly defined columns that are associated with each of the animal's whiskers. However, there are many connections between these columns as well, which are believed to aid in making associations between the information from the individual whiskers (Narayanan et al., 2015). Similar inter-columnar connections have been found in macaque motor cortex, possibly serving a function in coordinated muscle movement (Huntley and Jones, 1991). Importantly, intercolumnar connections can be asymmetric (Bernardo et al., 1990). Many of these features are potentially reproducible with our technique.

This structure of integration and segregation is persistent across the various scales of the nervous system. Individual brain regions are functionally segregated, clearly, as unless we have synaesthesia, we won't see what we hear, or hear what we taste, et cetera. At the same time, there's an immense cross-talk between different brain regions (you can describe in words what you see or hear, indicating that visual information has moved from your visual cortex to the various language centers), and disruptions between the strength of connections between distant brain regions has been implicated in various psychological conditions (Huang et al., 2023), (Chen et al., 2017). The ubiquity of these network features of the brain

-strongly interconnected modules with sparse interconnections- means that this could be a crucial structural feature if we want to improve our *in vitro* models.

To my knowledge, all microfluidic systems that have been developed to control neuronal connectivity do not attempt to produce a modular structure with both integration and segregation. The typical two-compartment design aims to achieve full integration from compartment A to B, and full segregation from compartment B to A (while both compartments are themselves fully integrated) (Pan et al., 2015), (Kanagasabapathi et al., 2011), (Gladkov et al., 2017). This way, you can have two separate cell types, and investigate on cell types influence of the other, without confounding cross-talk between the two (Neto et al., 2016). In cases where segregated clusters are produced by surface coatings (Marconi et al., 2012), or where small compartments are created with connecting tunnels (Yamamoto et al., 2018), integration is always symmetric - both clusters can connect with each other bidirectionally. In one experiment, many clusters were created using a similar perforated design as we have developed, but again, the clusters were connected with the goal of achieving very high segregation between them (Monma et al., 2025).

Our approach, however, allows for "soft" control of asymmetric integration and segregation, with close to arbitrary circuit control. When we propagation of action potentials, we used the channel design that we predicted to show the most selective propagation. As we can see, the similarity between action potentials coincided well with the morphological data, meaning that highly complex functional network architectures could be obtained by using any of the other patterns that we tested, or use combinations of them. There are, as I see it, two important limitations to this approach. The first is that neurite guidance isn't possible within the perforations, creating little isles of homogeneous circuitry. This may not be too big of a problem, however, as the cortex is built from highly interconnected columns. The second issue, is that it could be difficult to produce network where the "cross over", which could be a fundamental limitation in which topographies could be achieved. We also don't have selective control of axons and dendrites.

Neuronal ensembles, plasticity and sparsification in controlled artificial neuronal circuits (Paper IV)

29   Introduction

As I've mentioned, using electrical activity of cultured neuronal networks is highly appealing for many of the popular use-cases of *in vitro* models. Electrical activity is possible to assess non-invasively with micro electrode arrays, and over long periods of time. If you're investigating the effect of some compound on the nervous system, it is possible to record a neuronal culture live, as the compound is being applied, removed, or the long-term impact of a single or chronic administration of a compound (Chiappalone et al., 2003), (Tukker et al., 2018).

The read-out of microelectrode arrays are in most cases, simple assessments of their spontaneous network dynamics. As I have discussed throughout this text, the spontaneous network activity is likely a combobulation of a large array of underlying cellular processes, where sleep dynamics, plasticity, et cetera, are reflected. Spontaneous network activity can thus act as a unspecific reporter, where the exact cause of an observation can remain ambiguous. It is possible that the spontaneous network activity, seen in so many cultured neurons, are masking more subtle behaviors of the network.

30   Spontaneous activity of neurons with controlled network architecture

We applied our newly developed technique to guide neuronal network connectivity (Paper III) to analyse its effect on the spontaneous activity of neurons and their response to input.

We utilized the same iPSC-derived neurons and astrocytes as in previous experiments, and seeded them on standard microelectrode arrays and on microelectrode arrays with our perforated microchannel design, and cultured them for several months (Figure 15A). We were surprised to find that although network bursts were still present, they were complemented by oscillatory patterns of activity (Figure 15B) in microchannel cultures. These oscillations were always below 1Hz and persisted for long periods of time, up to minutes of oscillations without bursts in some recordings. It was clear that the binary partition of the network state into bursting or non-bursting would be inappropriate for describing the activity of these networks.

In addition to oscillating, these cultures exhibited clustered activity. By vectorizing neuronal spike-rates, we could measure correlations in spike rates between channels (Figure 15C). This way, we could see that neuronal activity was not uniform, but clustered into groups of correlated channels. Using a simple clustering algorithm, I used this information to see how these clusters were distributed on the microelectrode array (Figure 15D). In some cases, but not all, the clusters are clearly distributed along "columns", aligning with what we would expect given the uni-directional growth and spike propagation that the microchannel induces.

