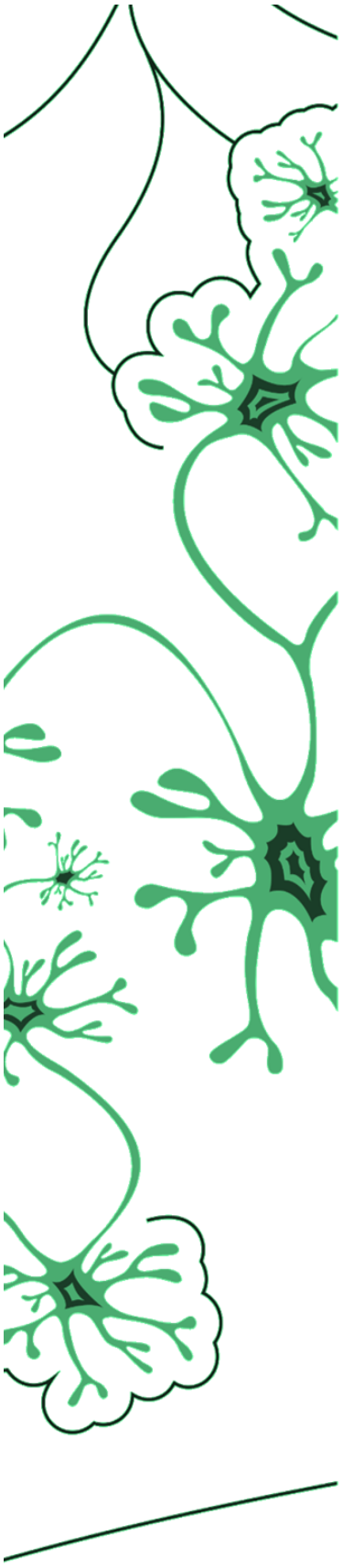

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The role of network bursts in memory

MSc Thesis

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To my mother and father

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Abstract

In the awake state, memory acquisition is thought to be underlined by high cholinergic cortical levels, which allow the incoming information to be encoded in the hippocampus. During slow-wave sleep, in a process known as systems consolidation, the encoded input is replayed by the hippocampus, which activates neocortical areas leading to the transfer of information to the cortex, where memories are permanently stored. This consolidation process, which results in persistent functional changes representing an experience in the brain, is believed to benefit from the oscillatory rhythms and the low acetylcholine availability observed in the neocortex.

Few studies have empirically tested the previous hypothesis, with an unceasing debate on the mechanisms behind memory consolidation and retrieval. Our goal was then to better clarify if cholinergic modulation and synchronized activity are indeed essential for memory consolidation. We mimicked the cue replay observed during systems consolidation through low-frequency electrical stimulation of dissociated cortical cultures bursting spontaneously, as observed during slow-wave sleep. We also applied the electrical stimuli replay in cultures treated with carbachol, a cholinergic agonist, to simulate the increased cholinergic tone observed in the awake cortex. We assessed the effect of both chemical and electrical stimulation on the activity and connectivity patterns of the cultures tested.

Our results show that carbachol administration transformed activity patterns from synchronized bursting into dispersed uncorrelated firing. In cultures without carbachol treatment, we observed significant connectivity changes upon first stimulus application ($p<0.05$), while subsequent stimuli did not perpetrate any further changes in network connectivity ($p>0.05$). Moreover, application of a different stimulus led again to significant connectivity changes ($p<0.05$), which did not erase the first alterations observed. Distinctively, in carbachol-treated cultures we did not observe any significant connectivity drives away from baseline values ($p>0.05$), although cultures still responded to stimulation throughout the duration of the experiments. These observations suggest that cholinergic activation and the absence of synchronous activity hamper memory consolidation in dissociated cortical cultures. The present findings represents a step forward towards more profound proofs-of-concept regarding the underpinnings of memory replay and consolidation during slow-wave sleep.

Contents

List of acronyms

ACh	acetylcholine
AChE	acetylcholinesterase
ANOVA	analysis of variance
BI	burstiness index
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CCh	carbachol
CFP	conditional firing probability
DIV	days *in vitro*
ED	Euclidian distance
EPSP	excitatory postsynaptic potential
FR	firing rate
hiPSCs	human induced pluripotent cells
LTP	long-term potentiation
MEA	microelectrode array
MFR	mean firing rate
MRS	magnetic resonance spectroscopy
MTL	medial temporal lobes
PFC	prefrontal cortex
PSTH	post-stimulus time histogram
REM	rapid-eye movement
SO	slow oscillations
SP	sleep spindles
STD	short-term synaptic depression
STDP	spike-timing-dependent plasticity
SWR	sharp-wave ripples
SWS	slow-wave sleep

Chapter 1

Introduction

1.1 Problem statement

Network or population bursts are a widely observed phenomenon in neuronal populations. This pattern of synchronized activity occurs during the early development of the brain, in certain stages of sleep and after some types of brain injury, including stroke and post anoxic encephalopathy.[1] Unperturbed *in vitro* cultures of dissociated cortical neurons also include this synchronous behaviour in their firing patterns.[2,3] Despite being the most striking display of spontaneous activity in cortical cultures, the importance of these oscillations is still not clear. Bursting patterns may have a specific function as cognitive performance or may be an epiphenomenon that occurs whenever networks are insufficiently activated.[4] Their role has only been speculated on, with no real evidence provided to support these hypotheses yet.

During immobility and slow-wave sleep (SWS), synchronous population discharges, also known as sharp waves (SWRs), are also observed to induce population bursts in region CA1 of the hippocampus.[5,6] These patterns are hypothesized to interact with other oscillatory rhythms, as sleep spindles (SPs) and slow oscillations (SOs), with their coupling believed to play a crucial role in memory consolidation.[7] After a memory trace is formed through the combined effort of several structures within the medial temporal lobe, particularly the hippocampus, and temporarily stored in this area (synaptic consolidation), the encoded information of the trace is then transferred to the neocortex, in a process known as systems consolidation.[7,8] In this latter consolidation stage that occurs during SWS, memory traces are replayed by the hippocampus, which repeatedly activates neocortical areas allowing information to be permanently stored in the neocortex.[9] Apart from the oscillatory patterns detected during SWS, the cholinergic levels in the neocortex and hippocampus also decrease to their minimum throughout this sleep phase. Conversely, a high cholinergic tone is observed in these brain areas during the awake state.[38] This cholinergic modulation is thought to be essential for declarative memory formation and consolidation, with high acetylcholine (ACh) concentrations supporting memory encoding and in turn hampering memory consolidation and retrieval.[10, 11,12]

Despite the enormous body of work regarding systems consolidation during SWS, little is currently supported by empirical evidences.[13] The underlying mechanisms behind memory encoding, consolidation and retrieval as well as the role of network bursts and acetylcholine in both formation and consolidation of a memory trace are still a matter of debate and urging for experimental data to allow the drawing of increasingly meaningful conclusions.[13] Solving these conceptual issues underpinning memory will also allow for a better understanding of certain memory-related pathologies as amnesic syndrome, Alzheimer's and Parkinson's disease.[14]

Several studies have proposed *in vitro* models such as dissociated cortical cultures platted on microelectrode arrays (MEAs) as useful platforms to answer the still pending questions surrounding memory.[44, 45,46] Cultures bursting spontaneously are hypothesized to develop a connectivity <> activity balance in the absence of external input, where occurring activity patterns support current connectivity.[4] By mimicking the replay of information that takes place during systems consolidation, previous studies have shown that repeated electrical stimulation leads to the formation of memory traces in these cortical cultures.[8] The external cues seem to disturb the initial established balance by changing the connectivity of the network and driving it

to a new equilibrium. The connectivity changes observed in the networks were then interpreted as formation and consolidation of persistent memory traces.[8]

Nevertheless, these experiments only aimed at inducing memory in dissociated cortical cultures with no cholinergic input[8] and so, bursting spontaneously as it is observed during SWS. The naturally occurring bursts may be suppressed by ghrelinergic or cholinergic activation[15], with the aim of mimicking the high cholinergic tone and absence of synchronized oscillations observed in the awake cortex. Combining this pharmacological manipulation with electrical stimulation to induce memory traces in the dissociated cortical neurons would elucidate to which extent the formation and consolidation of a memory trace *in vitro* is influenced by external input and intrinsic network patterns.

1.2 Research goal

The aim of this project is to assess if synchronized activity and cholinergic modulation are essential for memory consolidation in networks of dissociated cortical neurons. We will then appraise the role of bursting patterns and the effect of cholinergic treatment in memory trace stabilization. To accomplish this goal, we will electrically stimulate cortical neurons receiving or not receiving cholinergic input (through carbachol administration) and analyse the data obtained in terms of the activity and connectivity patterns of the networks. The end goal is to establishing relations between the given stimuli (electrical and chemical), neurophysiological activity and connectivity and relate the observations with memory consolidation mechanisms.

Chapter 2

Background

2.1 Memory

The quest for how experiences are stored in the brain has preceded modern science and psychology.[16] For centuries, philosophers and scientists have wondered what underlies cognition and perception and how knowledge is built up into learning and memory. Plato believed that *"there exists in the mind of a man a block of wax (…) harder, moister (…) a gift of memory"* [17] sharing with René Descartes the vision that truth is innate to one self, in the sense that knowledge is remembered and recalled through intelligence.[18] On the other side, Aristotle and later, John Locke and David Hume, defended that knowledge comes from experience, with the model of accumulated sensory experience being currently the dominating one.[13] The Renaissance witnessed a dawn for experimental science, anatomy and physiology, allowing the search for memory and knowledge to be transferred from the realm of philosophy and divinity into the scope of behavioural neuroscience. By the 19th century, the nervous system and the brain were placed as the central figures in perception, memory and cognition. With Richard Semon in the beginning of the 20th century, the notion that experiences could modify and create physical memory traces (the so-called *engram*) within the brain, ignited the search for empirical evidences of learning and memory within the nerve hub.[13] Only in the last century, the believe that synaptic plasticity and connectivity play a major role in memory formation and consolidation has been a matter of investigation, with speculating theories on their underling processes still fiercely debated.[19]

2.1.1 General definition

Memory is currently defined as the process by which new information and skills, acquired during experience (learning), are stored and retrieved.[20] Sensory input will become part of a memory if it results in persistent structural and functional changes that represent an experience in the brain, with this underlying ability for change called plasticity. The retention, reactivation and reconstruction of an experience implicates that, if a memory is acquired, it should then be expressed through behaviour, with observed physical neural changes within individual neurons or altered strengths of the synaptic connections.[16, 20] Some authors suggest the distinction between three different stages of memory (see *Appendix A.1*) in regard to the duration in which information remains accessible: immediate, short-term and long-term memory.[20] The flow of information through these different memory stages is dependent on encoding, retrieval and consolidation mechanisms, essential for the construction of an experience within the brain.

2.1.2 Encoding, consolidation and retrieval

After an event is experienced and sensed by the immediate memory and driven by attention to the short-term memory, some of its aspects are encoded by plasticity mechanisms, that allow the establishment of connectivity patterns to encode the received information.[7, 22] The spiking of individual neurons induced by the sensory input will persist after that input disappears, being incorporated in the new activity patterns of the network.[4] After this initial encoding within the hippocampus, the information to be preserved and transferred to the long-term memory is determined by memory consolidation mechanisms.[7, 22]

Two different processes are thought to underlie declarative memory consolidation: synaptic consolidation, in which memories are temporarily stored in the hippocampus after learning, and systems consolidation, in which memories are gradually transferred from the hippocampus to the neocortex.[7,23] The consolidated memory trace can be recalled and made again liable, with this reactivation maintaining, strengthening, changing or disrupting the trace.[22, 24]

Long-term potentiation (LTP) is thought to be the underlying mechanism in synaptic consolidation, in which enhanced synaptic transmission at some synapses in the hippocampus potentiates the consolidation of the memory trace.[20, 25, 26] The processes behind systems consolidation are suggested to require repeated activation of cortical areas by the hippocampus, allowing the transfer and permanent storage of information in the neocortex.[27, 28, 31] This is believed to occur during immobility and certain sleep stages, with several authors stressing the crucial role of sleep in consolidating the formed memory traces.[7, 29]

2.2 Sleep and memory consolidation

Sleep is believed to be essential in systems consolidation, specially the oscillatory rhythms observed during slow-wave sleep (SWS). These patterns include the hippocampal sharp-wave ripples (SWR), the slow oscillations (SO) in the neocortex and the thalamo-cortical sleep spindles (SP).[32] Sharp-wave ripples (SWR) are observed during the activation of neocortical areas and the prefrontal cortex (PFC) by the hippocampus, where the replay and cortical reactivation of awake neural patterns occurs.[34] SPs in turn are thought to promote and trigger synaptic plasticity, modifying neocortical synapses by LTP and allowing the consolidation of memory traces in neocortical cells.[7,34,35] Finally, slow oscillations (SOs) are believed to facilitate the coupling between SWRs and SPS, which are temporally correlated (see *Appendix A.2*).[7] An extensive description of the coupling between these three oscillatory patterns is beyond the scope of this work, so a more detailed analysis can be consulted in recent reviews on the topic.[34,35] The interactions between SOs, SWRs and SPs and their synchronization are believed to underlie memory reactivation and consolidation[36], but these oscillatory rhythms are not the only phenomena thought to be involved in the complex process of memory stabilization.

