THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

# Microfluidics based technique for electrophysiological studies of cells

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## Abstract

This thesis work investigates the application of microfluidics to perform electrophysiological studies on cells, including investigations of the effect of cholesterol on the dynamic ion permeability of TRPV1 ion channels, and the application of a microfluidic device, the multifunctional pipette, in electrophysiological studies on brain slices. In the first part of this thesis, Chinese hamster ovary (CHO) cells overexpressing the TRPV1 ion channel were used in a dynamic ion permeability study, where the activation properties of the TRPV1 ion channel were investigated using the patch clamp technique after depletion of membrane cholesterol. The dynaflow system, an open-volume multichannel microfluidic system, and the multifunctional pipette, a freestanding microfluidic device utilizing hydrodynamically confined flow for spatially confined solution exchange, were used to deliver chemical stimuli exclusively to the patched cell. The result showed that the depletion of membrane cholesterol impaired the dynamic permeability of large cations in TRPV1 in low calcium solutions. The second project focused on the application of the multifunctional pipette in neuropharmacological studies of the brain slices. We developed an experimental setup, performed feasibility studies, characterized the device performance and compared it with common superfusion techniques, using extra- and intracellular electrophysiological recordings of pyramidal cells in hippocampal and prefrontal cortex brain slices from rats. The multifunctional pipette was used in these experiments for highly localized delivery of the competitive AMPA receptor antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) to selected locations on the slices. By applying multifunctional pipette, we achieved a multifold gain in solution exchange time and more efficient drug delivery compared to whole slice perfusion. The amount of drugs required in the microfluidics-supported experiments was by several orders of magnitude smaller. The multifunctional pipette enabled selective perfusion of a single dendritic layer in the CA1 region of hippocampus with CNQX, without affecting other layers in this region.

# List of publications

This thesis is based on the work contained in the following articles:

**I. Effect of cholesterol depletion on the pore dilation of TRPV1**

Erik T Jansson, Carolina L Trkulja, Aikeremu Ahemaiti, Maria Millingen, Gavin DM Jeffries, Kent Jardemark and Owe Orwar

*Molecular Pain 2013, 9:1.*

**II. A multifunctional pipette for localized drug administration to brain slices**

Aikeremu Ahemaiti, Alar Ainla, Gavin D.M. Jeffries , Holger Wigström, Owe Orwar, Aldo Jesorka, Kent Jardemark

*Journal of Neuroscience Methods 219 (2013) 292– 296.*

**III. Spatial characterization of a multifunctional pipette for drug delivery in hippocampal brain slices**

Aikeremu Ahemaiti, Holger Wigström, Alar Ainla, Gavin D.M. Jeffries, Owe Orwar, Aldo Jesorka, Kent Jardemark

*Submitted to Journal of Neuroscience Methods.*

# Contribution report

**Paper I.**      Took part in planning and performing the experiment and participated in writing the paper.

**Paper II.**     Planned and performed the experiments, and participated in writing the paper

**Paper III.**    Planned and performed the experiments, and was the main writer of the paper.

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# 1. Introduction

The cell is the smallest functional unit of all known living organisms. In complex multicellular organisms, such as the human, the action of a single organ, or the whole system, depends on cooperation and interaction of different cells. It involves cell-to-cell communication, which is critical for normal functioning of the body [1-3]. This is especially obvious in the brain, where the neurons, the principle functioning units of the brain, are extremely specialized to perform cell communication [4]. Thus, studying the cellular interactions and communications in the neural network of the brain is essential for understanding the principles of brain functions, and for discovering the mechanisms and origins of brain disorders.

Brain slice *In vitro* is a valuable experimental model system for studying neural communication in an environment where the original cellular network is preserved. Compared to *in vivo* studies on the brain using live anesthetized animals, experiments on brain slices eliminate the influence of anesthetics on cellular functions. Additionally, the external environment, including ion concentrations, oxygen levels and the supply of nutrition can easily be controlled and accurately adjusted, which can hardly be achieved to the same extent in live animals. Brain slices are more easily accessible by probes and various imaging techniques, which enables precise physiological and pharmacological studies of the functions and properties of the neuronal networks present in these slices.

The cellular arrangement of a brain slice is not homogenous. It consists of various distinct substructures. For example, the hippocampal slice contains the hippocampus and dentate gyrus, the latter being part of the Hippocampal Formation. The hippocampus can be divided into three different regions, CA1, CA2, and CA3, where each region has several different layers (*e.g.* the CA1 has five different layers), each of them with different connections to other brain regions [5, 6]. This implies that neural activity in different parts of a slice may convey different functions. Thus, precise experimental control of the chemical environment at a certain location in the brain slice is important for understanding the function of a certain neural network, which is of value in pharmacological studies. Thus, focal perfusion, *i.e.,* changing the solution environment locally, is necessary for achieving the localized application of active compounds on brain slices. Conventional bath perfusion, a common method to exchange the solution around the entire slice, cannot fulfill this requirement, since the whole slice, and not only the region of interest, is affected. Micro-perfusion is necessary for restricting the region of drug application. One of most commonly used micro-perfusion devices is the glass micropipette, which can deliver biologically active substances to brain slices through a

micro-sized opening at the needle tip. However, flow control is quite limited, the operational volume is typically too small, and fabrication quality is not sufficiently controllable [7].

Microfluidics has emerged as a technology for handling the flow of liquid in devices with small dimensions (micrometer scale channels) and volume (microliters to femtoliters), which above all allows for precise control and manipulation of fluids[8]. With the advantages of physical dimensions comparable to cells, reduced sample consumption and waste production, and well controlled flow dynamics, microfluidic devices have become attractive tools in biological research [9].

With the development of the microfluidic techniques in general, an increasing number of microfluidic devices have been introduced to the field of brain slice studies, allowing spatiotemporal control over solution delivery to the extracellular environment of the brain tissue, for example focal perfusion within conventional slice chambers for improved control over the solution environment in a selected slice region [10]. Since the micro channels in the chamber are stationary, slices have to be accurately positioned with respect to the channels, especially when several channels are desired. Additionally, the re-adjusting the slice against the channels is challenging. Any movement to the slice fixed in the chamber introduces accumulative mechanical damage, which can reduce the viability of the slice.

A new promising approach is the use of open volume microfluidics, in particular hydrodynamically confined flow (HCF), technology for localized microperfusion. This allows for positioning the solution exchange device to a stationary sample, without the need of changing its location [11]. The channels can be adjusted to the selected region above the slice, eliminating many of the problems associated with microfluidic perfusion systems [7].

HCF devices even eliminate the contamination of remote regions of the brain slice, and ensure that superfusion only occurs locally. By continuous re-uptake of the delivered solution into the device, a small, rapidly moving volume of fluid is spatially confined within another, significantly larger fluid volume (*i.e.*, a virtual flow chamber) [11]. Localized drug delivery can be achieved by merely touching a selected area of the tissue slice with this confined fluid volume [12]. The principle is an extension of the "push-pull" arrangement of glass capillaries, where solution is delivered through one, and aspired by a second glass needle [13]. Consequently, micro fabricated devices abolish typical disadvantages associated with glass needles. Among others, they provide higher fabrication quality, allow for a broader choice of materials, and enable internal functionality, such as flow switching and gradient generation [14].

In this thesis work, a novel microfluidic technology was introduced, i.e. the freestanding multifunctional pipette (MFP), for superfusion studies of brain slices. This device was characterized

with focus on its performance and utility in neuropharmacology. As a HCF based microfluidic device, which is widely compatible with various imaging techniques and probes, the MFP shows promise and potential for application in advanced pharmacological research. It has provided an outstanding performance in single-cell studies [15, 16] and brain slice studies [17], and can be expected to open up new possibilities in neuropharmacological studies.

## 2. Ion-channels

### 2.1 The cell membrane potential and the Nernst equation

All living cells are enveloped by a membrane that acts as a barrier between the intracellular and the extracellular environment [18]. The main constituents of cell membranes are phospholipids, which contain both hydrophobic and hydrophilic (polarized) residues. The amphiphilic property of phospholipids determines the bilayer structure of the membrane, in which a hydrophobic environment consisting of fatty acid hydrocarbon chains is situated in between two layers of hydrophilic phosphate residues interfacing the aqueous phase [18, 19]. The cell membrane also contains proteins, which can be integrated with the lipid bilayer (e.g. ion channels) or may simply be associated with cell membrane. The phospholipid bilayer forms a particularly effective barrier to charged molecules [18, 19]. Thus, the cell membrane, together with the intracellular and extracellular fluids can be viewed as capacitor coupled in parallel to resistors (i.e. the ion channels). The ATP-driven potassium (K+)/sodium (Na+) pump in the cell membrane continuously transports $K^+$ into the cell and $Na^+$ out of the cell, which leads to concentration differences between the intra- and extra-cellular milieu, resulting in concentration gradients for these ions across the membrane (i.e. higher $K^+$ and lower $Na^+$ inside the cell, see Figure 1).

Thermodynamically, diffusion is a spontaneous process because it decreases the order in a system (increases entropy). This implies that diffusion releases energy. Walther Hermann Nernst quantified this energy as

$$\Delta G = -RTln\frac{C_{out}}{C_{in}} \qquad\qquad 4.1$$

where $\Delta G$ is the Gibbs energy released by diffusion, $R$ is the universal gas constant (8.314 $Jmol^{-1}K^{-1}$), $T$ is the temperature in Kelvin and $C_{out}$ and $C_{in}$ are the extracellular and intracellular concentration of the ion considered, respectively. The cell membrane is permeable to $K^+$ ions via the bidirectional $K^+$ channel. Driven by the concentration gradient, the $K^+$ ions migrate out of the cell, and the outflow of positively charged ions increases the negative charge of the cell. Increased negative charge attracts