Using vectorized spike-rates, we could also apply principal- and independent component analysis (PCA & ICA) to decompose the spike-rates. These two approaches are subtly different, but are both forms of decomposition, giving us components which can be inverted back to individual samples. In ICA, the components represent inferred sources, where statistical independence between components are maximized. Using this approach, we can inspect sources of neuronal activity (Figure 15E). The components of microchannel cultures typically show one of two things, diffuse global structure (probably associated with bursts) and local (often columnar) structure. Observing how these components vary in time (Figure 15E), we can see that they show some non-random distribution, often occurring in short bursts or spikes (Figure 15E). PCA, on the other hand, finds underlying axes of covariance. The components derived from PCA similarly show a horizontal organization (Figure 15F). In the example in figure 15F, you can clearly see how the components are related to the clusters in figure 15D. Taken together, clustering, ICA and PCA points to the existence of locally interconnected sub-networks, or modules, in these cultures, which exists hierarchically under a global network. However, these results were unfortunately not directly comparable to cultures on standard microelectrode arrays, since culturing neurons in our microchannel design inadvertently also increases signal-to-noise ratio and number of active channels (Paper III). The fact that clusters and PCA and ICA components are horizontally aligned, suggests that the microchannels are involved in formation of a modular network architecture.
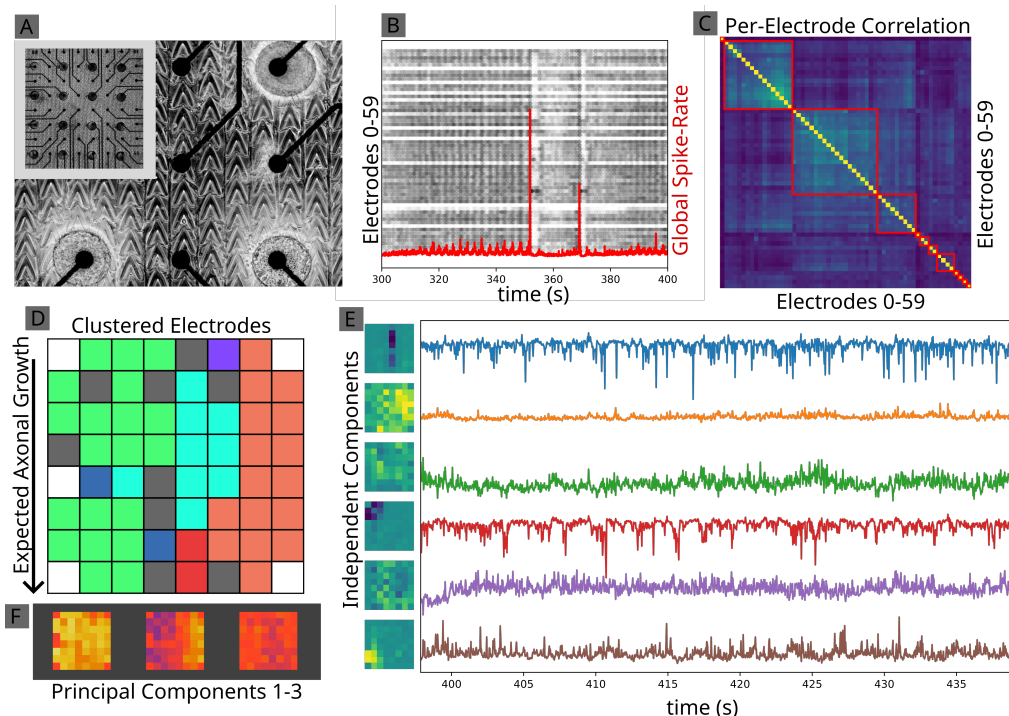
Figure 15: Spontaneous activity of neurons grown in perforated microchannels. A) Microchannel orientation on the microelectrode array. B) Raster plot of spikes black dots) from each channel of one microelectrode array, showing the massive numbers of spikes and active channels. The red graph shows the global spike-rate. We can see a clear oscillation persisting for many tens of seconds before two synchronized bursts occur. C) Pearsons correlation of spike-rates between all pairs of electrodes, showing at least three clusters of more strongly correlated channels. Red boxes indicates cluster as identified by dendrogram clustering. All cluster sized with fewer than 3 electrodes were discarded. D) The spatial distribution of the clusters from C on the microelectrode array. color indicates cluster identity. Microchannel layout is that of A. E) Example of independent components from independent component analysis, showing both columns and clusters of independent activity. Plotted by their relative strength in time, we see how these components occur in short bursts or spikes , consistent with neuronal cluster activity. F) Principal components from principal component analysis, showing the dominant axes of correlation. We can see how these are vertically oriented. The first component likely represent the globally synchronized bursts and oscillations. We can see how the two other components coincides well with the spatial distributions of clusters in D.