Fluctuations observed in the levels of certain neuromodulators, as acetylcholine (ACh), through different behavioural states are also suggested to mediate the information flow and consolidation of a memory trace.[33] ACh is a neurotransmitter and neuromodulator involved in arousal, attention and synaptic plasticity. [9, 11, 37] In the neocortex, the nucleus basalis of Meynert is the primary source of ACh, while its main projections to the hippocampus are the medial septum and the diagonal band of Broca.[11] Cholinergic levels appear to be high in the hippocampus and cortex during wakefulness and REM sleep, whereas a depletion in the ACh tone is observed throughout slow-wave sleep.[38] Several microdialysis studies assessing ACh availability in the cortex of cats and rodents during different stages of waking and sleep support this observation.[81, 82, 83] Decreased cortical ACh levels were measured during both SWS and quiet awaking while a rise in cholinergic tone was documented during active awaking, particularly during tasks involving increased attention to external stimuli, memory tasks and exploration of novel environments.[84, 85, 86, 87, 88]

The variation in ACh concentration is thought to be beneficial for both synaptic and systems consolidation, with the first requiring a higher cholinergic tone and the last a lower ACh level.[11] By potentiating synaptic plasticity during wakefulness, ACh is then hypothesized to facilitate the encoding of new information, but hampering memory consolidation and retrieval.[39] This

assumption is in agreement with a previous hypothesis on the role of SWS in systems consolidation, stating that reactivation of the neuronal networks which initially encoded the memory trace require an adjustment in network dynamics.[11] This is believed to be achievable by releasing the cholinergic suppression of recurrent neuronal feedback synapses in the hippocampus, allowing the flow of information to the neocortex during systems consolidation.[33] The reduction in ACh levels during SWS is then believed to enable memory consolidation, which is suggested to be further enhanced by a rising ACh tone in subsequent REM stages.[12]

2.3 Dissociated cortical neurons on the study of memory

The underlying mechanisms behind memory encoding, consolidation and retrieval as well as the role of synchronized oscillations and acetylcholine in both formation and consolidation of a memory trace are still a matter of ongoing debate.[13] With the technological advances in the last few decades, the information obtained from depth recordings in epileptic patients before surgery, as the famous case of Henry Molaison, is now reinforced by insights gained through optogenetics, transgene expression and electrical stimulation in mice, opening the path towards more proofs-of-concept regarding the underpinnings of memory consolidation during SWS.[13] Most studies focusing on synaptic plasticity and memory formation and consolidation have been performed *in vivo* in mice, due to the ethical and spatial resolution constraints of performing certain proposed paradigms in humans.[13] Nevertheless, it is hard to record simultaneous activity of multiple neurons *in vivo* and to provide accurate estimates of the synaptic coupling through this technique as well.[13] *In vitro* studies using dissociated cortical neurons plated on microelectrode arrays (MEAs) propose that this is a useful and promising platform to study neural networks in memory.[8]

2.3.1 Recording network activity using MEAs

A microelectrode array (MEA) is a device containing multiple microelectrodes that allow for parallel recording of neural signals, serving as the interface between neurons and electronics.[40] *In vitro* MEAs (with 60 electrodes in a typical conformation) can be applied in conjunction with dissociated cortical neurons, whose network properties and function seem to be retained from their *in vivo* counterparts.[41] Culturing these cells directly on a MEA device allows for a more controlled and simpler environment to study the patterns of action potentials generated by a relatively small and single layered neural network.[2] These devices have several advantages over other more traditional methods such as patch clamping, as the ability to perform non-invasive and long-term experiments, with repeated or continuous spiking activity recordings over several hours and the possibility to record and stimulate from multiple locations within the array.[40]

Dissociated cortical neurons become spontaneously active a week after being plated in a MEA, reaching a mature state approximately 3 weeks after seeding, where activity patterns and connectivity appear to stabilize.[43] Spontaneous activity of these cultures show periods of uncorrelated firing at some electrodes, which alternate with periods of short intense firing and variable quiescent phases.[43] The fast, intensified and synchronized firing events occur through the recruitment of many active sites, with almost all electrodes involved in the short-term discharges observed. These patterns are normally referred to as network bursts, present from 4-7 days *in vitro* (DIV) throughout the entire lifetime of cultures receiving no external input.[2,3,43]

Excitatory connections in neural networks of spontaneously active cells can then self-generate periodic activity.[52] This periodicity is thought to be underlined by the fact that each network

contains a critical number of excitatory connections.[53] The model proposed by O'Donovan (1997) suggests that the activity of a network with spontaneously active neurons connected recurrently through excitatory synapses will increase progressively until a threshold is reached. Upon this, a network burst will occur, leading to the depression of the network and consequently to a dramatic decrease in its activity (the quiescent phase), thought to be caused by neurotransmitter depletion.[52, 53] After this inactivation period, the cycle begins again, with the network slowly recovering and "building up" in activity until a new event of correlated firing occurs.

Networks bursts comprise many action potentials within a time window of approximately 200 ms, which was previously shown to induce spike timing dependent plasticity (STDP).[4] Activity patterns are not only determined by a certain connectivity but they also in turn affect connectivity through certain plasticity mechanisms. STDP follows that when an action potential is fired, synapses that were active just before that action potential occurs are reinforced, whereas synapses that were active after the action potential onset, and so irrelevant on its generation, are weakened.[13] These input deprived cultures are believed to develop a connectivity <> activity balance, where occurring patterns in spontaneous activity support current connectivity.[4] Although synchronized bursting patterns are thought to influence network connectivity, their importance to establish this connectivity <> activity balance is still not clear, with some authors speculating on their importance in cognition and early brain development while others suggest that they are a result of insufficiently activated networks. [4,52]

2.3.2 Electrical stimulation on memory trace formation

Previous studies have shown that perturbing dissociated cortical neurons through repeated optical or electrical stimulation leads to the formation of memory traces within these naturally bursting cultures.[44, 45, 46] Recurrent external input seems to disturb the naturally established connectivity <> activity balance, with changes in connectivity driving the network to another equilibrium.[3,8] This connectivity changes are then interpreted as evidence on the formation and consolidation of a persistent memory trace.[3, 8] Moreover, the response to the applied stimulus is believed to be included in the new palette of spontaneous activity patterns of the network.[3,8]

Several paradigms and parameters for electrical stimulation have been proposed to alter network connectivity. Most paradigms include rectangular and biphasic pulses, delivered either as current or voltage pulses that vary in duration from 200 to 400 µs.[47, 48] These pulses were mainly delivered as tetanic stimuli, with frequencies ranging from 20 to 250 Hz, inter-train intervals between 2 to 10 seconds and the number of pulses per train fluctuating between 10 to 100. [8,48,49] The electrodes chosen for stimulation also differed, ranging from random stimulation in all MEA electrodes[4, 50], stimulation at a single electrode[4, 44], stimuli delivered in randomly chosen pairs of electrodes[48, 51] or stimulation applied in two previously chosen electrodes accounting for the clearest network response.[8]

In a study conducted in our research group by le Feber et. al (2015), tetanic stimulation was shown to induce memory traces in seven cultures of cortical neurons, when stimulated through two different electrodes with 10 minutes of biphasic current train pulses. Parallel storage of the traces was observed, suggesting that different stimuli were able to induce connectivity changes in an independent manner. Low-frequency stimulation was also applied in four cultures, but only at a single electrode, yielding connectivity changes comparable with single electrode tetanic stimulation. Nevertheless, these experiments only included dissociated cortical cultures with no cholinergic input and so, bursting spontaneously as observed during SWS.

2.4 Research questions

The intrinsic mechanisms behind memory encoding, consolidation and retrieval are still urging for experimental data to support current formulated hypothesis.[13] Particularly, the role of slow-wave sleep in systems consolidation is still intensely debated, with some authors suggesting that the synchronized oscillations and the low cholinergic levels observed during this sleep stage are of utmost importance for memories to be consolidated in the neocortex.[7, 9, 12]

Recent works have proposed *in vitro* models as promising platforms to experimentally investigate memory mechanisms in neural networks.[8] The spontaneous activity of *in vitro* dissociated cortical cultures show the occurrence of network bursts, fast and intense discharges that encompass the synchronous recruitment of several active neurons, resembling the oscillatory patterns observed in the cortex during SWS. Although network bursts are a widely observed phenomena, their role is still not clear. Previous studies showed that these patterns can be suppressed in cortical cultures by cholinergic activation, mimicking changes observed in the awake cortex.[15]

Combining the administration of cholinergic input with repeated electrical stimulation of cortical neurons to induce connectivity changes in the networks, would elucidate to which extent the consolidation of a memory trace *in vitro* is influenced by external input and by intrinsic network bursting patterns. We hypothesize that high cholinergic levels and the absence of synchronized activity may facilitate memory encoding but hamper memory consolidation and retrieval. Therefore, the questions that guided our work may be drawn as follow:

- Does cholinergic input alter the spontaneous activity patterns of dissociated cortical cultures?
- Is it possible to form parallel memory traces through low-frequency electrical stimulation of spontaneously bursting cultures? *if so*
- Are network bursts essential for this memory trace consolidation? *and finally*
- Does cholinergic activation hamper memory consolidation in electrically stimulated cortical neurons?

In the following chapter, we will address the methodology applied to answer these proposed issues.

Chapter 3

Methods

3.1 Cell culturing

Cortical cells were obtained from Wistar rats at post-natal Day 1. After trypsin treatment, cells were dissociated by trituration with around 400,000 dissociated neurons (400 µL suspension) plated on a microelectrode array (Multi Channel Systems, Reutlingen, Germany), precoated with polyethyleneimine. This procedure lead to a cell density of approximately 5000 cells/mm^2, with aging cell densities gradually decreasing for approximately 2500 cells/mm^2. Neurons were cultured in a circular chamber with inner diameter $d = 20\ mm$, glued on top of a MEA with 60 titanium nitride (TiN) electrodes with a 30-µm diameter and 200-µm pitch. The culture chamber was filled with approximately 700-µL R12 medium and MEAs were stored in an incubator under standard conditions of 37°C, 100% of humidity and 5% of CO_2 in air. The medium was changed twice per week. After each experiment, the cultures were returned to the incubator. We used 23 cultures for 23 experiments, which were performed 26 ± 6 days after plating (culture age range from 19 to 36 DIV). Cultures used were considered to be in the mature phase of development, with spontaneous activity mainly dominated by network bursting patterns.[2,43] All surgical and experimental procedures complied with Dutch and European laws and guidelines.

3.2 Recording set-up

To assess cell activity, cultures were placed in a measurement set-up (Figure 3.1) outside the incubator, consisting of a MEA1060-Inv-BC preamplifier, a FA60 filter amplifier and a STG 1002 stimulus generator (all from Multi Channel Systems). Signals from all 60 MEA-channels were recorded at a sampling frequency of 16 kHz through a NI PCI-6071E analog-to-digital convertor board (National Instruments, Austin, TX), with noise levels typically from 3 to 5 μV_{RMS}. Before placing the MEAs in the measurement setup, culture chambers were firmly sealed with watertight but O_2 and CO_2 permeable foil (MCS; ALA scientific) and the temperature was kept at 37°C through a TC01 temperature controller (Multi Channel Systems). During recordings, a custom-made LabView (National Instruments) application allowed to set the three mass flow controllers (Vögtlin Instruments, Aesch BL, Switzerland) used to maintain the CO_2 level at approximately 5%, the humidity at 100% and N_2 at 0% during experiments. This gas mixture was flushed to an isolated Plexiglas flow hood where the measurement set-up was placed in, at a rate of 2 L/min. Recordings began after a 20-minute accommodation period. A custom-made LabView (National Instruments) application was used to drive the analog-to-digital convertor board for data acquisition, with all analog signals band-pass filtered (0.1 kHz–6 kHz) before sampling. A detection of action potentials was performed online on the recorded signals, through a predefined detection threshold set as 5.5 times the estimated root-mean-square noise level (rms). This threshold was continuously updated throughout the duration of the experiment, with 6 ms of data stored for each candidate action potential (time stamp, recording electrode and waveform of the potential spike). This detection allowed for data reduction and increased computational speed. To infer on the activity of the cultures, networks were considered active if more than 2500 spikes were recorded per 5 minutes of spontaneous activity, summed on all recording electrodes.