*2.1 The cell membrane potential and the Nernst equation*

the $K^+$ ions back into the cell. The electrical energy of this can be quantified as

$$\Delta G = -EzF \qquad\qquad 4.2$$

where $E$ is the electrical potential across the membrane unit, $z$ is the oxidation state of the ion under consideration and $F$ is the Faraday constant ($9.65 \times 10^4$ C mol$^{-1}$). When the attraction energy is oppositely equal to the diffusion energy, there is no net movement of ions and the two energies are in equilibrium

$$RT ln \frac{C_{out}}{C_{in}} = EzF \qquad\qquad 4.3$$

This relation can be rearranged to describe the equilibrium potential of the ion considered, leading to the important Nernst equation

$$E = \frac{RT}{zF} ln \frac{C_{out}}{C_{in}} \qquad\qquad 4.4$$

where $E$ is the equilibrium potential for the ion under consideration. Since $K^+$ ions can freely pass through the cell membrane and membrane is less permeable to other ions, the membrane potential, which is the sum of the equilibrium potentials of all the ions, is mostly determined by the equilibrium potential of the $K^+$ ion, which is at about -80 mV, and the cell membrane potential is typically -50 to -80 mV at resting state.
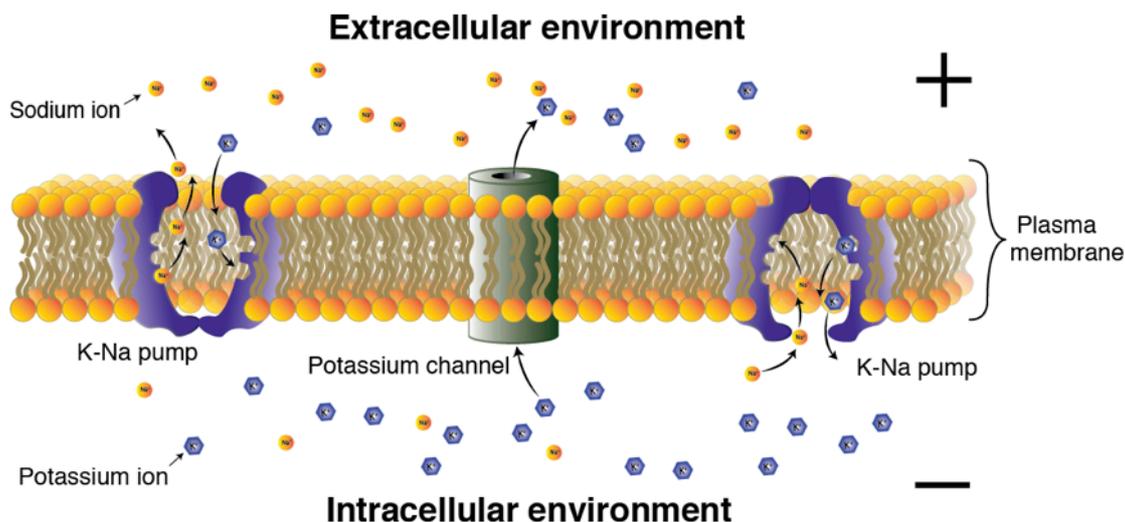
**Figure 1**. Illustration of the basic membrane potential generation process. Concentration gradients for $Na^+$ and $K^+$ across the membrane are generated by the K-Na pump, which transports $Na^+$ out of the cell and $K^+$ into the cell. Due to the selective permeability of the plasma membrane, only $K^+$ can freely pass through the membrane, and the efflux of the $K^+$ driven by a concentration gradient results in charge separation across the membrane, causing that the intracellular site of the membrane is more negatively charged than the extracellular environment.

The phospholipid bilayer is an effective barrier for charged chemical entities, thus the cell membrane is an insulator between two conductors (intra and extracellular aqueous salt solutions, which are very conductive to ions). However, this electrical insulation is not perfect: there are ion channels and transporters and there is also some leakage [20, 21], so the resistance of the cell membrane to the movement of ions across is finite. The current due to the flow of ions passing through the cell membrane is determined by the driving force and the membrane resistance. The driving force is the difference between the equilibrium potential and the membrane potential $E_m$. The bigger the driving force, the greater the net flow of ions. Thus, the current is proportional to the driving force, and limited by the resistance of the membrane, i.e., inversely proportional to the resistance. This can be stated as

$$I_{leak} = \frac{E_m - E_{rm}}{R_{leak}} \qquad 4.5$$

When the ion channel is activated, $I_{leak}$ is the current through the channels, $E_m$-$E_{rm}$ is the deviation from the resting membrane potential, and $R_{leak}$ is the resistance of the cell membrane against the ions passing through.

## 2.2 Properties of ion channels

Ion channels are pore-forming proteins in the plasma membrane, that open and close upon different stimuli (Figure 2). Ion channels are a crucial part of the cell membrane [22]. When the ion channels open, ions will flow in or out of the cell, depending in the concentration gradient across the cell membrane. This activity changes the ionic concentration difference, and also the electrical potential across the membrane. This membrane potential and the associated electrochemical gradients are substantially used by cells in their signaling and control systems [22].
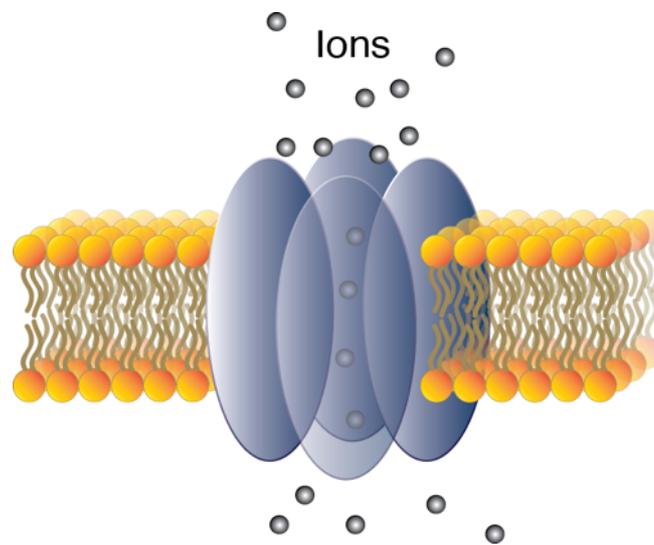


**Figure 2**. General illustration of an ion channel, which allows ions pass through the membrane (orange structures) while activated. The dark blue structures schematically depict the membrane proteins, which form the channel.

Ion channels are found in the membranes of all animal, plant and bacterial cells and play important roles in such diverse processes as nerve and muscle excitation, hormonal secretion, learning and memory, cell proliferation, sensory transduction, control of the salt and water balance and the regulation of blood pressure [23]. Ion channels also participate directly in cell apoptosis [24]. Considering their immense physiological functions and importance, it is not surprising that a considerable number of human and animal diseases are related to dysfunctions of ion channels.

Ion channels can be classified by their selective permeability to specific ions, such as $K^+$ channels, $Na^+$ Channels, $Ca^{2+}$ Channels, $Cl^-$ Channels and non-selective cation channels, or by the different

stimuli that activate the channels, e.g. voltage-gated ion channels, ligand-gated ion channels, or mechanical force-gated ion channels.

Ligand binding to the channel protein results in a conformational change of the protein, and this leads to pore opening (Figure 3A) [25]. Voltage gated ion channels have a structural motif,
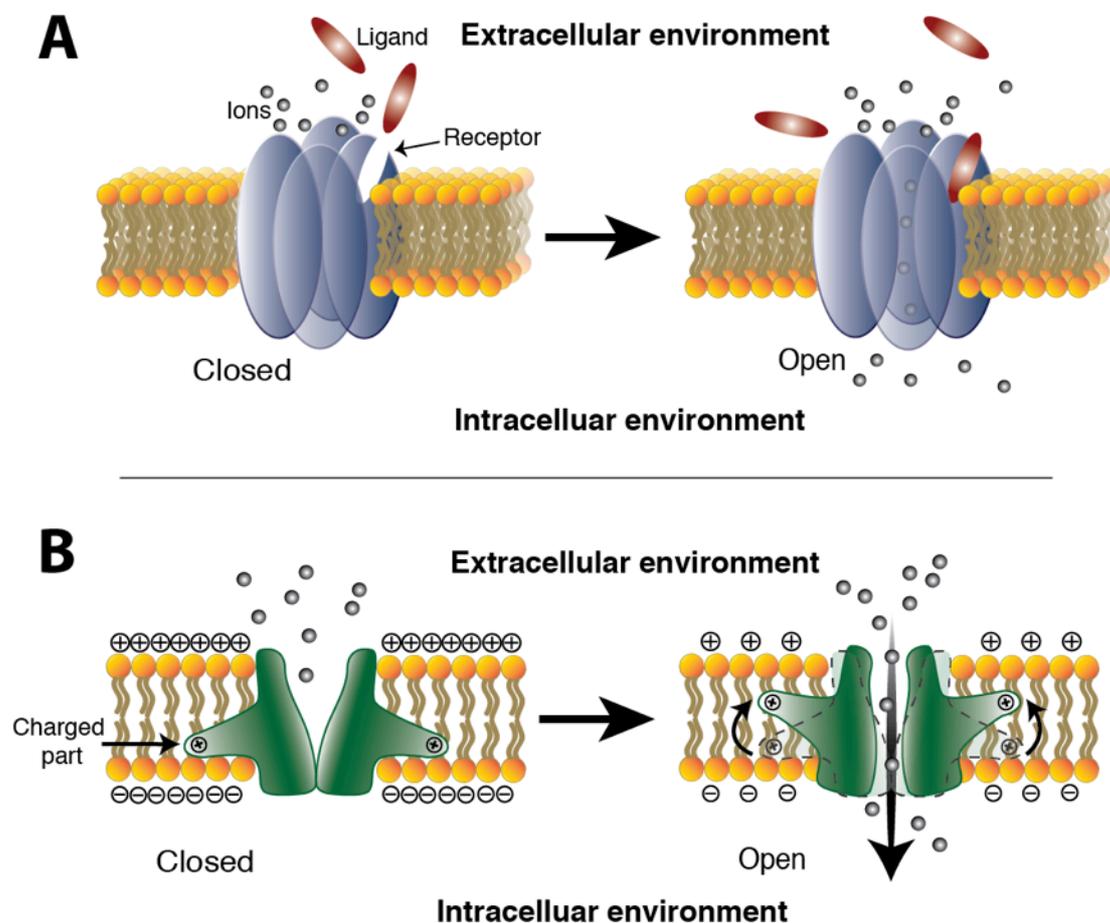


**Figure 3**. Schematic representations of different types of ion channels and their activating stimuli. A is a ligand-gated ion channel, which is activated by the binding of ligands to a specific binding site on the channel protein. B is a voltage-gated ion channel which can be activated by changes in membrane potential.

consisting of charged amino acids, which constitutes a voltage sensor (Figure 3B). The voltage sensors undergoes conformational changes when charged amino acid side chains respond to changes in potential, and consequently cause the opening or closing of the ion channel pore [26, 27]. Many ion channels also respond to other stimuli, such as mechanical force or temperature. Mechanosensitive ion channels [28] are the primary molecular biosensors in such diverse

physiological processes as touch, hearing, proprioception, or embryogenesis, as well as turgor control in plant cells and osmoregulation in bacteria [29]. Ion channels can be activated by a single stimulating factor, or by several different factors [30]. Temperature-sensing ion channels were the target channels in this thesis project. The transient receptor potential vanilloid 1 (TRPV1) ion channel studied in this thesis responds to different stimulating factors, including voltage, temperature, low pH, and a ligand.

## 2.3 The transient receptor potential vanilloid 1 (TRPV1) ion channel

### 2.3.1 The Transient Receptor Potential family

Transient receptor potential (TRP) ion channels are the guards of our sensory systems, responding to touch, temperature, pain, osmolarity, pheromones, taste and other stimuli [31]. They are important parts of the sensory apparatus of many multicellular organisms [32].

The TRP super family is divided into seven subfamilies, including TRPC, TRPV, TRPM, TRPN, TRPA, TRPP and TRPML [33], all of which have six putative transmembrane domains [32]. Some TRPCs may be store-operated channels, whereas others are activated by production of diacylglycerol or regulated through an exocytotic mechanism [32]. Many members of the TRPV subfamily function in sensory physiology and respond to heat, osmolarity changes, odorants, and mechanical stimuli [30]. The TRPM family functions as tumor suppressors and cold sensors [34]. The TRPN and TRPA include proteins with many ankyrin repeats. TRPN proteins function in mechanotransduction [32], whereas TRPA1 is activated by noxious cold and is also required for the auditory response [35]. TRPP and TRPML are distantly related to the other TRPs [32].