31 Stimulus responses and plasticity

We wanted to test the plasticity of the microchannel cultures. In the developing and mature brain, activity dependent plasticity shapes circuit formation and is often explicable by a Hebbian mechanism (Eysel et al., 1998). One important example is the source separation by Hebbian competition in the lateral geniculate nucleus, where asynchronous activity of the two retinas drives segregation of the synaptic fields of the two retinas (Penn et al., 1998). Similar processes are believed to take place in many other areas in the brain, underlying learning in adults. We wanted to see if neurons in microchannels could exhibit a similar behavior. It's possible that the functional segregation imparted by the microchan-

nels facilitates this process by limiting the spatial extent of stimulation pulses. We applied point stimulations to all channels of several cultures and measured the spatial propagation of single pulses (Figure 16C). Pulses invokes activity primarily along the horizontal axis of cultures, consistent with prior morphological findings, and these network's spontaneous activity. Interestingly, evoked activity was bidirectional along the horizontal axis, with pulses evoking activity along the entire column.

We also tested the ability for the neurons to respond to different random patterns of input, which we refer to as 'sources' (Figure 16A). Stimulating with different sources evokes distinct responses in the network, as seen in PCA analysis of evoked responses (Figure 16B). We stimulated with three random sources repeatedly (Figure 16D) and observed that responses to stimuli changed over time (Figure 16A). In this example, we can see that responses are initially quite similar, but grows distinct over time, until at 4-5 minutes, the responses become complementary - channels evoked by one pattern seem to be more weakly triggered by another.

We measured how the difference in responses changed over repeated stimulation of random sources, and found that there's a consistent increase in response differences when stimulating for 5 minutes (See paper IV). We thought that a possible explanation for this could be that the background excitability simply decreased, unmasking the intrinsic differences in evoked responses. To account for this, we extended stimulus times to 4 hours, after which we changed two of the sources for two new ones, and stimulated for another 4 hours, while we recorded for 5 minutes at the beginning and end of each session. Presumably, the culture would by this time have adapted to being constantly stimulated, and any further changes to stimulus responses wouldn't simply be due to a decrease in background excitability. Also, since we saw that differences between responses increased over time, we wondered if these changes would continue to increase over prolonged stimulation.
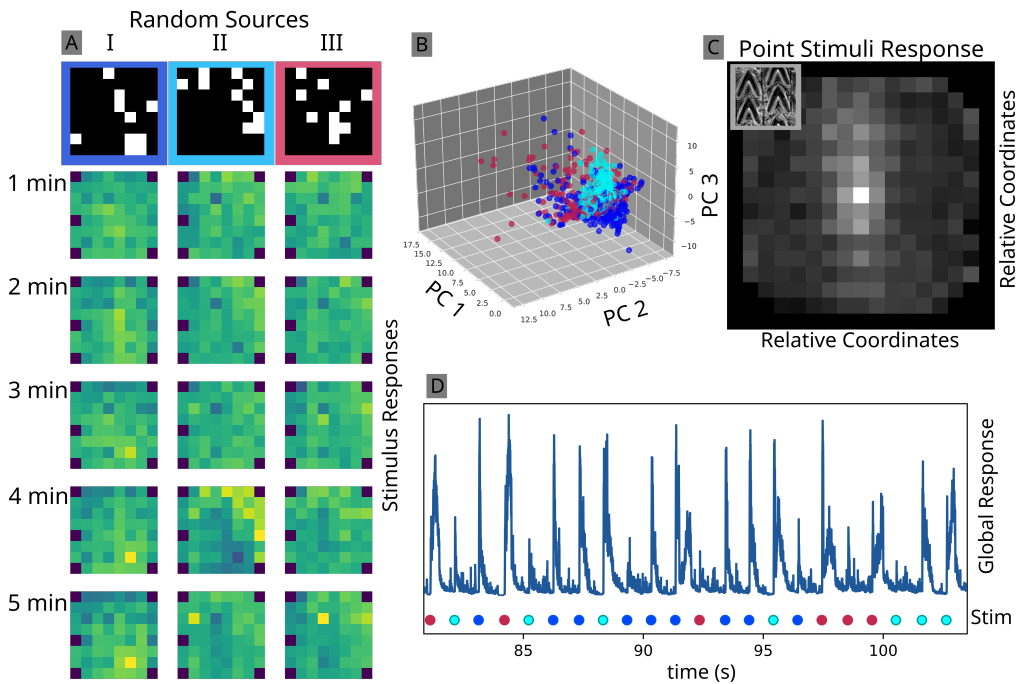
Figure 16: A) Three random patterned stimuli and how these were distributed across the microelectrode array. The following rows show the stimulus responses of a neurons over repeated stimulation of these random patterns. Evoked responses are initially quite similar, but become more distinct over time. B) Principal component analysis of evoked activity patterns. color indicates which of the random stimuli was presented. This shows that the different patterns produce distinct responses. C) Responses from single electrode stimuli. Inset indicates microchannel orientation. Responses are stronger along the vertical axis. D) Global responses to stimuli. colored dots indicates which pattern was presented.