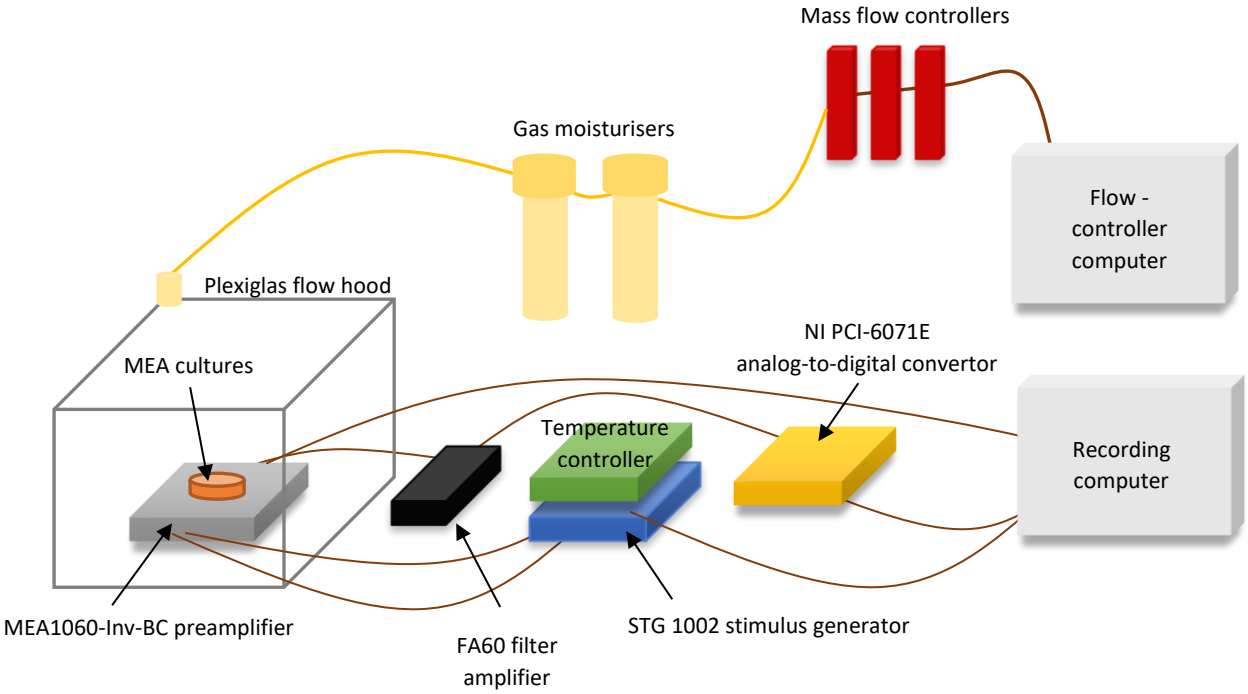

Figure 3.1 – Schematic of the measurement set-up used to record neurophysiological activity and control the atmosphere surrounding the cultures. The left bottom corner shows the flow hood placed on top of the preamplifier/headstage MEA1060-Inv-BC, which contains the cultures plated on a MEA and assures 100% humidity and 5% CO_2 on the atmosphere surronding the cultures. The gas mixture is monitored through 3 mass flow controllers programmed by a LabVIEW application. Data acquisition is controlled by the NI PCI-6071E analog-to-digital convertor, which bandpasses the signal pre amplified by the FA60 filter and directs it to the recording LabVIEW software.

3.3 Culture stimulation

3.3.1 Pharmacological manipulation

Carbachol (CCh, Sigma-Aldrich, St. Louis, MO, USA), a selective cholinergic agonist, was prepared in a stock solution of 400 µM in PBS and applied in half of the cultures to supress naturally occurring bursts. To determine the carbachol concentration needed for this suppression, 4 different CCh concentrations (5, 10, 20 and 40 µM) were administered in 3 spontaneously bursting cultures (35 ± 3 DIV). This range of concentrations was based on values in previous studies[15] that also aimed to disrupt the spontaneous synchronicity of the network. After a 30-min baseline recording of spontaneous activity, the concentrations were tested as follows: starting with 5 µM, the following concentration was always larger than the previous one by a factor of 2. The 30-min recordings of spontaneous activity in between administrations allowed to infer on network activity, which was quantified through the burstiness index (BI), a measure defined in section 3.5.2 of this chapter. The concentration showing the lowest BI among the 4 concentrations tested was chosen to further infer on its long-term effect on the activity patterns of the cultures. Experiments aimed at inducing memory traces through electrical stimulation lasted up until 15 hours, meaning that the CCh concentration administered should sustain burst suppression throughout the duration of the procedure. The long-term effect of the chosen concentration was assessed in 4 spontaneously bursting cultures (30 ± 5 DIV), through 15-hour continuous recordings after CCh administration. BI values for each hour of the procedure were again computed and compared with baseline values.

3.3.2 Electrical stimulation

Biphasic rectangular current pulses of 200 μs in each phase (starting with the negative phase first) were applied to all dissociated cortical cultures at a low frequency of 0.2 Hz. After an all-electrode probing procedure (see following section), pulse amplitudes were chosen between 12 and 36 μA, which typically allowed for more than 50% of the stimuli to trigger responses but still avoided electrolysis due to high voltages. These values for the stimulus parameters were based on previous studies[4,8] using low-frequency current stimulation, in which memory trace consolidation and learning was accomplished in cortical cultures bursting spontaneously.

3.4 Experimental design

The dissociated cortical cultures tested were randomly distributed in two different groups, the control (non-CCh treated cultures) and the CCh-group (CCh treated cultures), with the experimental procedure followed for each set presented in Figure 3.2. The total experimental time was kept as short as possible (around 15 hours), in an attempt to avoid spontaneous connectivity changes.[2]

In both groups, a 1-hour baseline recording began after a 20-minute accommodation period. After recording spontaneous activity, all electrodes were probed twice for each of the 3 predefined amplitudes (12, 24 and 36 μA), in random order. The 20-minute probing procedure allowed to find the amplitude and the two electrodes showing the clearest stimulus response, which were subsequently used for the stimulation protocol. This response was assessed through the post-stimulus time histogram (PSTH), derived by the custom-made LabView recording application (National Instruments). A PSTH curve depicts the total number of action potentials recorded at all electrodes, as a function of the latency to the given stimulus. This measure is further defined in section 3.5.2 of this chapter. In Figure 4.5 of the *Results* chapter, an example of such a network response to stimulation is depicted. Electrodes showing a greater area under the PSTH curve around latencies 20-100 ms were chosen as stimulation electrodes, with no constraints related to their location. The lowest amplitude to induce these responses through both stimulation electrodes was selected among the 3 tested.

In control cultures, the stimulation protocol started immediately after probing. Low-frequency pulses were applied through the first electrode for 10 minutes, followed by a 1-hour period of no stimulation. This period of spontaneous activity allowed to infer on functional connectivity. Each 10-min stimulation epoch and the subsequent 1-hour of spontaneous activity block were repeated 4 times. When finished, stimulation through the second selected electrode was applied with the same paradigm. Finally, the first electrode was again used to stimulate the network in the same fashion. In contrast to controls, CCh was firstly administered to each culture of the CCh-group after probing. A 1-hour baseline recording followed by a second 20-minute probing procedure were also performed prior to the beginning of the stimulation protocol, to allow connectivity comparison before and after CCh administration. The same stimulation paradigm (four periods of stimulation in each electrode) was applied as described for control cultures.

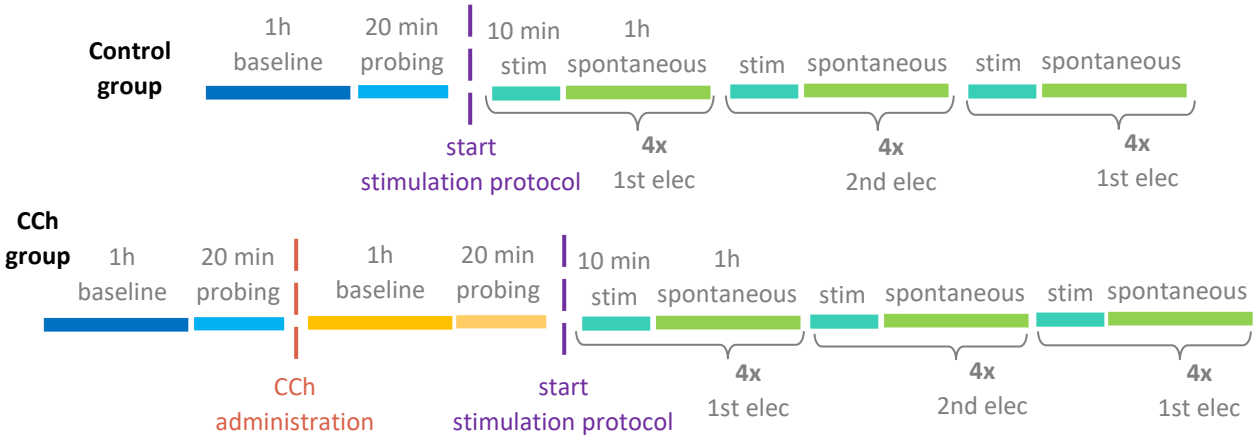

Figure 3.2 – Experimental design for both the control (top scheme) and CCh-group (bottom scheme). In both groups, after recording 1-hour of spontaneous activity (baseline), all electrodes were probed twice for each of the three predefined amplitudes (12, 24 and 36 µA) for 20-min, with the aim of choosing the 2 electrodes showing the clearest response to stimulation. In control cultures, the stimulation protocol began immediately after probing, with low-frequency pulses applied through the first electrode for 10 minutes, followed by a 1-hour period of no stimulation. These 2 blocks were repeated 4 times for the first electrode. When finished, stimulation through the second selected electrode was applied with the same paradigm and finally, the first electrode was again used to stimulate the network. In CCh-treated cultures, carbachol was administered to the bath after probing, with a second 1-hour of baseline and 20-min probing procedure after pharmacological manipulation. The same stimulation paradigm described for controls was then applied to CCh cultures.

3.5 Data analysis

The data obtained from the neurophysiological experiments previously described was analysed in terms of the activity and connectivity patterns of the cultures tested. When discussing the analysis methods used to assess these patterns, we will refer to the activity and relationships between MEA electrodes and not between neurons. Due to their size, recording electrodes might have been in contact with several units, meaning that the activity recorded per electrode is not representative of a single neuron but of the set of neurons that were closest to that particular electrode. As we want to infer on the activity and connectivity of the network rather than on single-unit behaviour, we do not discriminate between the activity of this individual neurons and so we will further refer to the recording electrodes in our analysis.

All data analysis was performed in MATLAB R2018a (MathWorks, Massachusetts, USA), with custom-made scripts and dedicated functions for each outcome variable extracted.

3.5.1 Artifact detection

To remove possible artifacts recorded together with real cell activity, an offline artifact detection analysis adapted from Wagenaar et al.[57] was performed. In short, a candidate action potential was considered valid if within a 1 ms window around the main peak of the waveform, no other peaks with equal or higher amplitude were recorded. Moreover, if lower amplitude peaks were recorded within that window, their amplitudes could not be 90% or 50% the amplitude of the candidate event. Duplicated spikes were also removed.

3.5.2 Activity measures

To analyse the activity of the cultures, four outcome measures were derived: the raster plot, the mean firing rate (MFR), the burstiness index (BI) and the post-stimulus time histogram (PSTH).

3.5.2.1 Raster plot

A raster plot depicts the activity of a group of neurons recorded by each MEA electrode, with the y-axis displaying the activity recorded in each channel over time (x-axis). This means that each thick in the graph corresponds to a spike detected by a particular electrode at the correspondent time stamp. A raster plot allows for a first glimpse on the activity of the cultures in each phase of the procedure.

3.5.2.2 Mean firing rate (MFR)

The mean firing rate (MFR) was computed as defined by Bologna *et al*. (2010).[54] In short, the firing rate (FR) of each single channel was firstly obtained as

$$FR = \frac{\int_0^T (\sum_{s=1}^{N} \delta(t - t_s))\, dt}{T} = \frac{N}{T} \qquad (1)$$

where *N* stands for the number of spikes recorded at that specific electrode at time t_s (the timing of a spike), *T* the duration of the recording and *δ(t)* the Kronecker delta function. FRs were then averaged across all active electrodes of the MEA, to obtain the MFR. A MFR rate value was calculated for the baseline and for each of the 1-hour of spontaneous activity recordings in between stimulation periods. Electrodes were considered active if more than 200 spikes were recorded at that particular channel in the 1-hour defined bins.

3.5.2.3 Burstiness index (BI)

The synchronicity of each culture was assessed through the burstiness index (BI), a measure introduced by Wagenaar *et. al* (2005) and expressed as

$$BI = \frac{f_{15} - 0.15}{0.85}. \qquad (2)$$

Briefly, each 5 minutes of a recording were divided into 300 1-second long time bins, with the number of spikes across all electrodes counted in each bin. Then the fraction of the total number of spikes accounted for the 15% of bins with the largest spike counts, f_{15}, was computed. [55] The BI is a normalized measure between 0 and 1, meaning that if a recording is mainly dominated by bursting patterns, its BI value will be close to 1, whereas a BI equal to 0 indicates absence of synchronized firing.

3.5.2.4 Post-stimulus time histogram (PSTH) and mean area under the curve

Electrical stimulation has been shown to induce a response in two different phases: the first phase (early response) is thought to result from direct activation of the neurons in the proximity of the stimulation electrode whereas the second phase (late or network mediated response) is believed to result from synaptically propagated signals of the neurons which fired in the first phase to their connected neurons, creating a wave of activity in the network.[44] To evaluate these

two responses of each network to the stimuli applied through a specific electrode, post-stimulus time histograms (PSTH) of all stimuli within each stimulation period were computed. Succinctly, for each stimulus applied, a time window of 300 ms before and after stimulus onset was defined. Within these latencies, the number of spikes contained in each 5 ms bin were counted and summed across all stimuli. A curve of the number of spikes per 5 ms bin averaged across all active electrodes was then obtained for each stimulation period.