Temperature activated TRP ion channels, which can be dubbed as thermo TRPs, have the distinctive feature that they can be activated alone by temperature [36]. Thermo TRPs detect almost the entire range of temperature sensed by most mammals [37, 38]. Ion channels TRPA1 and TRPM8 are cool sensors activated by cooling, while TRPV1, TRPV2, TRPV3, TRPV4 are heat sensors activated by heating [38]. The TRPV1 target is the target ion channel in this thesis.

### 2.3.2 TRPV1

The transient receptor potential vanilloid 1 (TRPV1) ion channel is a nociceptor ion channel, which is found in the peripheral nervous system (PNS), brain, spinal cord, skin, tongue and bladder [38], and it is predominantly expressed in small diameter dorsal root ganglia (DRG) and trigeminal ganglia (TG) neurons [39]. The TRPV1 ion channel can be activate by various stimulations, including heat (> 42 °C), protons, voltage and ligands [40-43]. It is a non-selective ion channel, which is permeable not only for monovalent cations, but also for $Ca^{2+}$ and relatively large cations [40]. TRPV1 has been shown to possess a dynamic selectivity for ions during stimulation similar to what has been observed for P2X purinoceptor channels, and later also for the TRPA1 ion channel [44-46], *i.e.*, a time- and agonist concentration-dependent increase of the relative permeability of the ion channel to large cations. The TRPV1 ion channel shares the putative configuration of the TRPs family that the ion channel is a tetrameric protein. Each protein monomer is composed of 6 transmembrane domains with large N terminal and C terminal [47]. The transmembrane domains S5 and S6 with the pore helix between them compose the ion conduction pore (Figure 4) [48]. The conformational changes in the pore loop region might be integrated in channel activation, and might also contribute to the dynamic ionic selectivity of the ion channel [44].

The TRPV1 ion channel is involved in the transmission and modulation of pain (nociception), and in the integration of diverse painful stimuli [49]. Pharmaceutical blocking of TRPV1 presents a new strategy to release the pain by silencing the pain sensor instead of stopping the propagation of the pain, as most of the traditional pain-killers do [50]. In addition to its role in the PNS as a nociceptor, the function of the TRPV1 ion channel in the brain has also shown correlation with complex brain functions, as addiction, anxiety and learning [51]. Thus, studying the dynamic ionic selectivity characteristics of the TRPV1 ion channel may reveal valuable knowledge for understanding the mechanisms of its various functions.
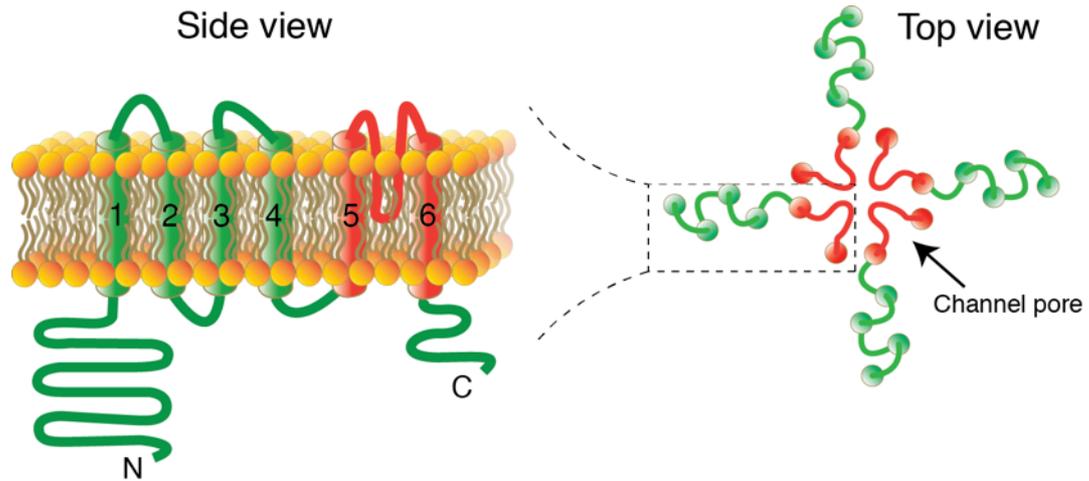
**Figure 4**. Schematic drawing (side and top view) of the TRPV1 ion channel. It is a tetrameric protein, where each monomer has 6 transmembrane domains. Transmembrane domains 5 and 6 (red) with the pore helix between them compose the pore of the ion channel.

# 3. Neurotransmission

## 3.1 Neurons

Neurons (nerve cells) are the fundamental units of the brain. In general, nerve cells are similar to all the other cells in the human body, but they are specialized in information transmission to and from other neurons or body organs [4]. As the neuron is the basic functional building block of the brain, understanding neurons is one of the essential prerequisites for understanding the brain.

There are about $10^{11}$ nerve cells in the human brain [52]. Each neuron consists of a *cell body (soma)* and numerous membrane extensions aroused from soma. One of these neurites is called axon, also known as nerve fiber, which transmits nerve signals to other neurons, muscles or glands. The neuron uses the other membrane extensions, which are known as *dendrites*, to receive signals from other nerve cells [53].

A typical neuron is depicted in Figure 5. The cell body contains the same organelles as other cells. However, the cell membrane is specialized to transmit information. There is only one axon (*cell projection*) coming out of soma (*cell body*), and it may branch and send out additional fibers, which transmit information to different target cells at terminal junctions, the *synapses*. All the other membrane extensions are the *dendrites*, which give a neuron its characteristic shape [54].

### 3.1.1 Axon

The axon has two fundamental functions in the neuron. One is to trigger the synaptic transmission via an action potential initiated from the soma. The other one is to transport chemical substances between the cell body and the synaptic terminals.

The connecting part of the axon to the cell body (soma) is called axon hillock, where the action potential is originated. Both inhibitory postsynaptic potentials (IPSPs) and excitatory postsynaptic potentials (EPSPs) are summed in the axon hillock. When the depolarization is sufficient enough to open voltage-gated sodium channels at the axon hillock, an action potential is generated and propagates along the axon down to the synaptic terminals [55].

The resting membrane potential of a neuron is usually -65 mV. At resting state, an influx of $Na^+$ into the neuron through open non-gated sodium channels is balanced by an efflux of $K^+$ through open non-gated potassium channels. The membrane potential remains constantly closer (but not equal) to the $K^+$ equilibrium [56]. When the cell receives excitatory input, the neuron membrane is depolarized. The depolarization opens up some voltage-gated sodium channels, which are normally closed at the resting potential. Activated sodium channels allow sodium ions to enter the cell and further depolarize the membrane. When the membrane potential is beyond the *threshold potential*,
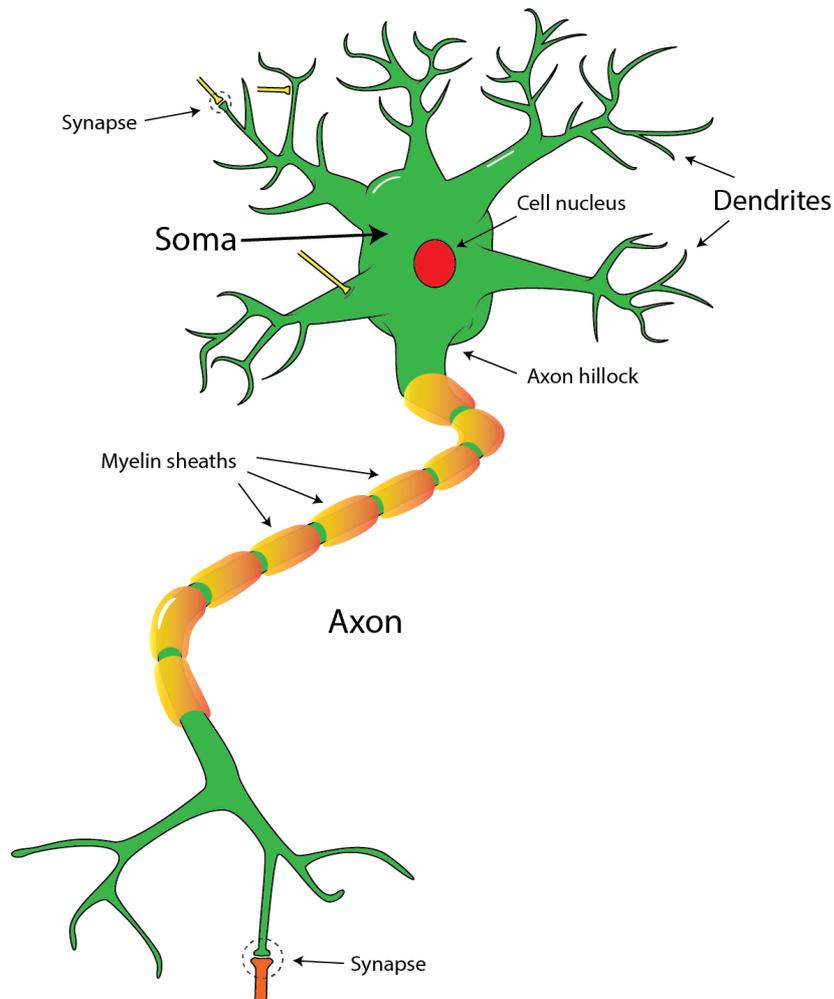


**Figure 5.** Schematic representation of a neuron. It is composed of three parts: the soma (cell body), the axon and the dendrites (specialized cell projections). The soma of the neuron is quite similar to other cells, while axon and dendrites are special to neurons. The axon can branch into many smaller fibers and form synaptic connections with other cells. Some axons are also equipped with myelin sheaths, which electrically isolates the axon and accelerates the signal transferring through the axon. The dendrites are simply the extensions of the membrane, by which extensive amounts of synaptic connections can be made with the axons from other neurons. The dendrites give the neuron its typical shape.

sufficient amounts of voltage-gated sodium channels open and the relative permeability of the membrane becomes higher to $Na^+$ ions than to $K^+$ ions, which generates the action potential. Sodium ion influx depolarizes the membrane (rising phase of the action potential) until it approaches the $Na^+$ equilibrium potential at around +40 mV, where sodium channels are inactivated and the influx of $Na^+$ through these channels is stopped (peak phase) [54].

After the peak phase of the action potential, the membrane is repolarized by the activated voltage gated $K^+$ channel, which increases the $K^+$ ion efflux from the neuron (falling phase of the action potential). The voltage gated $K^+$ channel is also activated by depolarization of the membrane, but the channels open with a delay (about 1ms). The $K^+$ channels are hence called delayed rectifier $K^+$ channels [54]. At the end of the falling phase, the membrane potential is more negative than the resting potential (after-hyperpolarization). This is because opening of the delayed rectifier $K^+$ channels increases the permeability of the membrane to $K^+$ ions in addition to the (already existing) resting permeability to $K^+$ ions through open non-gated potassium channels. The hyperpolarized potential is closer to the equilibrium potential of $K^+$, since there is little $Na^+$ permeability during this period. The resting membrane potential is restored, as the voltage gated $K^+$ channels are closed when after-hyperpolarization occurs [55, 56]. It should be noted that there are great $Na^+$ influx and $K^+$ efflux during the action potential. However, the $Na^+$ - $K^+$ pump is working all the time, even during the action potential, to maintain the ionic concentration gradients across the cell membrane [54].