We tested this for all cultures, using two different stimulus frequencies and amounts of current (Figure 16A-B). Again, we found significant increases in response differences, but somewhat surprising, the response differences returned back to initial values after repeated stimulation. After the first 4 hours of training, when we apply new sources, we again saw that the response differences increased over repeated stimulation.

Before training, after the first 4 hours of training, after training, and twice more with 8 hours of rest in between, we recorded spontaneous activity. We observed a decrease in synchronized activity in these recordings, and we quantified this by using PCA, and measured the "dimension irreducibility" of the components. This can be interpreted as the diversity of covariance axes, or simply the network asynchrony (a higher value meaning more globally asynchronous activity). This value increased in all cases of long-term training, and stayed high after rest, perhaps trending toward a time-dependent decrease (Figure 16C-D). If we look at the correlation matrix of spontaneous activity before and after training, we can see that there's no fundamental change to the correlation structure following training. It seems like the main correlation clusters are still present, but with less background cor-

relation (Figure 16E). The same effect -decreased synchrony- could also underlie the effect seen when inspecting independent components (Figure 16F), where several new patterns seem to emerge from training. Again, this could simply be due to reduction in synchrony, and doesn't necessarily have to imply the formation of new neuronal connections.

After training sessions, I usually did a few absent-minded stimulus presentations by hand (the stimulus software binds stimuli to keystrokes), just to see if anything interesting would happen. To my surprise, I found that quite often, the sources had a clear ranking in how strongly they excited the network as a whole. Usually, one of the patterns was much more competent to provoke large network burst events than the other sources. We measured this effect as the difference in magnitude of global responses (Figure 16G) and how that changed from the first 5 minutes of training, to responses after training. This is a highly noisy reading, but indicates that the network has a whole become more selective to one of the sources.
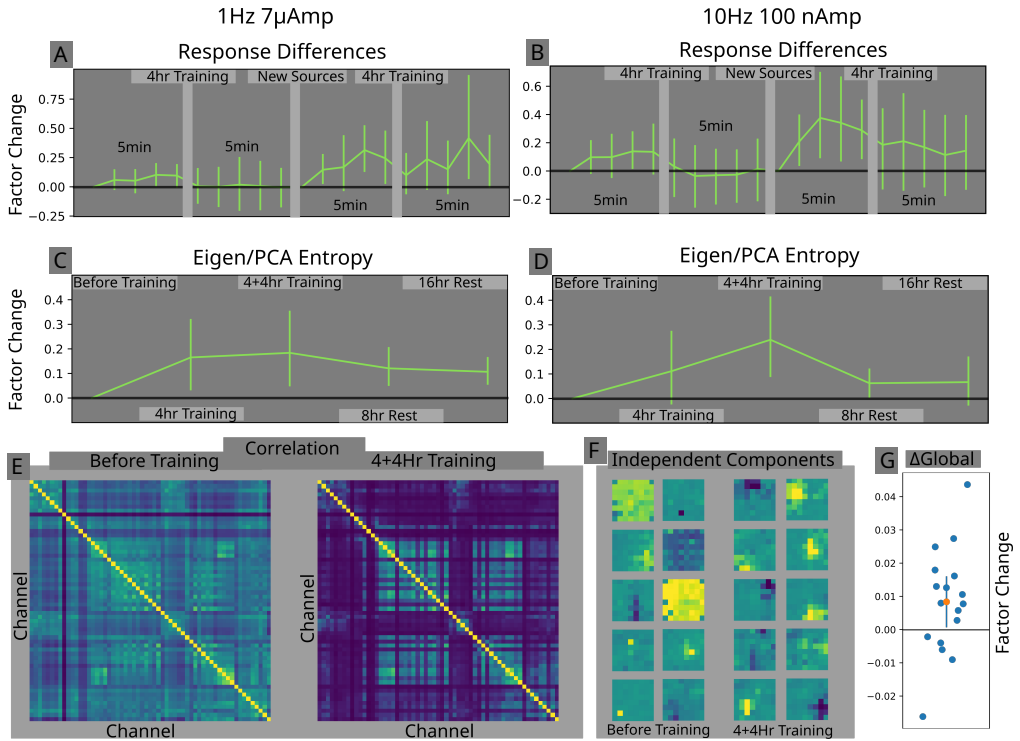
Figure 17: Results from long-term stimulation of patterned stimuli. A) Response differences during the first and last 5 minutes of a 4 hour training session where random sources were presented at 1Hz and with 7μAmp current per electrode. After 4 hours, two of the random sources were replaced by two new ones, and the process was repeated. Error bars indicated 95% confidence intervals. B) same as A but with 10Hz stimulus frequency and 100 nAmp current per electrode. C) Changes in PCA entropy activity after 4 hours of training, 8 hours of training, after 8 hours of rest and after 16 hours rest, according to the stimulus protocol in A. The PCA entropy indicates how much of the activity was dominated by a single principal component. A higher value is readily interpreted as less global synchrony. D) same as C, but with the stimulus parameters from B. E) Pearson correlation between all pairs of electrodes before and after training. This shows how a lot of unspecific correlation is removed, while the underlying strong correlations are retained. F) Independent components before and after training, showing how many new activity patterns emerge while the global activity patterns are greatly reduced. G) Change in difference between global responses evoked by the different patterns before and after training. The bar in the middle indicates the 95% confidence interval. As this has increased, it can be interpreted as as that the network has become sensitised to one pattern.