The area under each PSTH curve was also computed and averaged for each stimulation epoch, to assess whether the amount of action potentials in response to stimulation varied across the several stimulation periods. It should be noted that these areas were computed excluding the counts in the first 5 ms bins of each PSTH curve (as they mainly contained stimulation artifacts) and that background activity after stimulus onset was removed by subtraction of the activity already present prior to stimulation.

3.5.3 Connectivity measures

To analyse network connectivity, baseline and spontaneous activity epochs recorded in between stimulation periods were divided in data blocks of 2^{13} spiking events. Cultures were only included in the study if there were enough spiking events recorded to create a minimum of a data block per hour of the recording. This value was long enough to obtain multiple data blocks in all experiments, which were used to determine functional connectivity through conditional firing probabilities (CFPs) and to assess the magnitude of connectivity changes within the network through Euclidean distances (ED). An electrode was considered active if it recorded more than 200 action potentials within a data block.

3.5.3.1 Conditional firing probabilities (CFPs)

Based on previous work by Le Feber *et. al* (2007)[56], for all possible pairs of active electrodes, conditional firing probabilities (CFPs) were computed as the probability to record an action potential at electrode *j* at $t = \tau$ (with $\tau > 0$) knowing that one action potential was previously recorded at electrode *i* at $t = 0$. The function fitted to the probability curve can be depicted as

$$CPF_{i,j}^{fit}[\tau] = \frac{M_{i,j}}{1 + \left(\frac{\tau - T_{i,j}}{w_{i,j}}\right)^2} + offset_{i,j} \qquad (3)$$

in which $M_{i,j}$ represents the strength of a connection, $T_{i,j}$ its latency, $w_{i,j}$ the width of the distribution peak and $offset_{i,j}$ the offset of the curve, which depends on unrelated background activity. Figure 4.6 of the *Results* chapter illustrates one of these probability curves.

If a CFP distribution was not flat, the two electrodes were then considered functionally connected. The measure provided by this technique for the strength of a functional connection was further used to follow the connection development throughout the different phases of the experiment over time. The average number of functional connections was counted before and after CCh administration as well as the number of connections existing prior to the stimulation protocol and after its completion. These values were compared between control and CCh cultures.

3.5.3.2 Euclidian distances (EDs)

The strengths of all connections calculated through the CFPs were combined into a connectivity matrix $S_{i,j}$ for each data block. To assess the magnitude of changes between subsequent data blocks, the Euclidian distance (ED_0) between connectivity matrices at time t and time t_0 can be expressed as

$$ED_0(t) = \sqrt{\sum_{i=1}^{n}\sum_{j=1}^{a}\left[S_{ij}(t) - S_{ij}(t_0)\right]^2} \quad (4)$$

with $t > t_0$. In the case of baseline recordings, t_0 was chosen as the first data block of the recording, while in the case of stimulation at each electrode, t_0 was chosen as the last data block before stimulation at that specific electrode. ED_0 values were normalized ($ED_{0,\,norm}$) to the mean strength of all connections in the data block chosen for t_0 and averaged for each 1-hour of spontaneous activity. These values were then compared with baseline connectivity.

3.6 Statistical analysis

Different statistical tests were applied to study the relevance of the results obtained. The parameters used for statistical testing were the MFR, the BI, the mean PSTH area, the number of functional connections, the average connection strength and the Euclidian distances. The normal distribution of the data was checked using a Shapiro-Wilk test before conducting further statistical analysis, using a significance level of 5%. In the case of normally distributed data, two-sample t-tests, one-way repeated measures ANOVAs or two-way repeated measures ANOVAs were applied. The homogeneity of variances using Levene's test with 5% significance and the normal distribution of the residuals using a Shapiro-Wilk test and Q-Q plots were assessed prior to ANOVA testing. A Mann-Whitney Wilcoxon test was applied in case of non-normally distributed data.

In all graphs, the depicted results show the mean and respective standard error of the mean (SEM). All statistical testing was performed with SPPSS (IBM, New York, USA) and Origing2019 (OriginLab, Massachusetts, USA), using a significance level of 5%.

Chapter 4

Results

4.1 Carbachol concentration

4.1.1 Variation of administered concentrations

Before conducting the experiments aimed at inducing memory traces in networks with and without cholinergic input, we first assessed which carbachol concentration would be necessary to alter the natural synchronicity of 3 dissociated cortical cultures, by applying different CCh concentrations to the culture bath.

Figure 4.1 displays 5-min raster plots from 3 of the 5 spontaneous activity recordings of one of the cultures tested. The first plot depicts the activity patterns before pharmacological excitation (1) while the following two present the activity of the culture after 5 and 10 μM of CCh delivery (2 and 3, respectively). Carbachol induced activity pattern transformation, from seemingly uncorrelated spiking and synchronized network bursts during baseline, into more dispersed firing after each CCh application. We observed this alteration in all CCh concentrations tested (5, 10, 20 and 40 μM).

Normalized BI values are in line with this observation, with a decrease in more than 70% of spikes occurring within bursts after CCh treatment (panel A of Figure 4.2). Although all BI values after each concentration administered significantly differed from baseline (two-sample t-test, $p < 0.01$), the BI after applying 10 μM of CCh showed the lowest absolute value (0.21 ± 0.01) and p-value (paired sample t-test, $p < 0.001$) among the concentrations tested. Therefore, we chose this carbachol concentration to further evaluate its long-term effect in the cultures.

4.1.2 Long-term assessment of concentration effect

We administrated 10 μM of CCh in 4 spontaneously bursting cultures to verify if the pattern alteration observed previously was sustained through long periods of spontaneous activity recording. The results of BI normalization are shown in panel B of Figure 4.2. Differences between BI values before and after CCh administration are statistically significant (one-way ANOVA, $p < 0.01$) throughout the entire duration of the 15-hour recording procedure.

Following these outcomes, we selected a carbachol concentration of 10 μM to further induce bursting suppression in cultures submitted to electrical stimulation for memory trace induction.

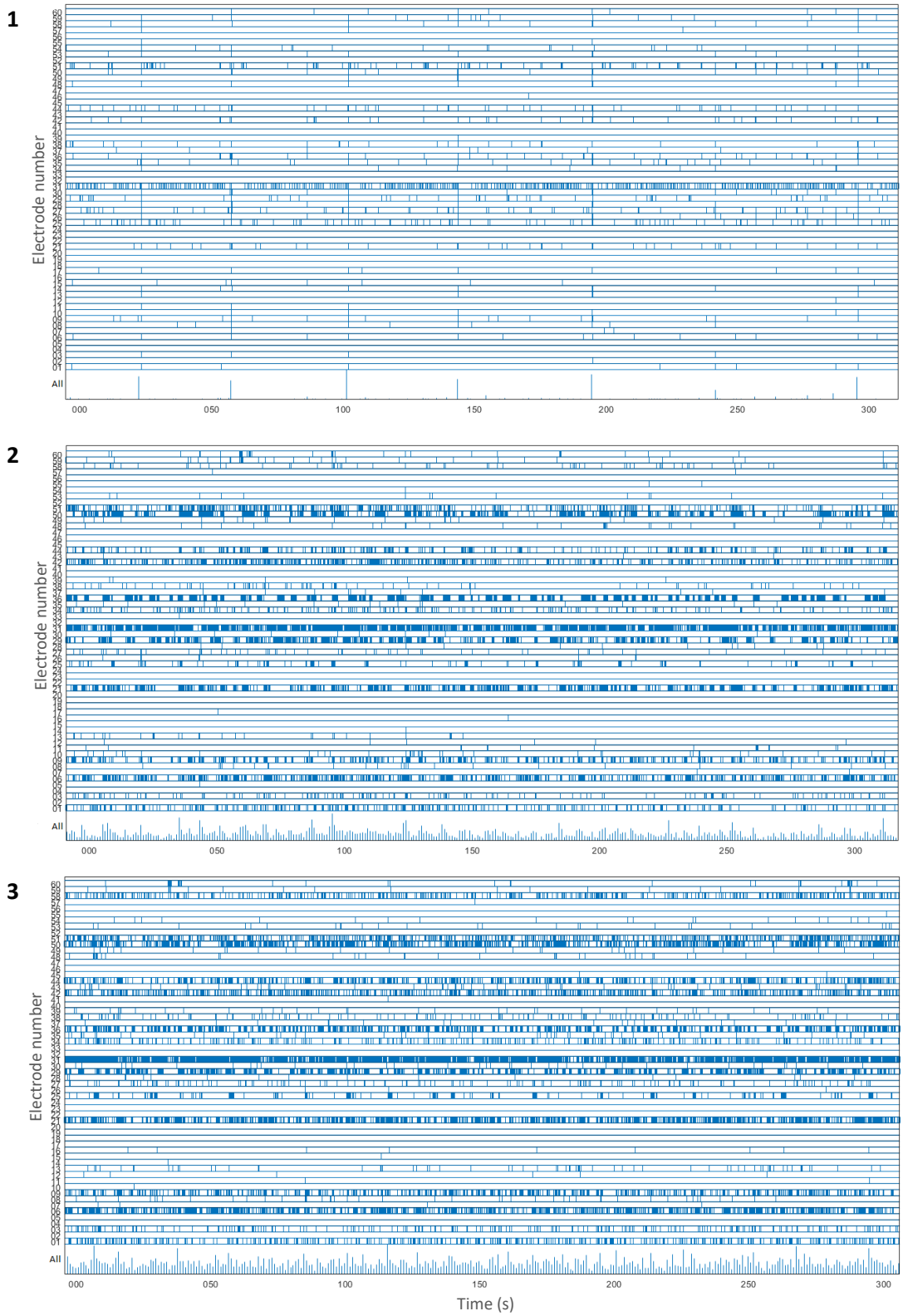

Figure 4.1 – Example of CCh excitation on the firing patterns of a dissociated cortical culture. The upper horizontal traces show the activity at the recorded electrode number whereas the bottom trace shows the summed activity of all electrodes. Each blue tick corresponds to an action potential. Activity before CCh administration (1) is mainly dominated by synchronized network bursts while CCh administration transforms these patterns into more dispersed firing, for both 5 and 10 µM (panel 2 and 3, respectively).

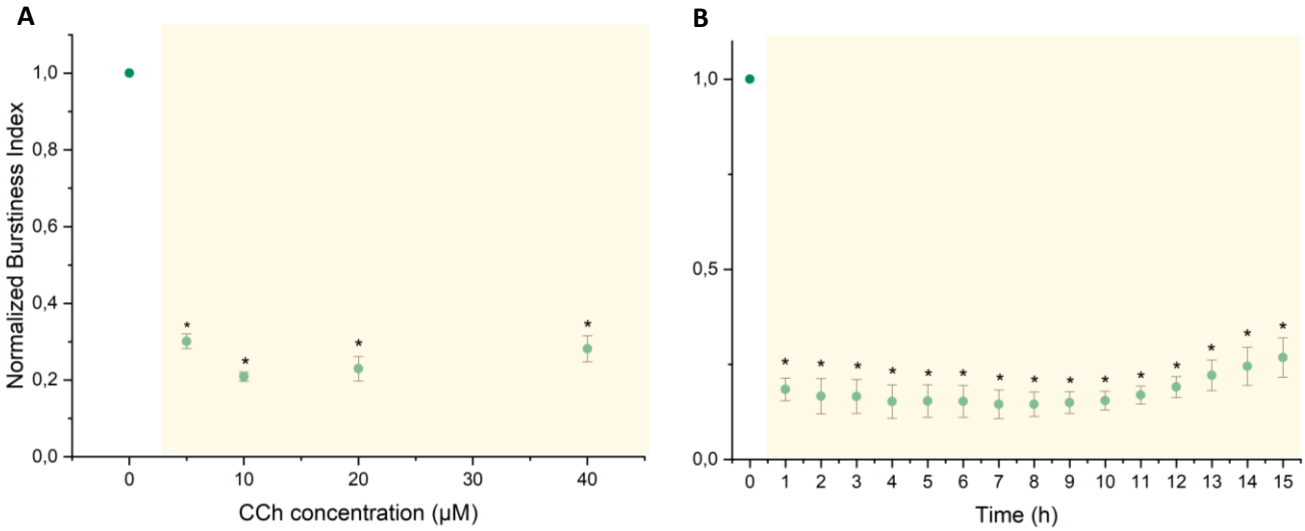

Figure 4.2 – Panel A displays the normalized burstiness index (BI) values ± SEM from baseline to each of the 4 CCh concentrations tested (5, 10, 20 and 40 µM) in 3 cultures. Significant differences ($p < 0.01$) were found in all BI values post-CCh administration when compared with the baseline. The BI for 10 µM showed the lowest p-value ($p < 0.001$) among the 4 concentrations tested. Panel B depicts the normalized burstiness index (BI) values ± SEM for each hour of the 15-hour recording procedure, after administration of 10 µM of CCh in 4 dissociated cortical cultures. All BI values post-CCh administration are significantly different ($p < 0.01$) when compared with the baseline, showing that burst suppression induced by carbachol administration is sustained throughout the experiment duration.