The action potential activates the voltage-gated $Ca^+$ channel on the presynaptic membrane. The $Ca^+$ influx mediates the release of the transmitters stored in the presynaptic terminal [57]. Certain chemical transmitters are manufactured in the cell body by the *endoplasmic reticulum*, and transported along the axon to the synaptic terminals via microtubules extending from the cell body all the way to the synapses. This is called *anterograde* (forward) *transport,* while *retrograde* (backward) *transport* refers to the transportation of substances from the synapses to the cell body [54].

### *3.1.2 Dendrites*

Except for the axon, all the major protrusions extending from the soma are dendrites, which give a neuron its characteristic shape [53]. The primary function of the dendrites is to increase the signal receiving area of the nerve cell. The cytoplasmic composition in the dendrites is similar to that of the

soma. Therefore, dendrites can be thought of as extensions of the cell body. Both the dendrites and the cell body receive information through synaptic connections from other neurons [54]. In the cerebral cortex, the dendrites of many neurons are covered with thousands of small projections called *dendritic spines* [53]. Each of these spines, which are part of a synapse, further enhances the synaptic surface area of the neuron. Dendritic spine synapses are thought to be excitatory synapses [53].

## 3.2 Synapses

The axon transmits a signal to the target cell via synapse, where the plasma membrane of the axon terminal comes into close proximity to the target cell membrane. In most of the synapses, the presynaptic site is located on an axon [54], but in some cases, the presynaptic part is also located on the dendrites and the soma [58]. The same possible locations apply to the postsynaptic part. There are two fundamental types of synapses: the *electrical synapse* and the *chemical synapse*.

### *3.2.1 Electrical synapses*

The electrical synapse, as the name indicates, uses electrical transmission between nerve cells (figure 6A). The narrow space (3-3.5 nm) between pre and postsynaptic membranes in the electrical synapse is called *gap junction*, which is connected by numerous *gap junction channels*. The pore of the gap junction channels is wide enough (1.2-2 nm) to allow ions and even medium-size molecules to pass through, hence connecting the cytoplasms of the two cells. When presynaptic voltage gate channels are activated, the generated current flows from the presynaptic cell directly to the postsynaptic cell, therefore the signal transmission at electrical synapse is rapid (< 0.1 ms). In some electrical synapses, the current can only pass in one direction, which is called *rectifying* or *unidirectional synapses*, while *nonrectifying* or *bidirectional synapses* allow current to pass in both directions [59].

In the adult mammalian CNS, signal transmission is conducted via electrical synapses where high synchronization in neuron activities is need for neighboring neurons [60]. For example, hormone-secreting neurons in the hypothalamus are connected with electrical synapses and fire almost simultaneously when the activation signal arrives. Thus a burst of hormone is secreted into circulation. Electrical transmission is more common in non-neural cells, such as cardiac muscle cells,
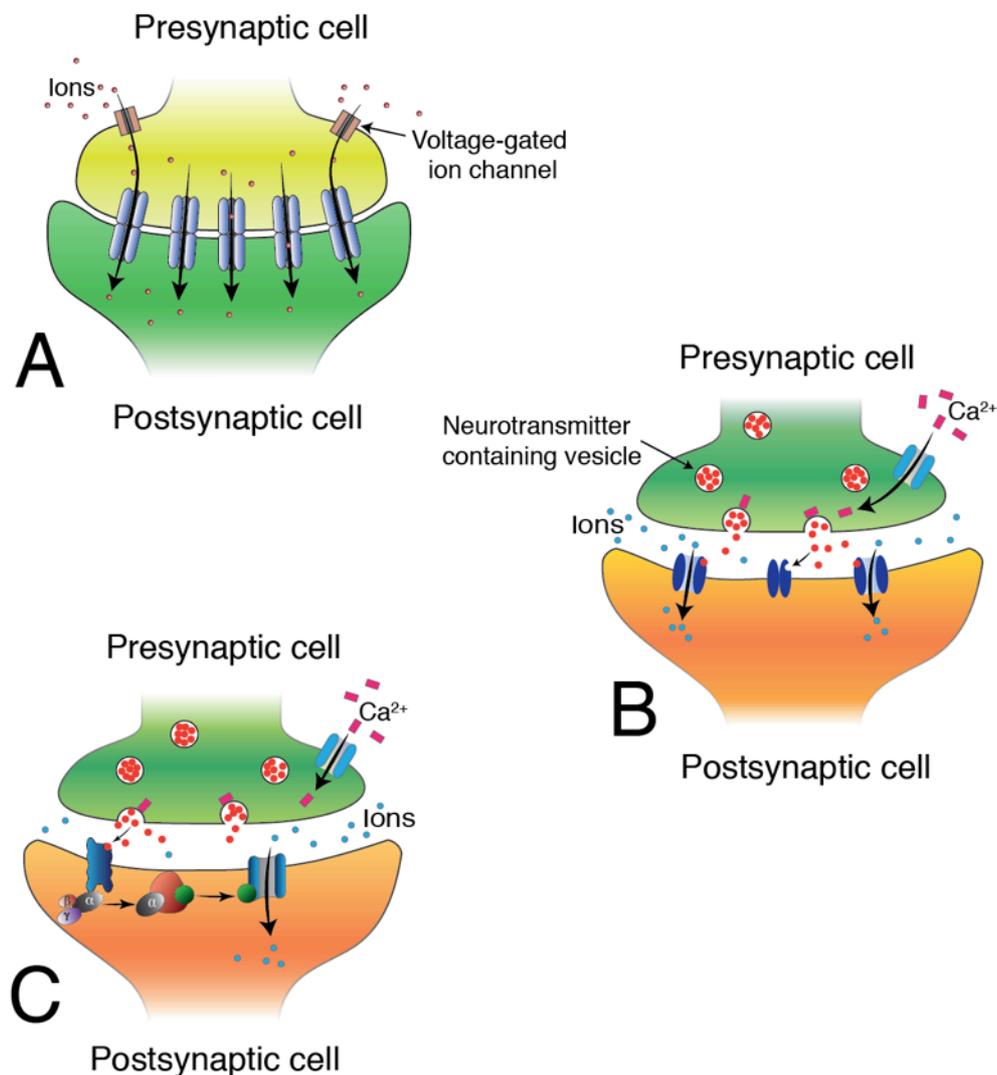
**Figure 6**. Schematic representation of different types of synapses. A is an electrical synapse, where the synaptic signal is transferred by the gap junction channels, which are formed by two hemichannels, one in the presynaptic membrane and the other in the postsynaptic membrane. When the presynaptic site is activated, the presynaptic current flows into the postsynaptic cell at very rapid speed (< 0.1 ms). B and C are chemical synapses, where the synaptic signal is transmitted by the neurotransmitters released from a presynaptic axon. In synapse B, binding of the neurotransmitters to the postsynaptic ionotropic receptors results in direct opening of the ion channels, which allows ions passing through the postsynaptic membrane, whereas in synapse C, the ion channels are activated by the complex G-protein coupled receptor-mediated intracellular system.

epithelial cells, liver cells and glia, but relatively rare in the mammalian nervous system [54].

*3.2 Synapses*

**3.2.2 Chemical synapses**

Chemical synapses are predominant in the mammalian brain. Unlike in the electrical synapse, the cytoplasm of pre and postsynaptic cells is not connected via channels. Instead, cells are separated by a tiny space (20 - 50 nm) called synaptic cleft, which is filled with extracellular solution. The pre and post synaptic membranes are connected by a matrix of extracellular fibrous protein in the synaptic cleft [53].

The chemical synapse use neurotransmitters to transfer signals from the presynaptic cell to the postsynaptic cell. Neurotransmitters can be categorized into three major classes: small molecule transmitters, neuroactive peptides and gaseous neurotransmitters [54]. Neurotransmitters are synthesized in the presynaptic cytoplasm or in the cell body, and transported via the axon to the synaptic terminals, where they are stored in vesicles.

When an action potential arrives at the axon terminal, the presynaptic nerve terminal is depolarized, which activates voltage-gated $Ca^{2+}$ channels. $Ca^{2+}$ ions flow into the terminal through the open $Ca^{2+}$ channels and cause vesicles to fuse with the presynaptic nerve membrane [61]. Neurotransmitters in the vesicles, then, are released into the synaptic cleft via *exocytosis* [56]. The released neurotransmitters diffuse through the synaptic cleft and interact with specific receptors in the postsynaptic membrane (figure 6B, C).

**3.2.3 Synaptic plasticity: LTP and LTD**

Synaptic plasticity, or altering of the synaptic strength, is believed to be a possible mechanism of learning and memory. Long-lasting increase of synaptic strength was first demonstrated in the dentate gyrus of the rabbit hippocampus by stimulating the entorhinal cortex with *high frequency stimulation* (HFS) for several seconds. After a short term of HFS, which is also known as tetanization, part of the recorded synaptic response increased to a much greater amplitude than normal. The phenomenon is called *long-term potentiation* (LTP) [62]. LTP refers to a long lasting increase, which is more than an hour (in-vitro) or even weeks (in-vivo). Evidences showed that changes in both presynaptic terminal (increase of neurotransmitter release) and postsynaptic structure (up regulation receptors) are the contributing factors for the LTP [63]. Its long duration after a short induction period (seconds), as well as the input specificity, cooperativity and associativity indicate that LTP is a possible molecular substratum of memory [64].

In contrast to LTP, a long-lasting decrease in synaptic strength, which is called *long-term depression* (LTD), was observed in both the hippocampus and the cerebellum. The LTD can be induced by a long period of *low frequency stimulation* (LFS), for instance, several hundred stimulation pulses at 1Hz [65, 66].

If LTP is the memorizing process, LTD is the forgetting process. The co-existence of LTD and LTP may have advantages of regulating memory storage in the brain. For example, LTD may help to delete some old memories, which could prevent synaptic saturation, and allow new memories to be stored. Conversely, it might be that LTD could conduct the memorizing process while LTP is responsible for forgetting [67]. It has also been suggested that LTD could increase the contrast between active and inactive areas, which is quite beneficial for the visual system [68].

## 3.3 Receptors

The interaction between transmitters and receptors either directly or indirectly facilitate specific ion channels to open or close, and as a result, specific ions enter or leave the cell. The receptors, which directly facilitates the ion channels, are called *ionotropic* or *ligand gated receptors*, while the receptors, which indirectly facilitate the ion channels, are called *metabotropic* or *G-protein coupled receptors* [54].

Ligand gated receptors are ion channels, which usually consist of multiple transmembrane protein subunits. Each subunit contributes to the pore formation of the ion channel. The neurotransmitters bind to the ligand gated receptors and directly facilitate the pore formation (Figure 6B). The activation of the receptors is rapid and for short duration [69]. The process of action potential induced $Ca^{2+}$ influx, neurotransmitter release, transmitter diffusion across the synaptic cleft and binding to the receptors gives the impression that the neurotransmission process may take a rather long time to occur. But the time from arrival of the action potential at the presynaptic terminal to activation of the ion channels in postsynaptic membrane takes as little as 0.2 milliseconds [53]. Thus, the chemical synaptic transmission via ligand-gated receptors is known as fast synaptic transmission. The synaptic transmission can be either excitatory or inhibitory [54].