32 Discussion

As I've mentioned throughout this text, neurons in culture develop synchronous network activity which resemble several different brain mechanisms but without having a direct *in vivo* counterpart. I believe the best explanation of the origin of synchronized network bursts is that they arise from synaptic homeostasis, which in the absence of input stimulation and network structure leads to excessive recurrent connections. Unlike cultured neurons, the mature cortex is sparsely connected, and stimulation and spontaneous activity typically involve smaller populations of neurons (Yoshida and Ohki, 2020), (MacLean et al., 2005). By growing neurons in microchannels, it was possible to overcome this and induce formation of modular network activity. Although, this alone doesn't mean that the network hosts a more sparse network, only that there's heterogeneity in the network. However, such modular and asymmetric connections are ubiquitous in the cortex and underlies much of the complex function of the cortex (Meunier et al., 2010), (Mignard and Malpeli, 1991), (Mountcastle, 1997). Growing neurons with a modular architecture may be an important step in improving our *in vitro* models.

During development, spontaneous patterned activity drives the formation of microcircuits (Ge et al., 2021), (Burbridge et al., 2014), which become further refined by sensory experience (Rochefort et al., 2009), (Thompson et al., 2017) and remain plastic throughout maturity (Eysel et al., 1998). These processes are often explicable by a Hebbian plasticity mechanism, which probably underlies higher cognitive function as well (Lim, 2021), (Rolls, 2021). Stimulating cultured neurons typically induces activity in large populations of neurons (Ruaro et al., 2005), (Isomura et al., 2015), (Isomura et al., 2023), (Pasquale et al., 2017), possibly reducing their representational capacity (Foldiak, 2003), possibly resembling the primitive unspecific cortical responses to visual input in the immature cortex (Colonnese et al., 2010).

Our experiments showed that long-term stimulation provoked increase in stimulus-evoked specificity, which is consistent with previous findings (Isomura et al., 2015). This increase was consistently transient, and not persisting after long-term training. This can be explained as a form of pattern specific adaptation. When switching to new patterns after 4 hours of training, these could once again support an increase in stimulus-evoked specificity, indicating that any adaptation was specific to a particular set of patterns.

Long term stimulation also reduced synchrony in cultures. This effect is perhaps most likely resulting from a homeostatic mechanism, reducing the overall neuronal excitability. It's important to note that this doesn't necessarily equate to a true sparsification of the neuronal network, but could be due to non-specific overall scaling of neuronal activity rather than a specific pruning of synapses. We also didn't see any major differences in the correlation structure of the network, indicating that most of the original network structure

was still intact. A similar change could also be induced by cholinergic stimulation, which shows that these changes can be produced by modulating of neuronal excitability.

In these cases of observed plasticity, the exact mechanisms are not clear from this simple experiment. There are many approaches for elucidating these mechanisms, however. Initially, it could be useful to use pharmacological blockade of NMDA receptors, which ought to suppress plasticity of they are indeed occurring via NMDA-dependent plasticity. Blocking NMDA receptors in cultured neurons, however, can cause large changes in network activity in cultured neurons (Parodi et al., 2024), with possible ramifications for analysing stimulus responses. Other approaches could be to use reporters for EIGs, which have been used to fluorescently label neurons which exhibit long-term potentiation *in vivo* (Xie et al., 2014). This could be very interesting to use *in vitro* as it could allow for real-time visualization of long-term potentiation with single-cell resolution. Another potentially important technique would be to use calcium indicators in parallel with microelectrode array recordings and stimulation. These two techniques utilized concurrently could allow for the high temporal resolution of microelectrode arrays and superior spatial resolution of calcium imaging, and could be used to investigate how neuronal representations evolve through training. Lastly, it could be highly relevant to use patient-derived, or knock-out cell lines with a genetically deficiency for long-term potentiation or other forms of plasticity, or other conditions which are reflected in abnormal neuronal connectivity such as schizophrenia (Huang et al., 2023) or autism (Minshew and Williams, 2007). Ultimately, it's these experiments which could provide real value for the field in a wider sense, as they would provide a proof of concept for utilization of controlled-architecture and experience dependent plasticity *in vitro* models such as the one we have developed.