4.2 Memory trace experiments

We explored the effects of carbachol administration and repeated electrical stimulation on the activity and connectivity of 23 dissociated cortical cultures. 13 cultures (25 ± 6 DIV) were randomly assigned to the control group while the remaining 10 (27 ± 6 DIV) were treated with CCh. 3 cultures were excluded from the control group after the recordings, due to abnormal activity caused by bacterial infections or to data loss related with storage issues of the recording setup. In the CCh group, 2 cultures were also excluded *à posteriori*, as the spontaneous activity recorded was not enough to infer either on activity or connectivity patterns. We then performed activity and connectivity analysis for 10 control and 8 CCh cultures meeting the inclusion criteria.

4.2.1 Activity patterns

4.2.1.1 Mean firing rate (MFR)

Figure 4.3 shows the network mean firing rate for each hour of the experimental procedure, in both control and CCh groups. Baseline values did not differ significantly between controls and CCh cultures (two sample t-test, $p > 0.05$). The MFR of control cultures appeared rather constant around this baseline value during stimulation, with no significant differences across the 12-hour procedure (one-way ANOVA, $p > 0.05$).

In contrast, the MFRs of the CCh group increased immediately after carbachol administration, differing significantly over time from its baseline value prior to pharmacological manipulation (one-way ANOVA, $p < 0.05$). We also found significant differences between MFR values in control and CCh cultures during stimulation (two-way repeated measures ANOVA, $p < 0.01$), with time not significantly affecting these observed differences ($p > 0.05$).

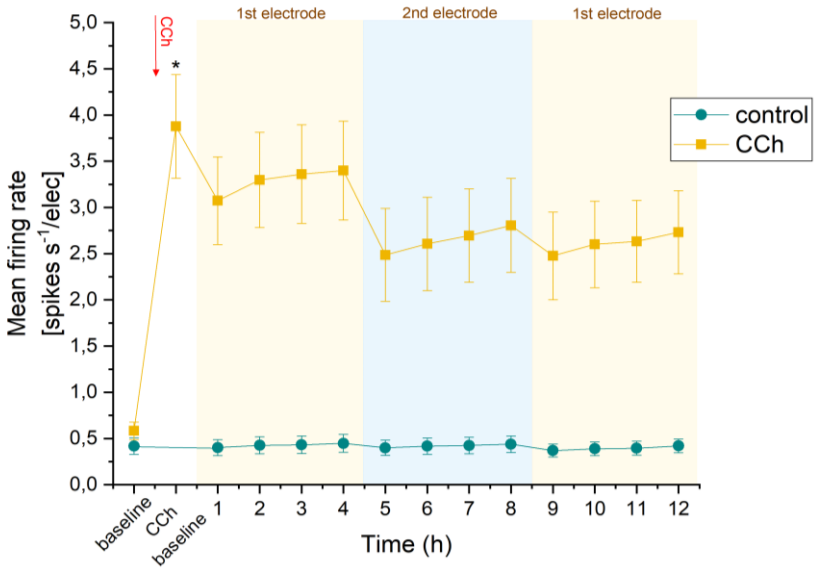

Figure 4.3 – Mean firing rates of control (blue) and CCh (yellow) cultures. Error bars represent the standard error of the mean (SEM) and coloured panels each stimulation phase. Baseline values between groups did not differ significantly ($p > 0.05$), as the MFR of controls across the 12-hour procedure ($p > 0.05$). CCh administration (represented by a red arrow) resulted in a significant ($p < 0.05$) increase in the MFR in the CCh group, which was kept throughout the experiment. MFR values differed significantly between groups ($p < 0.01$), with no time dependency on the observed differences ($p > 0.05$).

4.2.1.2 Burstiness index (BI)

To assess the burstiness of the cultures tested, we averaged BI values per hour of the procedure, with the results for both control and CCh cultures presented in Figure 4.4.

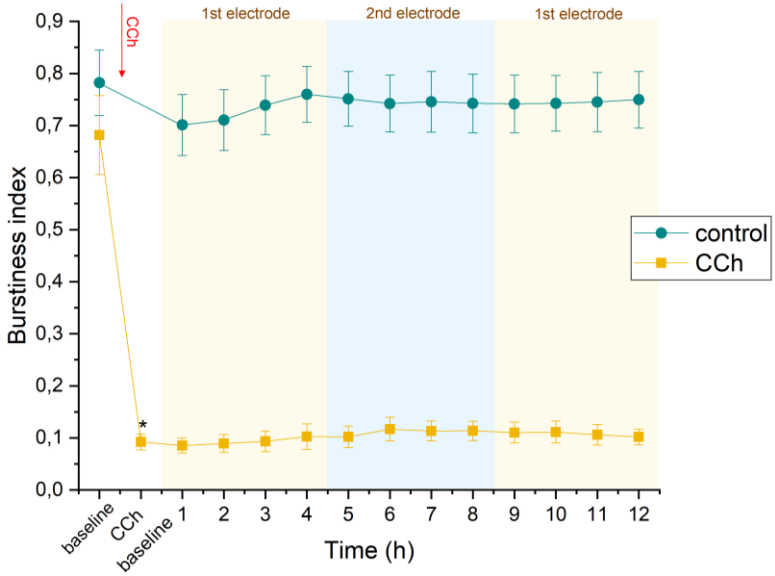

Figure 4.4 – Burstiness indexes of control (blue) and CCh (yellow) cultures. Error bars represent the standard error of the mean (SEM) and coloured panels depict each stimulation phase. Baseline values between groups did not differ significantly ($p > 0.05$) as the BI of controls across the 12-hour procedure ($p > 0.05$). CCh administration (represented by a red arrow) resulted in a significant ($p < 0.05$) decrease in BI values of the CCh group, which was kept throughout the experiment. BI values differed significantly between groups ($p < 0.01$), with no time dependency on the observed differences ($p > 0.05$).

Mean BI values at baseline for the control and CCh groups were 0.78 ± 0.06 and 0.68 ± 0.08, respectively, with no significant differences found between the two groups (two sample t-test, $p > 0.05$). This value was maintained in control cultures, with no significant differences found in BI values across the procedure's duration (one-way ANOVA, $p > 0.05$).

In carbachol-treated cultures, BI values dropped significantly after CCh administration (one-way ANOVA, $p < 0.05$), when compared with the average BI prior to pharmacological manipulation. This decrease in BI values to around 0.09 ± 0.02 was sustained throughout the stimulation periods. We also found significant differences between control and CCh BI values during stimulation (two-way repeated measures ANOVA, $p < 0.01$), with no effect of time in the differences observed ($p > 0.05$).

4.2.1.3 Post-stimulus time histogram (PSTH) and mean area under the curve

Network responses to electrical stimulation at a specific electrode can be observed through PSTH curves. Figure 4.5 shows three different examples of these curves during a 10-minute stimulation period (panels A, B and C).

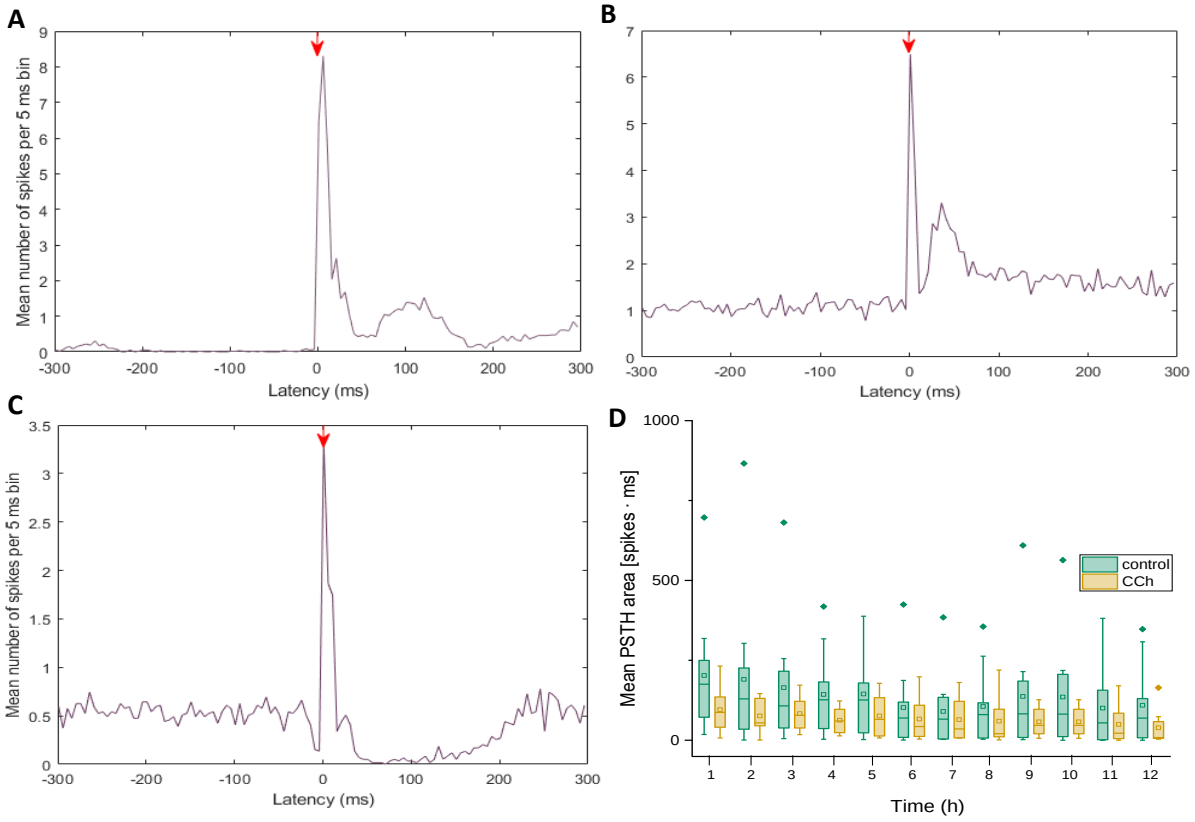

Figure 4.5 – Post-stimulus time histograms for a control (panel A) and two CCh cultures (panels B and C), during a 10-min stimulation period. The red arrow marks the stimuli onset centred at t=0. In both A and B, cultures responded directly and indirectly to the stimuli applied, with clear peaks around 10-50 ms and 60-150 ms. Background activity was more dominant in CCh cultures. In panel C, an example of network activity depression in a CCh culture after a direct response to stimulation, with the recovery to background activity around a latency of 200 ms. Panel D shows a boxplot of the mean PSTH areas under the curve for controls (blue) and CCh cultures (yellow). On each box, the central line represents the median, the small square the mean, the edges of the boxes are the 25th and 75th percentiles, the outliers are plotted individually, and the whiskers extend to the most extreme data points. The PSTH areas remained unchanged throughout the stimulation procedure, with no significant differences found across both control and CCh cultures ($p > 0.05$).

Panel A represents a typical response of a control culture, with almost no background activity before and after stimulus onset and a clear direct (from 10 to 40 ms) and indirect response (from 60 to 150 ms) to the stimuli applied at t = 0. CCh-treated cultures also show these two different responses but with enhanced background activity (see Panel B), which was already present in the latencies before stimulus onset. All control cultures displayed both a direct and indirect response to stimulation while only 75% of CCh-treated cultures showed a clear indirect response to the stimuli applied. Particularly in 2 CCh cultures, we observed an almost complete depression of network activity after the indirect response to stimulation, with the activity going back to baseline values at a latency of approximately 200 ms (see panel C).

The mean areas under the PSTH curves for each stimulation period of both control and CCh cultures are presented in panel D of Figure 3.5. For each group, we did not find any significant changes over time in the computed areas (two-way repeated measures ANOVA, $p > 0.05$). When comparing the PSTH areas of both groups, no significant differences were again observed throughout the duration of the experiments (two-way repeated measures ANOVA, $p > 0.05$).

4.2.2 Connectivity patterns

4.2.2.1 Conditional firing probabilities (CFPs)

To infer on functional connectivity, we computed a CFP for each pair of active electrodes *(i,j)* per culture, with an example of such a distribution presented in Figure 4.6. Most distributions obtained in control and CCh cultures (approximately 70%) displayed the observed non-flat cross-correlation. In CCh cultures, the $offset_{i,j}$ increased to higher values when compared with the ones obtained for control cultures. In contrast, the maximum of the cross-correlation peak decreased in CCh CFP distributions when compared with CFP curves for controls. We used the function fitted to the probability curve (as described in Equation 3) to obtain values for the strength ($M_{i,j}$) and latency ($T_{i,j}$) of the connection between each pair of active electrodes. Strengths of functional connections in control cultures were of the order of 10^{-3} to 10^{-2} whereas CCh functional strengths were of the order of 10^{-4} to 10^{-3}.