### *3.3.1 Fast synaptic excitation*

*3.3 Receptors*

The synaptic excitation refers to the synaptic activities that depolarize the postsynaptic membrane, with which action potential (firing) can be generated in the effector cell. The basic mechanism of the synaptic excitation is due to the opening of the ligand gated ion channels in the postsynaptic membrane by binding of specific transmitters to the receptors on the channels. The ion channel opens for a very short time (usually only a few milliseconds) and causes only a small depolarization on the cell membrane. Thus single synaptic excitation is not big enough to evoke an action potential. Multiple synapses on one cell can be excited at the same time. The sum of these small depolarizations is called *excitatory postsynaptic potential* (EPSP). The EPSP is graded, which means that the amplitude of the EPSP is proportional to the number of synapses activated at the same time. In the other words, the more axonal input, the bigger the EPSP. When the depolarization of the cell membrane due to the EPSP reaches and exceeds the threshold of the action potential, the effector cell fires an action potential [53, 54]

### 3.3.2 Fast synaptic inhibition

The process from arrival of the action potential to release of the transmitters in the synaptic inhibition is similar to the excitatory synaptic transmission. The only difference is the ion permeability specificity of the receptors. In fast synaptic inhibition, the neurotransmitter binds and activates the receptors, which allow $Cl^-$ passing through the membrane. The influx of $Cl^-$ ions due to the concentration gradient hyperpolarizes the membrane towards the equilibrium potential of $Cl^-$, at which the cell becomes less excitable[54]. The hyperpolarization is called *inhibitory postsynaptic potential* (IPSP). Interestingly, in some invertebrate neurons the IPSP depolarizes the membrane potential, due to that the equilibrium potential of $Cl^-$ is higher than resting potential. This may also occur in vertebrates during development or in certain cell populations. However, a depolarized IPSP can still exhibit an inhibitory synaptic effect, which decreases the EPSP response of the cell. The effect of the IPSP, whether it hyperpolarizes or depolarizes the membrane, is more powerful than the simple addition of negative and positive voltages of IPSP and EPSP respectively [53]. There are two possible reasons for strong effects of the IPSP. One is that, during IPSP, the $Cl^-$ channels open and the membrane is completely permeable to $Cl^-$ ions, which competes against the $Na^+$ channels under excitation and keep the membrane potential towards the equilibrium potential of $Cl^-$. The other reason can be that the inhibitory synapses tend to locate near the axon on the cell body, where the action potential is generated. Hence, the IPSPs can exert a stronger effect than the EPSPs, which usually originate from more distant synapses [53]. The excitatory or inhibitory responses of the

postsynaptic neuron do not depend on the chemical nature of the neurotransmitter, but rather on the type of receptors on the postsynaptic site.

### 3.3.3 Metabotropic/ G-protein coupled receptors

Unlike the ligand gated receptors, metabotropic receptors do not directly activate an ion channel, or do not have the ion channel as a part of the receptor. One or more metabolic steps are needed for the receptor to activate the associated ion channel. The metabolic steps for the activation involve intermediate molecules called *G-proteins*, which consist of three subunits (α,β,γ) and bind to the intracellular part of the metabotropic receptors (figure 6C) [54]. This is why metabotropic receptors are also called G-protein coupled receptors (GPCRs). The metabotropic receptors consist of only one transmembrane protein, rather than multiple protein complexes as in ionotropic receptors. Binding of transmitter to the metabotropic receptor dissociates the G-protein from the receptor. The dissociated subunits of the G-protein then react with effector molecules and generate secondary messengers. The secondary messengers stimulate certain enzymes, which then activate appropriate ion channels [54]. The activation and duration of the metabotropic receptor mediated postsynaptic response is longer than in the ligand-gated receptor. For comparison, the metabotropic receptor mediated synaptic transmission is called *slow synaptic transmission*.

### 3.3.4 Recycling of synaptic vesicle membrane and neurotransmitters

During exocytosis, the vesicle membrane fuses with the plasma membrane of the presynaptic terminal. Therefore, new membrane is added to the presynaptic membrane. The fused vesicle membrane, then, is retrieved back into the presynaptic terminal by a process called *endocytotic budding*. Most retrieved vesicle membrane is recycled and reloaded with neurotransmitters in the synaptic terminal to prepare for the next release. Some retrieved vesicle membrane is transported back into the cell body and either recycled or degraded [54].

The released neurotransmitters are removed from the synaptic cleft by different pathways. Most transmitters are reuptaken by the presynaptic terminal via specific transporters and degraded or reused [70]. Some transmitters are reuptaken by glia cells, where they are transformed into a non-active form and transported into the presynaptic terminal to be converted back into the active form

[71]. Certain enzymes in the synaptic cleft inactivate or degrade specific transmitters. The postsynaptic terminal can also take up and metabolize certain transmitters. The transmitters diffused into the circulation are eventually destroyed in the liver [54].

## 3.4 Glutamate receptors

Glutamate (glutamic acid) is one of the non-essential proteinogenic amino acids. Despite the role as a building block for proteins, glutamate is also the main excitatory neurotransmitter of the brain, and also the precursor for the brain's main inhibitory neurotransmitter, γ-aminobutyric acid (GABA) [4, 72]. The role of glutamate in excitatory postsynaptic activation is conducted by glutamate receptors (GluRs), which play important roles in neural communication, learning and memory [73, 74]. GluRs are also linked to many psychiatric and neurodegenerative diseases [75-78].

Based on the mechanism of activation mediating the postsynaptic excitation, glutamate receptors can be divided into two groups, which are ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) [4].

### *3.4.1 Ionotropic glutamate receptors (iGluRs)*

iGluRs are tetrameric, ligand-gated, cation-selective ion channels, which exhibit fast response to glutamate and mediate EPSPs [79]. Numerous studies have revealed the structure, pharmacology, function and regulation of iGluRs in great detail, and iGluRs have become the best-understood types of receptors [80]. iGluRs have not only been studied as important pharmacological targets for many human neurological disorders, but also used as an illustration model for pharmacological topics, such as agonist versus antagonist and receptor desensitization [81].

The ionotropic glutamate receptor subunits are classified into four subfamilies according to their distinct response to certain small molecule agonists and sequence homology: AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors (GluA1-GluA4), NMDA (N-methyl-D-aspartate) receptors (GluN1, GluN2A-GluN2D, GluN3A-GluN3B), kainate receptor (GluK1-GluK5) and Delta receptors (GluD1-GluD2) [82, 83]. Delta receptors have been referred as orphan glutamate receptors, because, unlike the other receptors, they do not bind to glutamate analogs [83]. GluD1 is highly expressed in hair cells of the auditory and vestibular systems in adult mice [84] and GluD2 is

predominantly expressed in Purkinje cells [85]. However, the mechanisms of their functions are not yet well understood.

*AMPA receptor*

AMPA receptors are present on virtually all neurons within the central nervous system, and responsible for most of the rapid excitatory neurotransmission [86, 87]. Note that AMPA-silent synapses are believed to exist and to play an important role during early development. AMPA receptors have four ligand binding sites, one for each subunit. The ion channel opens only when two or more sites are occupied. The AMPA receptor is permeable to $Na^+$ and $K^+$, but not to $Ca^{2+}$, unless the receptor lacks a GluR2 subunit [88]. The latter has shown to play a critical role in neurodegenerative diseases due to the calcium permeation of the receptor [89].

AMPA receptors also play important roles in synaptic plasticity. Up-regulation and down-regulation of AMPA receptors on the postsynaptic membrane have shown to correlate with LTP and LTD, respectively [90, 91]. Proper AMPA receptor function and regulation are critical to neuronal development and synaptic plasticity. Abnormal activations of AMPA receptors have shown critical correlations with many neurological diseases. AMPA receptors have also been used as an important neuropharmacological drug target for a number of brain-related disorders [92-95].

*NMDA receptor*

NMDA receptor is highly permeable to calcium ions, which indicates its important role in synaptic plasticity. The NMDA receptor ion channel is blocked by a magnesium ion at resting membrane potential. The membrane depolarization, which is induced by the AMPA receptor-mediated EPSP, unbinds the magnesium ion from the NMDA receptors. The activated, unblocked NMDA receptors allow $Ca^{2+}$ to flow into the cell [96]. The entered $Ca^{2+}$ undergo various intracellular pathways as second messenger, which regulates the AMPA receptor on the postsynaptic membrane. This property of the NMDA receptor covers many aspects of LTP and LTD [97]. Experiments suggest that activation of different subunits of the NMDA receptors play separate roles in inducing LTP and LTD [98, 99], which indicates that the activation pattern of NMDA receptors is crucial in determining LTP or LTD [100]. Two conditions, membrane depolarization and ligand binding,

should be met at the same time in order to open the NMDA receptor ion channel [101]. In addition to the main agonist (glutamate or aspartate), the NMDA receptor requires binding of the co-agonist glycine for efficient opening of the ion channel [102].

The modulatory function of the NMDA receptor on LTP and LTD makes it a very important factor in learning and memory. A large number of neurological and psychological diseases have been related to irregularity of the NMDA receptor. The NMDA receptor has also shown importance in neural survival. Blocking of the NMDA receptor render many types of neurons vulnerable to trauma [103], while intensely or chronically activated NMDA receptor can cause excitotoxic cell death [104].

*Kainate receptor*

Kainate receptors are distributed throughout the brain but, unlike AMPA and NMDA receptors, they act more as modulator of synaptic transmission and neuronal excitability rather than being the major postsynaptic target for glutamate [105]. Kainate receptors are present in both pre and postsynaptic membranes. Presynaptic kainate receptors modulate both excitatory (glutamate) [106]and inhibitory (GABA) [107] transmission, while postsynaptic kainate receptors regulate neuronal excitability [108]. The excitability enhancement of axons was also observed by the presence of kainate receptors [109]. Although the kainate receptors are categorized in the iGluRs family, some of their neuronal function is mediated through non-canonical metabotropic signaling pathways [110]. These modulatory functions make the kainate receptor attractive potential therapeutic target, and studies have shown a correlation of kainate receptors with many neurological disorders [111].

### 3.4.2 Metabotropic glutamate receptors (mGluRs)

mGluRs are GPCRs that widely spread in the central nervous system. In contrast to iGluRs, mGluRs exhibit slow response to glutamate via a second messenger system and modulate cellular excitability and synaptic transmission, playing an essential role in synaptic plasticity and memory formation [74]. mGluRs are expressed on both pre and postsynaptic membranes. The main function of presynaptic mGluRs is negative regulation of glutamate release. In the other words, activation of presynaptic mGluRs by glutamate inhibits the glutamate release [112]. On the other hand, function of the

mGluRs on the postsynaptic membrane depends on the activated mGluR subtype, accordingly, the activation of postsynaptic mGluRs either depolarizes or hyperpolarizes the membrane [113]. As a critical regulator for glutamatergic neurotransmission, mGluRs directly influence the induction and maintenance of both LTP and LTD [113]. Being intensely studied as an important therapeutic target, mGluRs have revealed critical correlations to many neurological and psychological diseases [112].