One of the strengths of using human *in vitro* models is the potential for creating cost-effective high-throughput screening experiments. If we want to utilize these tools for such experiments, scalability is key. The production of perforated microchannels, although being challenging to develop, is very simple once a working protocol was established. Creating the micropatterned silicon masters requires access to clean-room microfabrication facilities, but these days, companies also offer microfabrication of similar devices as a service. Once the masters are acquired, they can be used to mass-produce perforated microchannels. The perforated microchannels were effective in increasing signal-to-noise ratio and throughput of recordings, sometimes showing robust activity on every channel of the microelectrode array. This alone is a good reason for using them in cultured neurons, even if controlling network architecture isn't a wanted effect.

Conclusions and final thoughts

33 Summary

Here, we have explored the use of *in vitro* models, their limitations, and recent developments in improving their physiological relevance. We've reflected on four papers which have utilized cultured neurons to explore neuronal behavior and new approaches for creating advanced *in vitro models*.

In **paper I**, macroscopic organization of cells in a 3D fiber scaffold was described, where a simple bioprinter was used to create controlled deposition of human neural stem cells. This demonstrated the feasibility of using simple tools for creating fiber-gel hybrid cultures.

Paper II presented the self-assembly of 3D neuronal spheroids and how this process was associated with changes in network activity. Astrocyte concentration and cell density both affected the formation of clusters, which in turn influenced network activity, highlighting the potential for cluster formation as a confounding factor in experiments with neuronal cultures.

In **paper III**, a perforated microchannel device was developed which could produce high-fidelity control of neurite growth and influence how electrical activity propagates in a network of cultured neurons. The perforated design allowed large-scale cell entry into the microchannels, which determined their ability to form connections.

Paper IV show how these microchannels produce clustered and oscillating neuronal activity, and how patterned electrical stimulation can induce plasticity and long-lasting changes in spontaneous network activity.

34   A philosophical spin

I think a really important question, that perhaps we don't ask ourselves enough, is "why are we doing this?". It's easy to get drawn in by science for the mere sake of fascination of the natural world, or for intellectual stimulation or the awe and beauty of uncovering deeper fundamental truths about our universe. However, we also live in a world with tremendous suffering and injustice, and how are we helping the world with the science that we're doing? How can neurons grown in the lab be useful for the world? Let's start this journey from the very top of the ivory tower, and work our way down from there.

Neuroscience has an interesting history that is surprisingly intertwined with computer science and artificial intelligence. John Von Neumann, who was instrumental in developing modern computers, was influenced and inspired by the works of Warren McCulloch and Walter Pitts in their formulation of neuronal networks implementing propositional logic (Von Neumann, 2017). Further overlap includes the development of the perceptron, the convolutional neuronal networks or self-supervising algorithms like auto-encoders. In some sense, there's similarities to the fields of computer science, neuroscience and machine learning that transcends their individual particulars. They are all involved with information in some sense. Memory, object detection, inferring causes of observations, all these are shared topics in these fields. Information itself may even have a thermodynamic basis (Parrondo et al., 2015), and it's no coincidence that Claude Shannon, who more or less founded the field of information theory, called his measurement of uncertainty 'entropy'.

David Deutsch describes the concept of "knowledge" as "information with causal power" (Deutsch, 2011). In a world without knowledge creating entities, big things affect little things. The temperature of the surface of planet Mars is driven by the sun, and its interaction with the planets surface and atmosphere. Mars will stay this way forever, until the sun burns up all its hydrogen fuel and turns into a red giant, or if some high-energy freak event like Mars colliding with another celestial object occurs. Big things affect little things. On planet earth, this process is reversed. The arrival of oxygen producing cyanobacteria meant that the composition of the earths atmosphere was completely changed. You can imagine some distant alien civilization with technology similar to the James Webb telescope observing the earths atmospheric composition millions of years ago. They would perhaps note that over the course of millions of years, our planets atmospheric composition changed gradually. The same can be said about the recent increase in atmospheric carbon dioxide. Perhaps they understood that this was due to the arrival of life? Little things like cyanobacteria suddenly affect big things, and can be described as a "phase transition" in the universe. David Deutsch and Chiara Marletto developed the concept of contructor theory (Deutsch, 2013), which explains events like this as the product of knowledge. The cyanobacteria basically has the knowledge of how to use sunlight to create carbohydrates encoded in its genome. In theory, we could transcribe this knowledge and encode it on a hard drive, send

it off into space, and some alien civilization could receive it and recreate the knowledge of biological solar energy, and make new cyanobacteria that may once again poison the atmosphere of some planet with oxygen. David and Chiara reason that the universe can be divided into things that are possible or impossible, and knowledge is the key that can make something that can happen, happen. If it can't happen, then there simply must be some law of physics that prohibits this thing to happen. This is what they call the "infinite reach of knowledge".