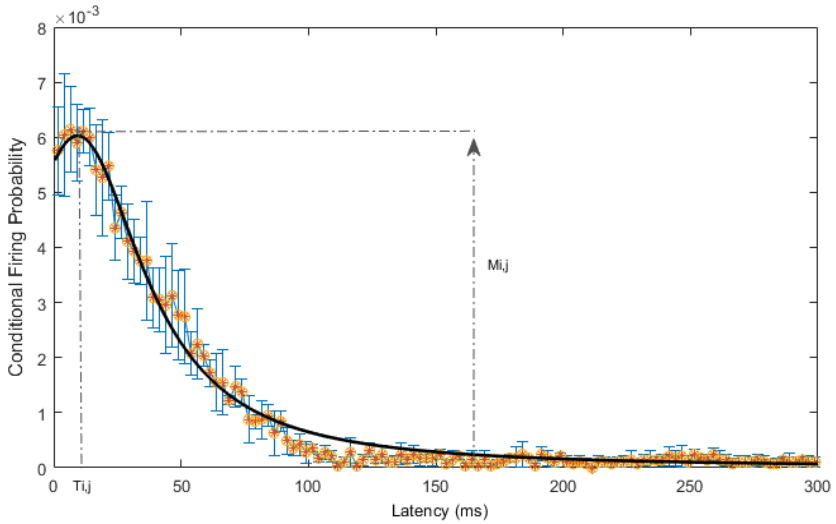

Figure 4.6 – Example of a conditional firing probability curve of a control culture. The means ± SD of values calculated in 0.5 ms bins are depicted. The solid black line represents the function fitted (as defined in Equation 3 of the *Methods* chapter) that was used to obtain the values for the strength ($M_{i,j}$) and latency ($T_{i,j}$) of the functional connections between pairs of electrodes *(i,j).*

4.2.2.2 Development of functional connections

From the function fitted to the CFP curve (Equation 3), we grouped the values for the strength ($M_{i,j}$) of the connections between each pair of active electrodes in *M* matrices, allowing for an overview of all relationships in each culture over time. Upon CCh administration, the average number of functional connections decreased non-significantly when compared with the average values before manipulation in CCh-treated cultures (two-sample t-test, $p > 0.05$). Moreover, the number of functional connections existing prior to the electrical stimulation protocol did not differ significantly (two-sample t-test, $p > 0.05$) between control (173 ± 25 connections) and CCh cultures (132 ± 23 connections). The stimulation protocol applied yielded significant changes (Mann-Whitney Wilcoxon, $p < 0.01$) between the number of connections maintained after stimulation in controls (70 ± 8%) and in CCh-treated cultures (45 ± 13%).

To follow the development of connection strength over time, we computed the mean strengths of the connections along the different phases of the procedure for both control and CCh cultures, with the results depicted in Figure 4.7. No significant differences were found between the mean connection strength at baseline between the control and the CCh group (two-sample t-test, $p > 0.05$). In control cultures, the average connection strength remained rather constant during stimulation (one-way ANOVA, $p > 0.05$). In CCh-treated cultures, carbachol administration led to a significant decrease in connection strength, which was sustained throughout the stimulation procedure (one-way ANOVA, $p < 0.05$). During the stimulation protocol, we did not find any significant differences between the connection strength immediately after CCh administration (CCh baseline) and the subsequent 12-hour values (one-way ANOVA, $p > 0.05$). Mean connection strengths across the stimulation periods differed significantly between control and CCh-treated cultures (two-way repeated measures ANOVA, $p < 0.01$), with time not affecting significantly the differences observed ($p > 0.05$).

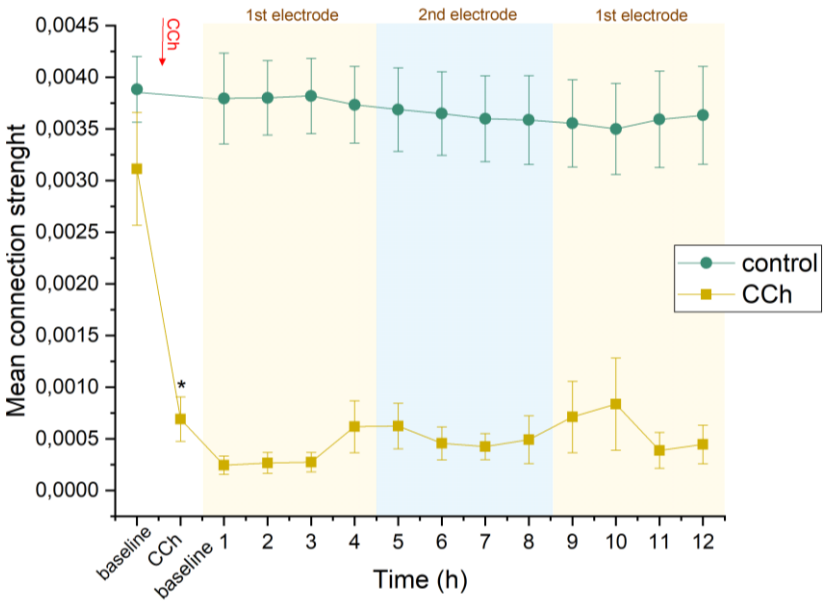

Figure 4.7 – Mean strength of control (blue) and CCh-treated connections (yellow). Error bars represent the standard error of the mean (SEM) and coloured panels depict each stimulation phase. Baseline values between groups did not differ significantly ($p > 0.05$) as the mean strength of connections in control cultures across the 12-hour procedure ($p > 0.05$). CCh administration (represented by a red arrow) resulted in a significant ($p < 0.05$) decrease in the mean strength values of the CCh group, which was kept throughout the experiment. Mean strength values differed significantly between groups ($p < 0.01$), with no time dependency on the observed differences ($p > 0.05$).

4.2.2.3 Euclidian distances

To assess the magnitude of changes between baseline connectivity and subsequent stimulation periods in each particular electrode, we computed normalized Euclidian distance values ($ED_{0,norm}$) for each 1-hour of spontaneous activity, with the results obtained presented in Figure 4.8. $ED_{0,norm}$ values during baseline were not significantly different between the control and the CCh group, not only before CCh administration (two-sample t-test, $p > 0.05$) but also after pharmacological manipulation (two-sample t-test, $p > 0.05$).

Stimulation yielded a significant increase in $ED_{0,\,norm}$ in the control group (one-way ANOVA, $p < 0.05$), with a larger distance between the connectivity before and after the first stimulation period than between subsequent stimulation periods. Switching to a second stimulation electrode yielded once more larger distances upon the first stimulation period, with non-significant changes after several stimuli applied (one-way ANOVA, $p > 0.05$). Stimulating the cultures again with the first electrode induced minor changes in $ED_{0,\,norm}$, which were similar to the ones observed between subsequent stimulation periods when stimulating with that electrode for the first time.

The effect of stimulation was strikingly different in CCh-treated cultures, in which we did not observe any significant increases in $ED_{0,norm}$ (one-way ANOVA, $p > 0.05$) across the stimulation protocol, meaning that differences in $ED_{0,norm}$ after the first stimulation or subsequent stimulation periods across both electrodes were non-significant (one-way ANOVA, $p > 0.05$) over time.

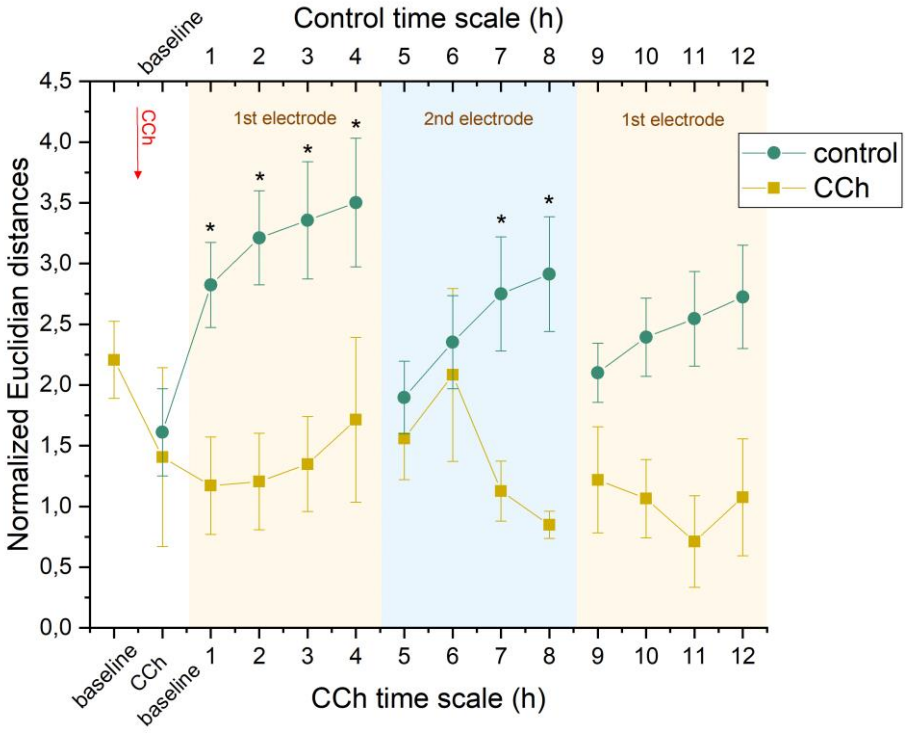

Figure 4.8 – Normalized Euclidian distances for control (blue) and CCh-treated cultures (yellow). Error bars represent the standard error of the mean (SEM) and coloured panels depict each stimulation phase with the respective stimulation electrode. The red arrow depicts the moment in which carbachol was administered in the CCh group. The lower x-axis shows the time scale for the CCh-treated cultures while the upper x-axis the time scale for control experiments. Baseline values between both groups did not differ significantly ($p > 0.05$). Upon stimulation, $ED_{0,\,norm}$ increased significantly in control cultures ($p < 0.05$) whereas we did not observe any significant changes in the $ED_{0,\,norm}$ of CCh-treated cultures ($p > 0.05$).

Chapter 5

Discussion

In this project, we aimed to untangle the role of network bursts and cholinergic activation in memory consolidation. To achieve this goal, we first assessed the effect of carbachol administration on the spontaneous bursting patterns of dissociated cortical cultures. Repeated electrical stimulation was then applied to cultures receiving this cholinergic input, with the effects of the given stimuli on the activity and connectivity patterns of these cultures compared with the effects of the same stimulation protocol in cultures with no pharmacological manipulation.

Our results show that low frequency stimulation leads to substantial changes in the network connectivity of spontaneously bursting cultures upon first stimuli application, with subsequent repetition of the cues not perpetrating any further changes in the connectivity of these cultures. Distinctively, in carbachol-treated networks we did not observe any drive in connectivity away from the initial balance during the entire duration of the stimulation procedure. These observations lead to the conclusion that in the presence of cholinergic input and absence of synchronized oscillations we could not observe consolidation of the stimuli presented, whereas cultures not treated with carbachol and so, bursting spontaneously, consolidated the given cues.

5.1 Effect of carbachol administration on the intrinsic patterns of dissociated cortical cultures

Dissociated cortical neurons in cultures with more than 3 weeks *in vitro* generate intrinsic synchronized rhythmic bursts of action potentials in the absence of external input.[2] These cultures are believed to be hyperexcitable, with a small stimulus or even no stimulus at all inducing bursts of activity in the entire network.[55] To disturb these well-established patterns, we pharmacologically manipulated dissociated cortical cultures by administering carbachol, a cholinergic agonist.

Carbachol treatment in these cortical cells transformed the spontaneous activity of the cultures from network bursting patterns into more dispersed firing. Our results are in line with previous studies, which showed a loss in the regularity and synchronization of network bursting patterns and the occurrence of more isolated single spikes after CCh and ACh administration, using concentrations ranging from 10 to 50 µM in culture medium.[15, 58] Despite all concentrations tested demonstrating that intrinsic synchronized patterns massively disappeared after cholinergic activation, we only investigated the long-term effects of the administration of 10 µM of CCh. Although firing patterns seemed mainly dispersed after 5 µM CCh treatment, we noted that rasterplots still included few occasional bursting events. 10 µM was then the lowest concentration among all tested that robustly displayed a complete pattern transformation. Continuous 15-hour recordings of spontaneous activity in cultures treated with 10 µM of CCh showed a significant decrease in the burstiness index values for each hour of the procedure. The effect of cholinergic activation in abolishing synchronized bursts was then sustained throughout the 15-hour recording period. These observations also agree with previous literature, in which similar CCh concentrations were reported to change activity patterns up to 24 hours after carbachol administration.[15]

These findings served as the base for our stimulation experiments, in which we applied the previously derived concentration of 10 µM of CCh in the culture bath of 8 spontaneously bursting networks. Prior to stimulation and upon CCh administration, a significant decrease in BI values was again observed, accompanied by a decrease in the average strength of functional connections and a simultaneous increase in the mean firing rate to almost 6 times its baseline value. Previous findings support our observations, reporting similar trends for the three parameters analysed.[15, 58]

We administered CCh in our cultures to study the effect of cholinergic input in cortical cells rather than ACh, as previous works concluded that the desynchronization effects of both CCh and ACh appeared to be comparable at medium concentrations.[58] ACh is rapidly hydrolysed after its release in the synaptic cleft, with early microdialysis studies reporting concentrations between 0.1 and 6 nM in the cortex of awake rodents.[89, 90] Most of the studies measuring basal ACh apply acetylcholinesterase (AChE) inhibitors (as neostigmine and physiostigmine), which enhance the levels of ACh in the extracellular space.[91] Some authors argue that this manipulation might not reflect the real ACh concentration *in vivo*, and so approaches such as magnetic resonance spectroscopy (MRS) have been proposed to tackle this issue.[92, 93] MRS only targets extracellular choline, which is both a precursor of ACh and a metabolite of its hydrolysis.[93] In the choline cycle, the re-uptake of this metabolite by the pre-synaptic cell is not as fast as the hydrolysis of ACh, making its concentration more assessible in the synaptic cleft.[93] Previous studies suggest a proportional correlation between choline levels and ACh availability in the cortex, reporting choline concentrations between 1 and 5 mM in the cortex of healthy volunteers and between 0.6 and 13 µM in the cortex of awake rat and mice.[89, 90, 93, 94] We administered 10 µM of CCh in our dissociated cortical cultures, which is within the latter range of choline concentrations observed in rodents. CCh is a synthetic derivative of choline and, contrarily to ACh, is resistant to hydrolysing enzymes as acetylcholinesterase, remaining present in the culture medium throughout the duration of the experiments.[95] As a cholinergic agonist, CCh activates directly acetylcholine receptors whereas choline, by participating in ACh synthesis, intervenes indirectly in this excitation. We then believe that our administered CCh concentration is comparable to the observable levels of choline in *in vivo* rodent models and in turn, indirectly equivalent to the ACh availability in the awake cortex.