# 4. Cellular networks

In the structurally and functionally complex multicellular organism, cells are differentiated into various types with particular functions. In mammals, major cell types are skin cells, blood cells, neurons, muscle cells, fibroblasts, stem cells, and others. Different cell types have different appearances and functions, yet they are genetically identical [3]. All cells are surrounded by a cell membrane, enclosing their intracellular structure and composition, establishing their identity, and separating their interior from the extracellular environment [1]. The cell membrane constitutes a functional subunit of the cell, where many biochemical reactions that mediate cell-to-cell communications, are carried out, which are conducted either electrically or chemically via different functional units of the cell membrane, such as ion channels [2]. In unicellular organisms, cell-to-cell communication is crucial for cell survival, *e.g.,* spreading of antibiotic resistance genes among bacteria [114]. In multicellular organism, cell communication plays much more complicated functions, e.g. operation of the immune system is conducted by the signaling between different immune cells [115]; neuronal communication determines the function of the brain [54]; interactions between different organs and tissues are also based on cellular communication. Thus, studying the cells, especially the cellular networks, can provide valuable information for understanding the function of tissues and the pathological mechanism of diseases.

The brain as the control center of the body contains the most complex cell-to-cell communication networks, which determine the function of the brain and is responsible for most of the neurological and psychological diseases. Neurons are the core cells for brain function, and are uniquely specialized in communicating with other neurons (sending and receiving signals) or sending signal and information to other organs of the body. The brain can be divided into different major functional units with specific neural network and function: cerebral cortex, cerebellum, brain stem, midbrain, limbic system, thalamus, hypothalamus, and basal ganglia. Each unit can be further divided into several subunits.

As part of the limbic system, the hippocampus is the most intensely studied brain region and is thought to be involved in learning and memory. The well-mapped neuroanatomical structure and neural network of the hippocampus make it a good model for neurotransmission, a well-characterized platform for application of new technology to brain research, and an attractive pharmaceutical target for many neuropsychological diseases.

*4.1 Hippocampus*

The prefrontal cortex, the anterior part of the cerebral cortex, has extensive interconnections with much of the brain and forms complex neural networks. This region of the brain is considered as the "central executive" of the brain, which regulates working memory, decision-making, reasoning, problem solving as well as planning, and execution. One common symptom in various psychiatric disorders, including depression and schizophrenia, is executive deficits, which are attributed to either frontal lobe damage or dysfunction, or to breaking of the interconnection between prefrontal cortex and subcortical regions. Thus, studying the neural network of the prefrontal cortex has utmost importance in understanding the mechanism of many psychiatric disorders.

## 4.1 Hippocampus

Its striking appearance in the brain easily identified itself even to the early brain anatomists. The name "hippocampus" was first given by the Bolognese anatomist Giulio Cesare Aranzi (1587), who associated the three-dimensional appearance of the human hippocampus to the sea horse, while some others linked the structure of the hippocampus to ram's horn, and De Garengeot (1742) named the hippocampus "*cornu ammonis*" or "*Ammon's horn*" after the mythological Egyptian god Amun Kneph, whose symbol was a ram. The term Ammon's horn is rarely used now, but interestingly, even though the name "hippocampus" has become the standard term, the abbreviations of cornu ammonis (CA1, CA2, and CA3) are referred to the subdivisions of the hippocampus [6].

### *4.1.1 Hippocampus anatomy*

The hippocampus is part of the hippocampal formation, a functional system, which is comprised of several related brain regions. The other regions of Hippocampal formation are the *dentate gyrus, subiculum, presubiculum, parasubiculum* and *entorhinal cortex* [6]. The hippocampal formation is one of a few brain regions that can receive and manage highly processed, multimodal information from various neocortical regions [6]. The unique neuroanatomical structure of the hippocampal formation is the basis for its function, including its involvement in learning and memory, which has been under investigation by many neuroscientists [73, 116-118].

The hippocampal slice consists of hippocampus tissue, which can be divided into the three regions CA1-3, and *dentate gyrus* tissue. Figure 7 shows the transverse section of a rat hippocampal slice. It

can be seen that the pyramidal cells are densely packed into a single layer. The CA regions are also structured depthwise into several defined layers, for example, the CA1 region can be divided into five layers: axons (*alveus*), basal dendrites (*stratum oriens*), pyramidal cells (*stratum pyramidale*), proximal apical dendrites (*stratum radiatum*) and distal apical dendrites (*stratum lacunosum-moleculare*) [5, 6].
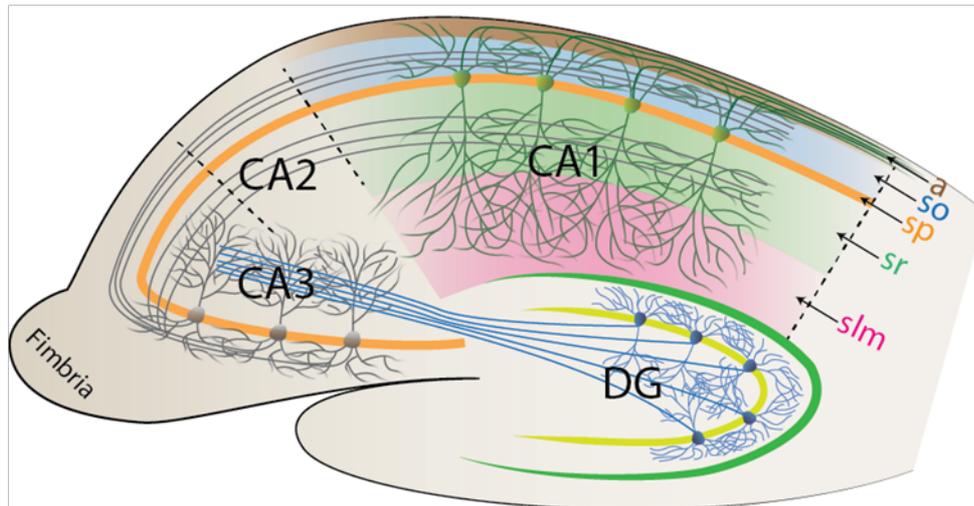


**Figure 7**. Schematic drawing of the transverse section of a rat hippocampal slice. Pyramidal cells in the hippocampus are packed into a distinguishable layer, *statum pyramidale* (sp). Neurons in the CA3 region project axons to the region CA1, where the excitatory synapses are formed with both basal dendrites and proximal apical dendrites of the neurons in the CA1 region at layers *stratum oriens* (so) and *stratum radiatum* (sr), respectively. Neurons in the CA1 region also receive projections from the *entorhinal cortex* in the layer *stratum lacunosum-moleculare* (slm), the most superficial layer closest to the *dentate gyrus* (DG). Pyramidal cells in the CA1 region send their axons out of the hippocampus through the layer *alveus* (a).

The pyramidal cells in the CA1 region are probably the mostly studied type of neurons in the brain. The pyramidal cells in the CA1 region receive both excitatory and inhibitory synaptic inputs [119]. The major excitatory signal resource of the CA1 is CA3 region. Pyramidal cells in CA3 region project excitatory axons (the Schaffer collateral axons) to the pyramidal cells in CA1 region to form synapses at both stratum oriens and stratum radiatum. The entorhinal cortex (EC) also projects excitatory axons directly to the CA1 region, and the projections selectively innervate dendrites in the stratum lacunosum-moleculare [6, 119, 120]. Glutamate, the major excitatory neurotransmitter, participates predominantly in the excitatory synaptic interactions of the hippocampus.

The inhibitory synaptic interactions, on the other hand, are mainly conducted by interneurons using inhibitory neurotransmitter, γ-aminobutyric acid (GABA) [6]. Neuroanatomical data indicate that virtually all hippocampal interneurons are GABAergic [121]. The principal cells of the hippocampus make synaptic contacts also on GABAergic interneurons to regulates the release of the GABA [121]. The neuronal interaction in the hippocampus is far more complex than simple excitatory and inhibitory interactions mentioned above. In addition to the glutamate and GABA, there are several other neurotransmitter systems in the hippocampus, which modulate the neural activities. For example, cholinergic afferents are present in every layer of the hippocampus, whereas noradrenergic innervation is mainly appears in the *hilus* of the *dentate gyrus* and *stratum lucidum* of the CA3 region [122]. The hippocampus also receives serotonergic and dopaminergic innervation modulating various neural activities [123][127].

Thus, this complex anatomical and histological organization of the hippocampal formation constitutes the basis for higher functions of this brain region that has been widely studied for many decades.

## 4.2 Prefrontal Cortex

The prefrontal cortex (PFC) is the anterior part of the frontal lobes of the brain, which receives projections from the mediodorsal nucleus of the thalamus [53]. The human PFC can be divided into three primary regions, which are *dorsolateral* region, *orbitofrontal* region, which is also described as the *ventromedial* prefrontal cortex, and the *frontal eye field* [124]. Different cortical regions of the PFC associate with different brain regions processing external information (all sensory and motor systems) and internal information (memory, reward and affect systems), The PFC is involved in emotional behavior and cognitive processes that includes behavior, speech and reasoning, planning and executive function.

### *4.2.1 The PFC: Anatomy and Signaling pathways*

PFC is differentiated into 6 distinguishable horizontal layers and the neurons in various layers interconnect to form intrinsic micro-circuitries called columns [125]. Layer I almost entirely lacks neuronal cell bodies, and mainly consists of apical dendrites and axonal ramifications from the upper

cortical layers, and interneurons [125]. It receives intensive input from M-type (matrix) thalamus cells, which strongly excites inhibitory interneurons of layer I, as well as forming excitatory synapses with dendrites of pyramidal neurons from upper layers [126].

Layer II and III contain mainly small and medium size pyramidal neurons, as well as non-pyramidal neurons [125]. Layer II and III received motor and sensory input signals from other area of cortex, and mediates the communications across cortical regions [127]. Layer II and III are also the main excitatory input resource to the layer V [128].

Layer IV contains stellate and pyramidal neurons, and it is the main target for thalamocortical input from C-type (core) thalamus neurons [125, 129]. Layer IV projects axons to layer II and III and layer VI [130].

Layer V contains large pyramidal neurons, which project excitatory axons to the thalamus. It forms intensive interconnection with layer II and III [125, 128].

Layer VI less intensively forms interconnections with other cortical layers. It contains variously sized and shaped pyramidal neurons as well as interneurons, which project both excitatory and inhibitory axons to the thalamus [125].

Different layers of the PFC have distinct roles in signaling pathways, which can be categorized in two classes: the ascending and the descending pathway [130].

Ascending pathways are also called bottom-up (or feedforward) pathways, The idea refers to the signal inputs coming from subcortical brain structures up to the cortical layers and from lower cortical layers to the higher cortical layers. Layer IV is the primary target of the ascending pathways. The layer IV receives excitatory inputs from the thalamus C-type neurons [131], and layer IV relays the signals to layer III and II, which further project excitatory inputs to the layer V. As mentioned above, layer I receives intensive excitatory input from M-type neurons in the thalamus to the inhibitory interneurons [53, 125, 132], which regulates the excitability of the layers II and III by directly forming synapses in layer I with dendrites of pyramidal cells in layer II and III [130].