Knowledge is produced by evolution, like in the case of cyanobacteria. Mutations can be seen as random guesses, and the ones that produce valid knowledge may become selected. Lifeforms on planet earth has an enormous amount of knowledge encoded in their genes. How to turn carbon dioxide and calcium into calcium carbonate, how to extract energy from carbohydrates, how to move through water with hydrodynamic efficiency, et cetera. This process is slow, takes millions of years, but the human brain produces knowledge very rapidly in comparison. The knowledge of how to make fire, or to write emails, or to sing, is knowledge that is transitioned from one generation to the next, but even as you are reading this text, or looking out the window, your brain is constantly generating knowledge. If you see a bomb outside the window, you may quickly rush out on the street and disarm it, preventing catastrophe. Like we said, knowledge is information with causal power, little things (photoreceptors on your retina, neurons in your head) affect big things (an explosion).

Carl Popper proposed that knowledge is created by a process of conjecture and criticism (Popper, 1963). In his view, knowledge is essentially created inside the brain and then tested and criticized. Why do I smell fire? Look in the kitchen. No. Look outside the window. No fire in sight. Could it be coming from another apartment? Go out and check the stairwell. Doesn't smell out here. And so on. This is the process by which knowledge is attained, and is how we do science, in Poppers view. This idea is remarkably similar to how evolution creates knowledge, only that evolution basically has to test randomly. How do I turn sunlight into carbohydrates? Mutation, mutation, mutation... Aha! The principle is the same in that knowledge arises internally, in the form of mutations, and is only later tested. It's obviously not the sunlight that produces the knowledge of how to turn solar energy into carbohydrates, just as in the the Popperian view it's not observations that creates knowledge (unlike what empiricists believe). The same could be true for how our brains form knowledge, as well. According to the Bayesian brain theory and predictive processing, causes are generated within the brain and are tested by forming predictions of what sensory input ought to arrive given the current prediction. Causes are similar to scientific *explanations* in that they *are* not what they depict. If I draw an atom on a piece of paper, that is clearly not an atom, it's an explanation, an expression of knowledge in the form of paper and ink. Similarly, the color "red" is only neurons and synapses, not the actual color that exists outside in the world.

What if we could uncover the neurological basis for how these guesses are made, a kind of "spark of creativity" in the brain? This would be truly invaluable to the field of AI research, given that we have reasons to believe that current AI models are more like very expensive imitators, rather than being able to create knowledge (Elton, 2021). Apart from striking some deep philosophical and technological notes, the idea of uncovering the neurological basis for knowledge creation can have massive implications for our understanding of mental illness. Take psychosis for example. In the light of Bayesian brain theory, psychosis can be interpreted as a failure of hypothesis testing (Corlett et al., 2009). This is evident in certain perceptual illusions, where people suffering from schizophrenia or psychosis seem to not make the same perceptual guesses as healthy individuals do (Dima et al., 2009). The 'hollow mask' illusion is an illusion where an image of a rotating "face mask" is perceived as a convex face, even when seen from the concave (hollow) side. This illusion doesn't work on some schizophrenic individuals. Perceptual distortions like this are also accompanied by delusions (Schmack et al., 2015), suggesting a similar underlying mechanism for both perception and formation of more abstract beliefs.

35   A few thoughts on *in vitro* models

Developing treatments for depression, schizophrenia or other disorders are clearly very important endeavours. The process of finding treatments is an incredibly painstaking effort. We can think about this as another case of creating knowledge. Creating knowledge both requires us to make very good and informed conjectures, which subsequently, need to be tested. *In vitro* models can be vital tools in both parts of this equations, both the understanding of basic neurological mechanisms, but also the testing of potential treatments. Take epilepsy for example, where neuronal cultures have helped in elucidating how plasticity, inhibition and excitation balance, or excitotoxicity are involved in the pathology of epilepsy. Using neuronal cultures to subsequently screen for treatments could be a much more efficient alternative to animal models, but also a more ethical one. The important part here, however, is that the neuronal culture models need to be a good predictor for drug outcome.