The switch from synchronized rhythmic bursting into more dispersed firing upon carbachol administration is thought to be caused by muscarinic receptor activation.[15, 37] Exogenous application of muscarinic agonists, such as carbachol, can result in facilitation or inhibition of synaptic transmission.[37] Upon CCh administration, we observed an increase in network activity (increased MFR) and a simultaneous decrease in network excitability (decrease in BI and average connection strength). Here we define network excitability as being the ease with which a network response can be induced by a stimulus, or in the case of unperturbed cortical cultures, the ease with which ongoing activity can induce synchronized bursting patterns.[15, 53] Several mechanisms as synaptic scaling, activation of the inhibitory system and increased short term synaptic depression (STD) have been hypothesized to affect network excitability.[15]

Homeostatic synaptic scaling is a form of synaptic plasticity that adjusts the strength of all excitatory synapses of a neuron up or down to stabilize firing. If the input to a neuron increases by long-term potentiation with a resulting increase in postsynaptic firing, synaptic scaling reduces the strength of synaptic input until the firing rate returns to normal levels.[65] As we observed both an immediate and sustained increase in network activity and an instantaneous

and long-lasting decrease in network excitability, it is improbable that synaptic scaling has taken place, as the time constant for this mechanism *in vitro* is in the order of a day.[59, 60]

Activation of inhibitory neurons in turn would be translated into a rapid inhibition in activity upon CCh administration, initially outweighing the excitatory effect of CCh. Our results show an immediate increase in network activity and sustained reduced excitability, seeming unlikely that induced inhibition would cause the effects observed. This is also supported by the observation that cultures in earlier stages of development show spontaneous dispersed firing patterns, even though their inhibitory system is still in a premature state.[61]

Our results comply with previous hypothesis stating that reduced network excitability may be due to an increase in the short-term depression of recurrent excitatory synapses.[15] Short-term depression is thought to be caused by a depletion of neurotransmitter, consumed during the synaptic signalling process at the axon terminal of a pre-synaptic neuron.[62] Previously observed cholinergic presynaptic suppression of the spread of excitation in the visual cortex[63], reduction of the size of excitatory postsynaptic potentials (EPSPs) in several cortical synapses by muscarinic receptor activation[64] and reduction in ESPSs by ACh administration in hippocampal cultures[68] support the view that short-term depression might be the underling mechanism of the observed decrease in synchronized firing in our cultures. In line with previous work, we then hypothesize that the desynchronization observed upon CCh treatment is most likely a network phenomenon.[15]

5.2 Effect of repeated electrical stimulation on the activity and connectivity of neuronal cultures

Unperturbed dissociated cortical cultures display rather stable activity and connectivity patterns[2, 43], which remain in balance due to their mutual interaction, leading to synchronized bursting events. Administrating carbachol in the culture bath of these networks disrupted the rhythmic patterns previously established, transforming spontaneous activity into dispersed firing. We then observed the effect of low frequency electrical stimulation in cultures treated with carbachol and compared these observations with the changes induced by the same stimulation protocol in cultures bursting spontaneously.

Stimulation did not significantly affect the bustiness index of both control and CCh-treated cultures after each stimulation period throughout the protocol. This suggests that after stimulation most spikes still occurred outside network bursts in CCh-treated cultures, with maintained dispersed firing throughout the procedure and reduced excitability. In contrast, the activity patterns of control cultures remained burst dominated in the full stimulation procedure. These observations agree with previous studies, where burst control was achieved with low frequency single-electrode stimulation. Their results show that when stimulating at a rate of 2 stim/s, the level of burstiness before and after stimulation remains unchanged.[55]

The mean firing rate after each 10-minutes of stimulation remained rather sustained as well, with no significant changes within each group. These results are in line with previous reports of maintained MFR after cessation of stimulation.[55] We also found significant differences across the entire procedure between the high values in MFR of CCh cultures and the lower values of control cultures, reflecting increased activity upon CCh administration.

Network responses to electrical stimulation at a specific electrode appeared to be quite similar in shape between CCh and control cultures. In both cases, a clear direct and indirect response to stimulation was evoked around latencies 10 to 150 ms after stimulus onset, agreeing with the shapes and latencies described in previous work.[4, 8, 67] All control cultures displayed both a direct and indirect response to stimulation while only 75% of CCh-treated cultures showed a clear indirect response to the stimuli applied. This decrease in the indirect response to stimulation in CCh-treated cultures might reflect the reduction in synaptically propagated signals of the neurons which fired in the early phase of response to their connected neighbours.[44] PSTH curves in CCh cultures also showed an increase in ongoing background activity before and after stimulus onset, in line with the increased offset in CFP curves, which also reflects an increase in unrelated background activity. These observations further support the switch in network activity to more dispersed firing patterns after CCh-treatment.[15, 67] In 25% of the cultures tested, we observed an almost complete suppression of network activity after the indirect response to stimulation, with the activity going back to baseline values at a latency of approximately 200 ms. This fast depression might be explained by the activation of the inhibitory system, which usually occurs in network bursts or stimulus responses[43, 68], and seems to neutralize the effect of CCh activation of the neurons in these cultures.

The mean area under the PSTH curves of each stimulation period did not differ significantly over time across both control and CCh-treated cultures, suggesting that the number of action potentials in response to stimulation was rather constant throughout the procedure, with the stimuli applied consistently evoking similar responses to stimulation in both control and CCh cultures.

Most conditional firing probability distributions obtained appeared to be non-flat (70%), suggesting that most active electrodes were functionally connected[56], with the number of functional connections prior to stimulation not differing significantly in both control and CCh-treated cultures. The maximum of the CFP curves of CCh cultures decreased when compared with the peak of the distributions in control cultures. This reflects the significant decrease in average strength observed after CCh administration, which was maintained throughout the duration of the procedure. Despite this decrease, the values for functional connection strengths were still within the normal range reported in previous studies also using CFPs to monitor network connectivity in spontaneously bursting cultures.[4, 8, 56] The mean connection strength in control cultures remained rather constant throughout the stimulation procedure, as reported in previous work.[4] This observation might be due to the fact that stimulation induced changes in the strength of the functional connections of the control group both up and down, and so the mean strength was not affected by the protocol, as also observed in the literature.[4]

In control cultures, we observed huge connectivity changes upon the first stimulation period, with $ED_{0,\ norm}$ increasing significantly when compared with baseline values. Subsequent stimulation periods with the same electrode induced only minor connectivity changes in the network, not driving the connectivity further away from the initial state. These results are in line with a previous study[8] that applies both low-frequency and tetanic stimulation in dissociated cortical cells. It is then hypothesized that external input (electrical stimulation) drives the network away from the initial balance towards a new equilibrium, due to mutually affective forces between activity and connectivity. Stimulation at a second electrode yielded once more large connectivity changes after the first stimulation period, with again non-significant differences after several stimuli applied in the following stimulation periods. The network was once more driven to another balance and was still able to adapt to a different external input,

meaning that the slight changes in connectivity observed after several repetitions of the first stimulus were not underlined by impeded network plasticity.[8] This previous work also suggests that the activity patterns of the network in the new balance include the response pattern to the stimuli applied.[8] Indeed, our results show that returning to the first electrode did not significantly alter network connectivity further from the initial state, with only slight changes in connectivity quite similar to the ones observed between subsequent stimulation periods when stimulating with that electrode for the first time. These changes appear to reflect fluctuations in connectivity around a stable point, seeming that the stimuli applied can no longer drive network connectivity further away from the equilibrium, as it did upon first application of the stimulus. The shape of our curves for $ED_{0,norm}$ are quite comparable with the ones obtained in this previous study for both low-frequency and tetanic stimulation.[8]

In CCh-treated cultures, the effect of electrical stimulation on network connectivity was notably different. $ED_{0,norm}$ did not change significantly across the entire stimulation period when compared with baseline values. The stimuli applied through the same stimulation protocol used as for control cultures seemed unable to drive network connectivity further away from the initial state, with only random fluctuations in connectivity around similar values prior to stimulation onset. These non-observed changes in connectivity were not caused by a fading effect of the stimuli applied, has we have noted before that the response of the networks to stimulation was rather sustained between subsequent stimulation periods. Moreover, the curve we obtained for $ED_{0,norm}$ is extremely similar in shape to the one presented in previous work for non-stimulated cultures, in which no significant connectivity changes were also observed.[8] We normalized ED_0 curves to the mean strength of all connections prior to stimulation in that particular electrode, to be able to draw fair comparisons between control and CCh connectivity curves.

Previous studies have shown that different stimulation protocols alter functional connectivity[4], and in our own protocol both CCh-treated cultures and controls were stimulated with the same stimulus parameters. This makes it unlikely that the delivered stimulus itself would be the cause for the observed changes in connectivity. It is also well known that both tetanic and low-frequency electrical stimulation can trigger network bursts with increased number of spikes when compared to spontaneously generated ones.[69, 70] Previous works have hypothesized that these evoked network bursts are needed to induce connectivity changes.[8, 61, 70] In our cultures, both CCh and control networks reacted similarly to the low-frequency pulses applied, with evoked network bursts triggered very shortly after stimulation onset. Despite this, in CCh-treated cultures this bursting effect was not sustained after stimulation was resumed as networks treated with CCh were not able to self-generate synchronized bursting patterns, due to the hypothesized increased short-term depression of recurrent excitatory synapses.[15] Ongoing activity patterns are thought to influence connectivity, with connectivity patterns, in turn, influencing activity. Carbachol induced increased activity and decreased network excitability which did not allow the networks to be driven away from the established equilibrium, with no detected changes in network connectivity. In spontaneously bursting cultures, a transition to a new balance was indeed achieved, with observable differences in the connectivity of the cultures upon first stimuli application and subsequent repetition of the cues not perpetrating any further changes in the connectivity of these networks.

5.3 How are these results related with memory consolidation mechanisms?

Previous studies have demonstrated that electrical stimulation can lead to the formation of memory traces within naturally bursting cultures.[8, 44] Literature suggests that a memory trace should be formed in an experience-dependent manner, it should be specific to the stimulus provided, it must outlast the period to which a network is exposed to stimulation and it should be reactivated after subsequent presentation of the stimuli that underlined its onset.[71] Our results in control cultures comply directly with the first three criteria and indirectly with the last one. Firstly, the connectivity changes we observed occurred upon external stimulation, which can be seen as an experience-dependent interaction. Secondly, stimulation through different electrodes induced different connectivity changes, with these changes being specific to each stimulus delivered. Thirdly, the connectivity changes withstood the stimulation period for at least the 12-hours of our protocol. Finally, the fact that connectivity stabilized after presenting again the first stimulus might indicate that reactivation of the trace occurred, with no more significant connectivity changes disturbing the established balance. Previous work support these observations using tetanic stimulation[8, 48, 51] and report parallel consolidation of the memory traces induced.[8] The relative small size of our network and the simplicity and long duration of our stimuli might have accelerated the trace consolidation process observed, which through systems consolidation *in vivo* is thought to require from several hours to days to occur.[24] We then believe that the connectivity changes observed in our spontaneously bursting cultures translate the parallel consolidation of induced memory traces.