In contrast to the notion of the ascending pathway, a descending pathway refers to the signaling from a higher cortical layer to a lower one and from a cortical area to a subcortical region of the brain. The descending pathway, also called top-down or feedback signaling pathway, is thought to play an

important role in cognitive control [125, 133]. Principle cortical target of the descending pathway is layer I, which receives axons mostly from upper layers, as well as some from deeper layers. Layer V and layer VI project axons to thalamus neurons. Layer V projection is mainly excitatory, and layer VI has both excitatory and inhibitory actions [125, 130].

## *4.2.2 The rat PFC*

The rat cerebral cortex is approximately 1000 times smaller than that of a human cortex, making a direct translation based on anatomy alone impossible [134]. The region in the rat cortex corresponding to the human dlPFC is the rat medial PFC [134, 135]. This notion is based on the fact that the rat mPFC, in similarity to the human dlPFC, builds extensive reciprocal projections from e.g. the mediodorsal thalamus, receives similar neurotransmitter input (e.g. noradrenaline from the LC, serotonin from the DRN, dopamine from the VTA) and expresses similar receptors as the human dlPFC. Thus, similar behaviors are mediated via these areas in humans and rats, respectively, such as attention, working memory and social interaction.

# 5. Superfusion systems

Superfusion is the technique of running a stream of liquid over the surface of a biological sample. The major purpose of the superfusion system in studies of isolated cells is transporting the active compounds to the surface of the cell. In ligand-gated ion channel studies, quick changing of the solution around the cell is desirable for understanding the function of the ion channel, such as ligand binding profiles and channel kinetics. Among all the approaches for the solution exchange, the open volume multichannel perfusion chambers, including the Dynaflow ® microfluidic device used in this thesis, have become the most commonly used superfusion systems for high throughput electrophysiological studies of ion channel [136].

Superfusion systems in physiological studies also have to provide constant supply of nutrition and oxygen, and aid in metabolic waste removal, for maintaining the physiological environment and the slice viability [7]. The most commonly used perfusion systems for brain tissue slices is the conventional perfusion chamber, where physiological solution enters the chamber from one side and leaves from another, so that the tissue sample placed in the chamber is continuously provided with fresh, oxygen and glucose enriched buffer solution. Biologically active compounds are typically delivered to the sample via the same flow. Various types of perfusion chambers have been developed and are commercially available for *in-vitro* studies. Some of them are useful for general purposes (e.g. QuickStage™ chamber, AutoMate Scientific), while others are designed for special purposes (e.g. field stimulation chambers, Warner Instruments). The major problem with the system is the non-localized perfusion, where the whole sample is submerged in the bath and perfused with the active compounds. All parts of the sample are equally exposed and thus become over time contaminate with the applied compounds, which reduces the reusability of the sample and, in the case of pharmaceutical studies on a tissue sample, makes it impossible to determine the focal effect of the active compound, which prevents revealing valuable information for understanding the biochemical role of the compound, and the mechanism of its action.

In comparison with the conventional perfusion chamber, the glass pipette, which is a widely used simple yet efficient perfusion device, delivers a drug much faster, and more localized. Glass pipette-based pressure controlled micro-perfusion systems are also commercially available. One commonly found system is the Picospritzer® (Parker Hannifin Corp). The glass pipette, to be used in the perfusion experiments, is pulled from a heated glass capillary. The tip diameter is, generally, in the micro- to nanometer size range. The glass pipette can be conveniently positioned to a certain region of the tissue or to a specific cell, and the solution filled into the pipette is injected directly

onto the target area. Different types of glass tubing systems have also been developed for quickly changing the solution around the cell with an improved switching profile (figure 8). For example, the Y-tube, which consists of two inlet tubes and one outflow tube, can rapidly change the solution around the cell by controlling the speed of flow in the inlet tubes [137]. Another noteworthy glass-tubing system for superfusion is the double-barreled pipette, which is a glass capillary that has a glass septum dividing the tube into two compartments, which can be filled with different solutions.
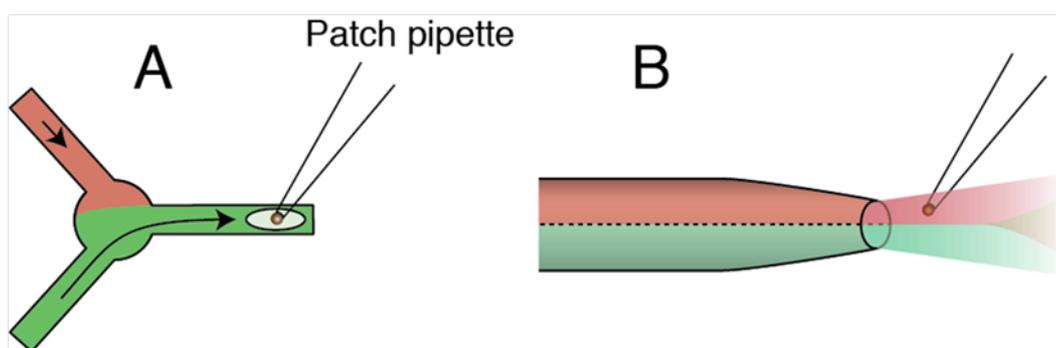


**Figure 8.** Two different types of glass superfusion devices. A) Y-tube. B) double-barreled pipette.

The concept was later adopted and extended in many other multi-barrel perfusion systems, including the Dynaflow® chip, one of the microfluidic devices used in this thesis work. When pressure is applied to the pipette, two solutions are transferred out of the pipette opening, and the interface of the two solutions is sharp, even in the open volume outside the pipette opening. The cell can be exposed to the two solutions simply by moving the pipette [138]. However in this, the flow originating from the glass pipette is difficult to control, and cannot be completely halted. Therefore, volume confinement cannot be fully achieved, and a build-up of the injected solution in the open volume occurs. Moreover, consistency in fabrication is challenging, and the tendency to break upon contact with a hard surface inside the chamber makes the glass pipettes often tedious to use. Progress in superfusion technology has been made through microfluidic devices, which have overcome several of the limitations and challenges with well-controlled flow dynamics and highly repetitive fabrication quality. Along with the increased popularity of microfluidics in analytical chemistry in the last decade, various microfluidic devices have also been integrated into experimental techniques for studies of biological material, particularly as highly efficient superfusion tools. In that context, microfluidics often improves and simplifies the standard techniques. Furthermore, the added benefit

of on-chip functionality, allowing, for example, for switching rapidly between different solutions (which is not possible with glass needles), has enabled new and more sophisticated experiments.

## 5.1 Microfluidics

The main ambition of microfluidics is managing and studying the flow of small volumes of fluids (microliters to femtoliters) in physical dimensions on the micrometer scale. Some properties of flow in microfluidic systems are, due to the small dimensions, quite different from the macroscopic flows (for example of water from a tap) what we can daily observe.

Flow can be characterized by the Reynolds number (Re), which is a dimensionless number associated with the Navier-Stokes equation,

$$\rho \left[ \frac{\partial v}{\partial t} + v \cdot \nabla v \right] = -\nabla p + \eta \nabla^2 v + F \qquad \qquad 5.1$$

where $\rho$ is the fluid density, $v$ is the fluid velocity, $p$ is the pressure, $\mu$ is the fluid viscosity, and the $F$ is external forces such as gravity. The Navier-Stokes equation can be viewed as an extension of Newton's second law for fluids. The left-hand side of the equation shows the mass per unit volume ($\rho$) times the acceleration of the fluid in both time and space and the right hand side consists of the forces per unit volume due to a pressure gradient ($\nabla p$), viscosity ($\eta \nabla^2 v$), and other external forces [139].

The Reynolds number represents the ratio of the inertial to the viscous forces. The Reynolds number for a fluid stream (for example in a channel) can be written as

$$Re = \frac{\rho v L}{\eta} \qquad \qquad 5.2$$

where $\rho$ is the fluid density, $v$ is characteristic fluid velocity, $\eta$ is the viscosity and $L$ is the characteristic size, which depends on the cross-sectional geometry of the channel through which

fluid flows. The laminar flow, which is characterized by low Reynolds numbers (Re << 1), is a smooth and predictable flow, where viscous forces dominate, while turbulent flow, which is characterized by high Reynolds numbers (Re > $10^3$), is chaotic and unpredictable, and the inertial forces dominate the fluid dynamics (figure 9). At low Reynolds numbers, the fluid dynamics is determined by the external driving forces (i.e. pressure) and fluid movement is well controlled.  In the low Reynolds number regime, a fluid is set to motion by applying an external force, and will stop when the external force is turned off. In contrast, turbulent flow (high Reynolds number regime) can be initiated by an external force, but cannot be stopped by simply eliminating the external force, as inertia contribute to the motion [139].
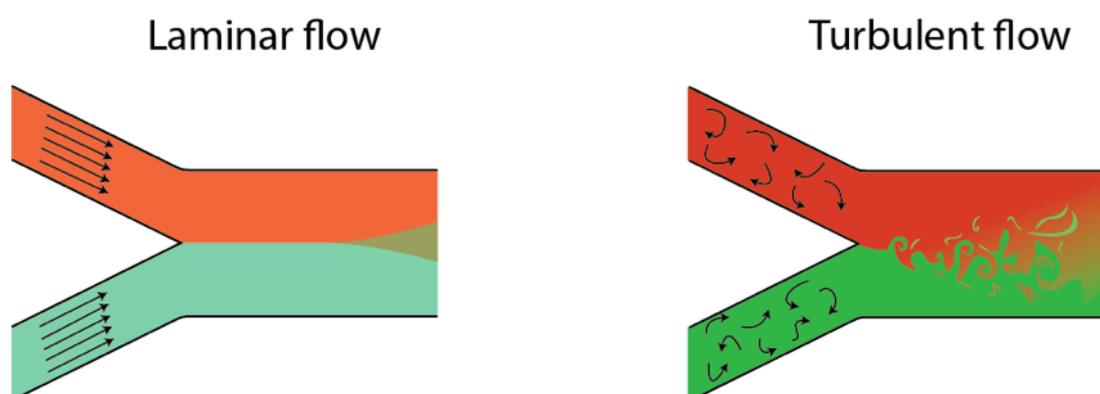


**Figure 9.** Schematic representation of laminar (left) and turbulent (right) flow in a Y-shaped channel.

Water or water-based solutions of similar viscosity as working fluid are in the low Reynolds number regime, when they pass through a channel with the size smaller than about 100μm, and at velocities on the order of μm/s. The fluid dynamics in microfluidic system is typically under low Reynolds number (Re) conditions, and laminar flow is an intrinsic characteristic of channel-based microfluidic systems [140].