Relying on electrophysiological readout from neuronal cultures is potentially a really efficient way of doing high-throughput screening experiments. Researchers are already using multi-well microelectrode arrays for this purpose, where several cultures can be recorded simultaneously (Tukker et al., 2018). Using electrophysiological readout also means that the response of a treatment can be observed in real-time, as a drug is being applied, and it is is being removed, and the long-term consequences of an acute treatment. This is in comparison with other readouts which constitutes end-points of a culture, such as mRNA sequencing, or morphological analyses. Most electrophysiological analyses, however, rely on looking at the dynamics of spontaneous network bursts. As we've discussed, these bursts

don't really have an existing in-vivo counterpart, but are more like an artifact of the particular circumstances of in-vitro conditions. On the other hand, spontaneous network bursts can be seen as unspecific reporters for a wide array of neuronal processes, making them useful in assessing if a compound is affecting the neurons in some way. However, as we saw in paper II, network bursts can also respond to irrelevant factors such as cluster formation, meaning that relying on network bursts could be a potential source of systematic errors or misleading results.

A contrary take on this is that synchronous network bursts are the enemy. From my own experience in analysing the responses to stimuli (Paper IV), network bursts are a frustrating source of noise. When stimulating neurons, network bursts will occur sporadically, making the network unresponsive to stimuli for many seconds. When doing point stimulation (Paper IV), I had to increase recording replicates by a lot in order to smooth out the noise that the network bursts create. Also, if we want to analyse connectivity by looking at spontaneous spike firing patterns, to which extent is this data being skewed by synchronous bursts? It is possible that network bursts are masking important features of the underlying circuit, as we showed in our carbachol treatments in paper IV, where new clusters of network activity emerge as the synchronous activity is removed.

As we saw in paper II, the formation of clusters alone could drive changes in network burst dynamics. Using a perforated microchannel device could be a useful way for reducing the formation of clusters, or at least confining them to the individual perforations. I feel like I can't "over sell" the serendipitous discovery of how much the signal-to-noise ratio, and the number of active channels increased when using perforated microchannels (Paper III and IV). Even if it turns out that guiding network circuitry isn't useful, or not relevant for a particular experiment, the use of perforated microchannels for the mere purpose of increasing experiment throughput could be very useful. I think this is especially true because manufacturing perforated membranes is relatively simple (once you figure out how to produce them), and materials for producing them is relatively inexpensive, making this technique deployable at scale.

Neurodevelopmental disorders and psychiatric conditions are complex pathologies which involves both the formation of neuronal network but also their plasticity and long-range interactions of neuronal subpopulations. Modelling complex pathologies like these may be limited by the relative simplicity of traditional cell cultures. Our results show several paths forward toward the goal of realizing the potential of human neuronal cell cultures. Using electrospun fibers remain an understudied method for controlling neuronal circuit architecture *in vitro*. Electrospun fibers potentially provide more control of circuit microarchitecture than microchannels or spheroids, as fibers can guide neurite outgrowth throughout the culture and also on a smaller feature scale. Elecrospinning is also particularly attractive as it's both an inexpensive manufacturing technique as well as hosting a broad repertoire of fiber morphologies that can be constructed. Electrospun fibers can also conveivably be

surface modified to incorporate molecular gradients to provide an additional handle on neuronal connectivity *in vitro*.

The simplicity and reliability of using spheroids and soft lithography with microelectrode arrays makes them attractive in controlling network architecture and microenvironment *in vitro*. Formation of spheroids on microelectrode arrays is a conveniently easy procedure for generating 3D tissue-like microenvironments, but alternative tools for obtaining reliable recordings are warranted. Several alternative microelectrode array designs have emerged, but perhaps a simpler approach for low-cost electrophysiology is possible? Using thin electrode wires, the type otherwise used for creating *in vivo* tetrode probes could perhaps be used to circumvent the need for expensive microfabricated approaches. Soft lithography turned out to be our most successful technique for controlling circuit architecture, and despite limitations in microenvironmment control, resolution of control and possible topographical limitations, these probably represent the most immediately lucrative approach for further research.

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List of papers

I. Hörberg, C., Sandberg, A., Arvidsson, M., O'Carroll, D.C., Johansson, F., Englund Johansson, U.(2025). Controlled Deposition of Human Neural Stem Cells on Fiber Substrates using Gel Encapsulation. (submitted).

II. Hörberg, C., Englund Johansson, U., Johansson, F., O'Carroll, D. (2022). Spontaneous Cell Cluster Formation in Human iPSC-Derived Neuronal Spheroid Networks Influences Network Activity. Eneuro, 9(5).

III. Hörberg, C., Beech, J.P., Englund Johansson, U., O'Carroll, D.C., Johansson, F. (2025). Population-Wide Control of Neuronal Networks in vitro. (manuscript).

IV. Hörberg, C., Beech, J.P., Englund Johansson, U., O'Carroll, D.C., Johansson, F. (2025). Plasticity, Sparsification, and Ensembles in Designed Neuronal Circuits in vitro. (manuscript).