Following this line of thought, the fact that stimulation did not induce any changes in the connectivity of our CCh treated cultures implies that these networks were unable to memorize and consolidate the given cues. Our assumption is supported by a vast literature focusing on the role of acetylcholine and synchronized neural activity in memory consolidation and formation.[6, 7, 9, 11, 31, 35, 32, 39, 72] It is generally supposed that an increase in the cholinergic input to the hippocampus is necessary for memory formation in the awake state, while a depletion in the ACh tone is required for memory consolidation in the neocortex during slow-wave sleep.[11, 39] Moreover, when memories are acquired during arousal, theta oscillations abound, while during systems consolidation in SWS, synchronized oscillations (hippocampal sharp-wave ripples, thalamo-cortical sleep spindles and neocortical slow oscillations) are thought to induce long-lasting forms of synaptic plasticity, allowing memory consolidation in the neocortex.[7,9] In previous experimental work, sharp-wave ripples were suppressed during sleep, after a memory training task, with impaired spatial learning observed[73], while an increase in SPs and SWRs densities during SWS was reported after a period of intense wordlist learning.[76] Enhancement of neocortical SOs and spindles by exogenous stimulation has also led to improved memory consolidation.[74, 75] Other studies have shown that blocking cholinergic transmission improved consolidation of declarative memory when subjects were tested after a period of slow-wave sleep[77] whereas cholinergic activation before SWS induced a deterioration in recalling a previously conducted memory task.[76] These observations suggest that high ACh levels and the absence of SWRs might facilitate memory encoding but hamper memory consolidation and retrieval.

Our results agree with this supposition, although we can only infer on memory consolidation and not on memory formation and retrieval, as our experiments were solely conducted in cortical cells. Indeed, in the presence of cholinergic input and absence of synchronized

oscillations we could not observe consolidation of the stimuli presented, whereas cultures not treated with this agonist and so, bursting spontaneously, seemed to consolidate the given cues. Nevertheless, this study has some caveats that should be explored in future work.

5.4 Study limitations and future recommendations

Despite the fact that our findings seem to be supported by an enormous body of work in the realm of memory consolidation and sleep, not only theoretically but also experimentally (with *in vivo* rodent models), only few studies actually tested the effects of carbachol administration in dissociated cortical cultures plated in microelectrode arrays.[15, 58, 67] To our knowledge, the present work seems to be the first study combining both electrical stimulation and cholinergic activation in dissociated cortical cultures plated in MEAs. To ensure that the results are reproducible in a wider variety of dissociated cortical networks, the sample size for both control and CCh-treated cultures should be increased in future studies.

Although recording neural activity through MEAs might pose an added advantage to patch-clamping or brain slices due to the possibility of multiple location long-term recordings, our networks are still simplified models of the real *in vivo* architecture of the brain. Declarative memory formation and consolidation are complex phenomena involving activation and inhibition of several brain areas and neural pathways, with intricate links between structures such as the hippocampus, the neocortex, the amygdala and the entorhinal cortex. Our results can only be translated to a certain extent into memory consolidation mechanisms, as the cultures used only included cortical neurons. To increase the complexity of the model, cortical cells could also be coupled to hippocampal networks, and so formation, consolidation and retrieval of the induced memory traces could be thoroughly investigated. This may be performed in 3D neuronal networks as well, with several cortical and hippocampal layers coupled to 3D *in vitro* MEAs, augmenting even further the complexity of the model towards a more resembling *in vivo* situation.

Memory acquisition and its further strengthening and recollection is also thought to occur in a relatively sequential order, with memories acquired during the awake state and posteriorly replayed and consolidated during slow-wave sleep. In our study, each culture was assigned to a different group, either control (mimicking the SWS state) or CCh-treatment (mimicking the awake state), and so the same network was never assessed with the stimulation protocol in those two different pharmacological conditions. It would be extremely interesting to assess first the effect of stimulation in a spontaneously bursting culture (control situation), then pharmacologically suppress its bursting patterns with carbachol administration (CCh-treatment situation) and stimulate through two different electrodes than the first ones chosen, washout the remaining CCh afterwards and then again stimulate the culture with the stimuli previously applied in the first two phases. Although we tried to apply this sequential rational in several cultures, the activity after washout was not enough to infer on network connectivity and continue the procedure. During washout, not only the remaining CCh was removed but also other neuronal substances important for network functioning. It might also be the case that the cultures were simply not strong enough to endure this procedure. A possibility to counteract this disadvantage might be to allow CCh to degrade naturally. In most CCh-treated networks, we observed that cultures regained network bursts after 24 to 48 hours upon the procedure completion. This trend should then be further investigated.

To better observe the stabilization in connectivity after application of subsequent stimuli, it would also be valuable to increase the number of stimulation periods through each stimulation electrode. Previous work has shown this stabilization effect in connectivity from the second simulation period up until the 9th epoch of stimuli delivery.[8] Furthermore, another recording of spontaneous activity could be added to the experimental protocol after probing all MEA electrodes, to ensure that this stimuli applied did not induce any connectivity changes before starting the stimulation phase. Despite previous studies reporting no significant connectivity changes after random stimulation[4], this observation should be confirmed.

Although we have observed that an increased cholinergic tone with subsequent absence of synchronized bursting patterns hamper memory consolidation in cultures that were bursting spontaneously prior to CCh administration, it would be of value to test the effect of electrical stimulation in cultures in the earliest stages of development, which do not include in their spontaneous patterns synchronized activity yet.[43] Stimulating these cultures with the same stimulation protocol for memory trace induction would allow to better discern the contribution of synchronized bursting in memory trace consolidation in our experiments.

We used CCh administration to disrupt the natural bursting patterns of the cortical cultures tested, but other stimulation techniques as optogenetics have been proved to succeed in desynchronizing neocortical networks by selective stimulation of cholinergic axons.[79] In practice, it should be feasible to test our protocol using light as an external input instead of CCh administration, with the end goal of comparing the outcomes obtained with our results.

Finally, our stimulation protocol may also be applied in neurons differentiated from human induced pluripotent cells (hiPSCs) plated on MEAs. Several protocols have suggested that these hiPSCs derived neurons appear to have activity and connectivity patterns comparable with rodent cultures.[80] A network model of human cells has the added advantage of being more easily comparable and translatable into a real neurophysiological human setting.

5.5 Conclusion

The synchronized patterns of unperturbed dissociated cortical cultures can be disrupted by cholinergic activation, which transforms spontaneous network bursts into dispersed firing. When stimulating these cultures through low-frequency pulses, the stimuli applied seem to be unable to induce connectivity changes in the networks. However, applying the same stimulation protocol to cultures with no cholinergic input appears to drive the cortical networks to a new balance, with major connectivity changes upon the first stimulus application and no connectivity changes observed after further presentation of the cues. These permanent changes in the functional connections of spontaneously bursting cultures can be translated into parallel consolidation of induced memory traces, as presentation of a second input did not erase the first trace induced. Cholinergic activation seems to hamper this memory trace consolidation, while synchronous bursting appears to favour this process. We conclude that high cholinergic levels and the absence of synchronized patterns impede memory consolidation in dissociated cortical cultures whereas networks bursting spontaneously are able to memorize the given cues.

Chapter 6

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Appendix A

A.1 Immediate, short-term and long-term memory

Three types of memory can be identified in different stages over a period of time (Figure A.1). When presented with sensory input, the immediate or sensory memory is capable of recalling ongoing experiences for a few seconds.[20] Attention to that input leads to short-term memory, in which few pieces of information are recalled in a time span from seconds to minutes.[20, 21] The hippocampus, the mammillary bodies and both the anterior and medial nuclei of the thalamus are thought to be involved in immediate and short-term memory.[20] Constant repetition of this presented stimulus may lead to the transfer of this information to a more permanent type of memory, that can last from some hours to an entire life-time, called long-term memory.[21] Information stored in long-term memory may be consciously accessible (declarative or explicit memory) or inaccessible consciously (non-declarative or implicit memory).[19] Facts and events are stored in the declarative memory, either in the semantic (facts and events independent of context, so abstract knowledge) or in the episodic memory (information specific to a particular context, normally key events related with a specific time and place).[16] The medial temporal lobe (MTL) and the diencephalon are regarded to account for declarative memory while non-declarative memory, depending of its type, either procedural, priming, non-associative or classical conditioning (divided in emotional or somatic) memory, is distributed through the striatum, neocortex, the reflex pathways and the amygdala and cerebellum, respectively.[16, 19]

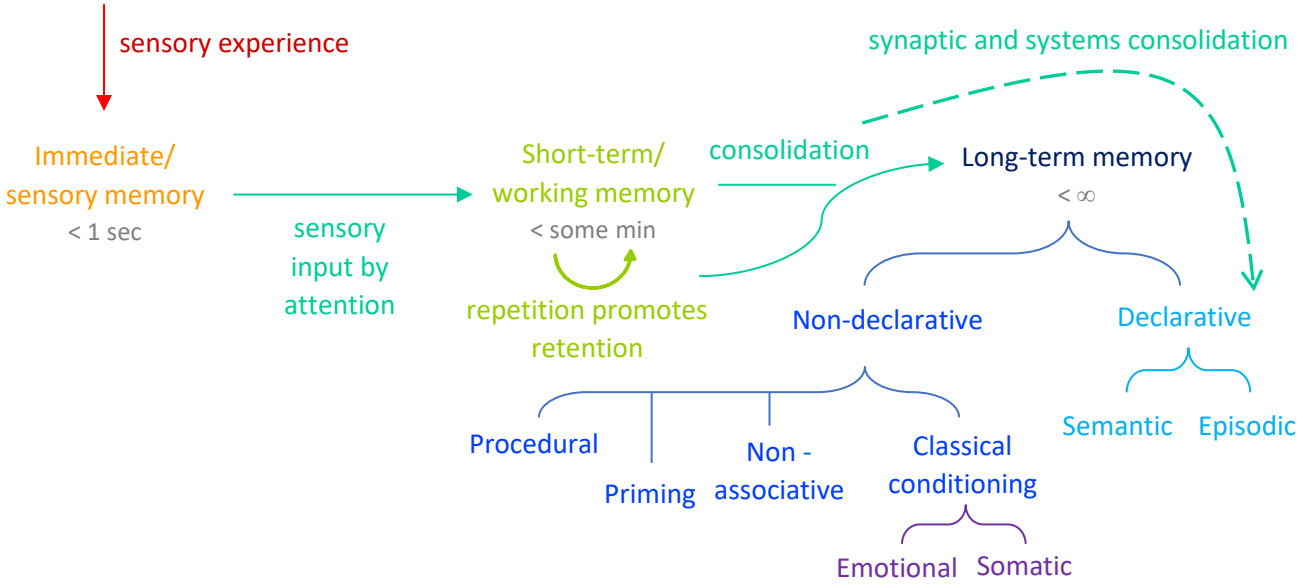

Figure A.1 – Memory stages from sensory input, through encoding in the short-term memory and to consolidation into different long-term memory types. Adapted from Squire (2004) and Clark (2017).

A.2 Slow oscillations, slow-wave ripples and sleep spindles

During behavioural states with absent external stimulation as immobility and SWS, the CA3 region of the hippocampus is released from subcortical inhibition, giving rise to synchronous network discharges or bursts (sharp waves). These waves propagate through the hippocampal CA1 region, originating extracellular ripple oscillations.[7] Sharpe-wave ripples (SWRs) and the spiking of several cortical neurons are correlated with sleep spindles (SP), regular and transient oscillations observed in the thalamus and neocortex during SWS.[35] The coupling of SWRs and SPs is hypothesized to be facilitated by slow oscillations (SO), cortically generated patterns that reflect the up- and down-state alternation of cortical networks.[35] Up-states reflect periods of membrane depolarization and increased cortical spiking whereas down-states are marked by membrane hyperpolarization and absence of cortical spiking activity.[35] SOs usually emerge in the frontal cortex and propagate as travelling waves towards the visual cortex and the MTL structures, interacting with SWR at the hippocampus and subsequently with SPs.[35] SWRs are more likely to occur in the alternation between down- and up- SOs states, and are synchronized with SPs (Figure A.2).[34]

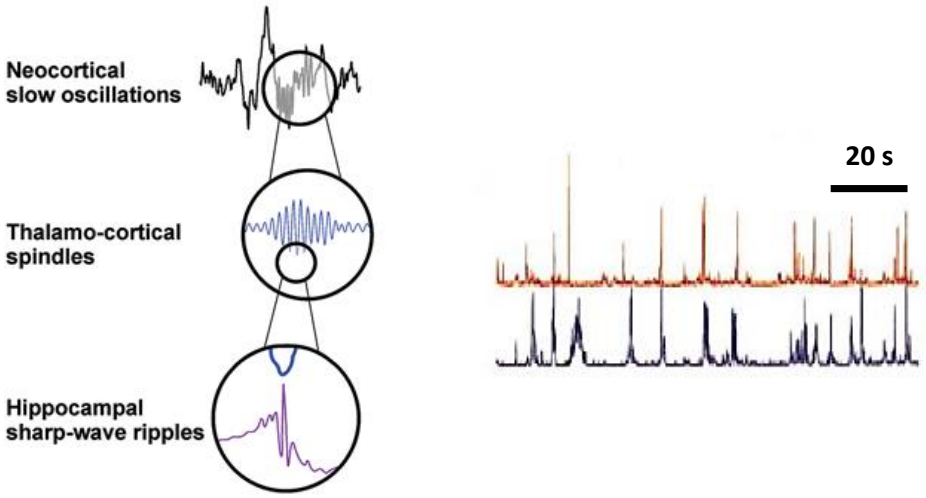

Figure A.2 – On the left, temporal relation of SOs, sleep spindles and hippocampal SWRs, adapted from Marshal et al. (2013). On the right, correlation between hippocampal ripples (in red) and neocortical sleep spindles (in blue) during SWS. Adapted from Axmacher et al. (2006).