## 5.2 Applications of microfluidics in biology

Since the inception of the microfluidic devices, intensive research has been conducted in the field of microfluidics. One major driving force for its continued development is the applicability of the

microfluidics in biological and biomedical research. Advantages of the well-controlled flow dynamics, small reagent volumes, high heat and mass transfer rate, fast response times, reduction or elimination of cross contamination and the ability to perform multiple processes simultaneously, make microfluidic devices suitable for biomedical analysis, chemical sensing, genetic analysis, metabolic monitoring, biomolecular separation, cell studies, high-throughput screening and drug delivery, among others. The integration of microfluidic techniques and devices in biological studies covers a wide variety of applications, which includes manipulating individual cells and the environment around them, and improving the viability of organotypic tissue slices (e.g. brain slices). Microfluidic devices have been used for monitoring growth, productivity, and heterogeneity on the single-cell scale [141], to analyze signaling compound transmission mediated cell-cell interaction[142], and for optimizing proliferation and differentiation of neural stem cells (NSCs) in culture by exposing the cells with a stable and reproducible concentration gradient of growth factors under continuous flow [143]. Microfluidic channels have also been used to physically resemble blood capillaries in studies on Plasmodium falciparum-infected erythrocytes [144], for inducing focal stimulation in one portion of a brain slice via a well-characterized laminar flow [10], and for localized drug administration to the brain slices by utilizing hydrodynamically confined flow [11]. With the development of microfluidic techniques, more and more microfluidic devices have been introduced and applied in various areas of biological research, and have shown to provide significant benefits. However, challenges and drawbacks still exist. Microfluidic devices do not have standardized components. This may impede their integration into existing laboratory environments and the cooperation with other techniques, such as patch clamp electrophysiology. Moreover, if specimens are deposited in microfluidic flow chambers, their accessibility to external probes is often problematic. In addition, microfluidic devices with fixed channel positions require positioning of the sample against the channels, for example by means of hydrodynamic focusing [10]. The increased sample movement required to achieve the required sample position may introduce mechanical damage to the sample, which may decrease the quality of the sample and of the collected experimental data, especially in case of brain slice studies.

## 5.3 Fabrication of microfluidic devices

The microfluidic devices described in the following sections were used in this thesis, and are produced with soft-lithography techniques (figure 10), using polydimethylsiloxane (PDMS). PDMS is a silicon-based polymer, which is commonly used in prototyping and fabrication. It is inert, non-
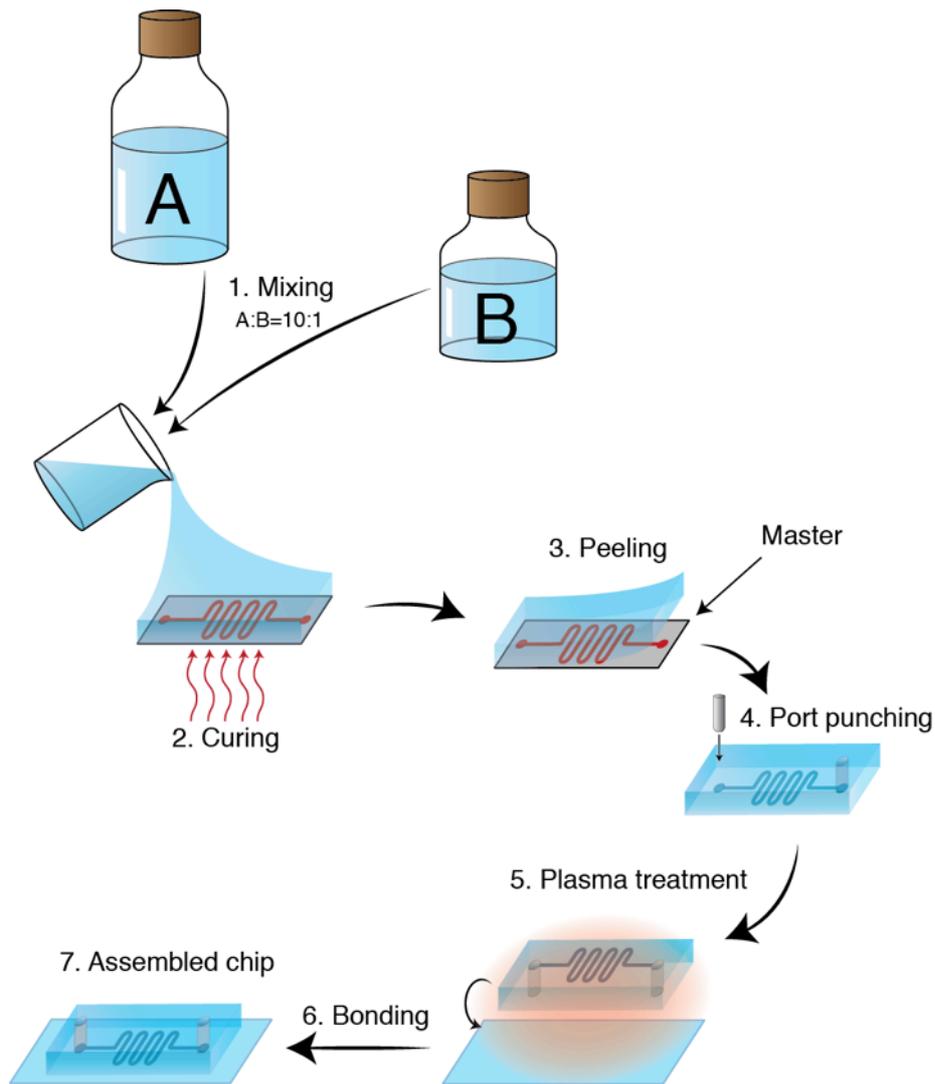
**Figure 10**. Soft lithography. The base component (A) and the curing agent (B) are mixed in 10:1 ratio. The well-stirred mixture is poured on a mask, where the microchannel structures are embossed, and cured in the oven. The cured elastomer is then peeled from the master and the connection holes are punched. Finally, the block is bonded to a glass slide after both bonding surfaces have been treated with oxygen plasma.

toxic, non-flammable and optically transparent, and particularly suited for the fabrication ofmicrofluidic devices [141]. PDMS devices containing microchannels can be prepared in a few steps from a liquid pre-polymer. The channel structures are embossed on a "master", from where they are transferred into the PDMS by replica molding. Two liquid pre-polymer components (base and curing agent containing a catalyst) are mixed, and poured onto the master, which is positioned at the bottom of a mold. The master consists of a layer of the negative UV-curable photoresist SU-8 on

a Si-wafer, produced by means of photolithography. After curing in an oven, the PDMS elastomer can be peeledoff from the master/mold. The surface of the PDMS block generated in this procedure can be activated by oxygen plasma treatment. Following such treatment, it can be bonded with other activated PDMS surfaces or glass. Since the bond is permanent and irreversible, it prevents communication or mixing of solutions between the channels [145]. Interfacing with tubes or other supply lines completes the microfluidic device. Depending on the intended application, silicon, glass and various kinds of plastics have also been used for fabrication.

## 5.4 Dynaflow® chip

The Dynaflow ® chip (figure 11) used in this thesis consists of a PDMS mold containing the channels, which is plasma-bonded to a glass slide. The chip has 16 microchannels, each connected to an on-chip solution reservoir. These reservoirs can be filled with different solutions, or solutions containing different concentrations of the same compound. A lid connected to a pump is tightly attached to the reservoirs so that the solutions in the reservoirs can be driven by pressure through the connected microchannels, which lead into an open bath with the dimensions $35 \times 20 \times 4$ mm ($w \times l \times h$). In the outlet region, the 50-µm-wide and 57-µm-high channels are closely spaced and separated by 22-µm-thick walls. Close to the channel outlets, the outflows from the channels form a patterned laminar flow inside the open volume, which maintains its sharp inter-solution boundaries for a distance of approximately hundred micrometers. The patterned flow volume can easily be accessed from the open bath. Scanning and positioning of micropipettes, electrodes, or patch clamped cells are possible in this chip. The cells can be exposed to a sequence of solutions precisely and with fast switching times in the range of tens of milliseconds. The cells are placed and patched in the open bath.
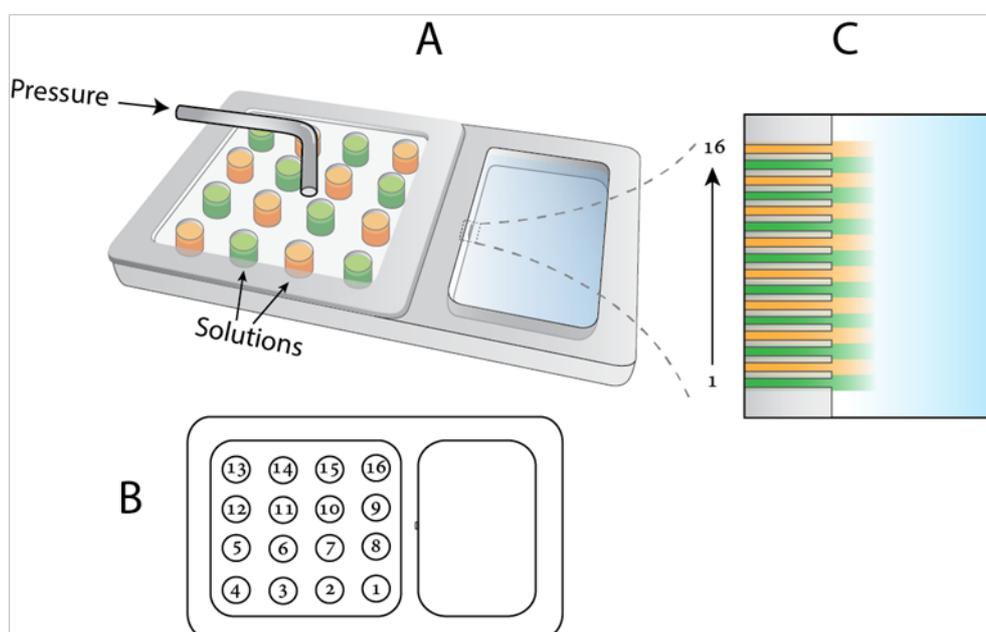
**Figure 11.** The Dynaflow® chip. This microfluidic chip has 16 on-chip solution reservoirs. When the pressure is applied to the channel via a sealed cover, the solutions in all reservoirs are simultaneously pushed through individually connected microchannels, and exit into the open bath. In the outlet region, the microchannels are closely spaced. The patterned laminar flow from the channel can be maintain for approximately hundred micrometers inside the open volume, which can be accessed by patch pipette from the open bath.

## 5.5 Multifunctional pipette

The multifunctional pipette used in the thesis work (figure 12) is a different open-volume microfluidic device. It also allows for dispensing solutions into an open bath, and is capable of superfusing cells and tissue samples. However, the multifunctional pipette features a *hydrodynamically confined flow* (HFC), which is achieved by injecting a fluid stream out of the tip of the pipette into an open volume, and aspirating it back into the device through two adjacent channels (recirculation). This generates a confined volume, i.e., a virtual flow chamber, featuring a sharp boundary between the injected and open volume fluid. Within 20 μm from the interface, the concentration of a compound in the confined volume is reduced by 90%. The pipette with the virtual flow cell at its tip can be then positioned to a single cell, or any other object or surface element inside the open volume for stimulation or analysis, without affecting the surrounding liquid or objects in the vicinity. Similar to the Dynaflow device, the pipette features on-chip wells. It has 8

solution reservoirs, four of which contain active solutions, the other four are needed to collect the device inflow (waste). Instead of having parallel channels, the pipette switches the solutions exiting a single channel, using on-chip circuitry. Each well is connected through a manifold to an individual channel in a computer-operated pressure controller, which sets the flow velocities for injection and aspiration in order to adjust the size and shape of the HCF volume. The manifold for addressing the individual reservoirs is integrated into the upper part of a pipette holder, which presses against the PDMS to seal each well individually.
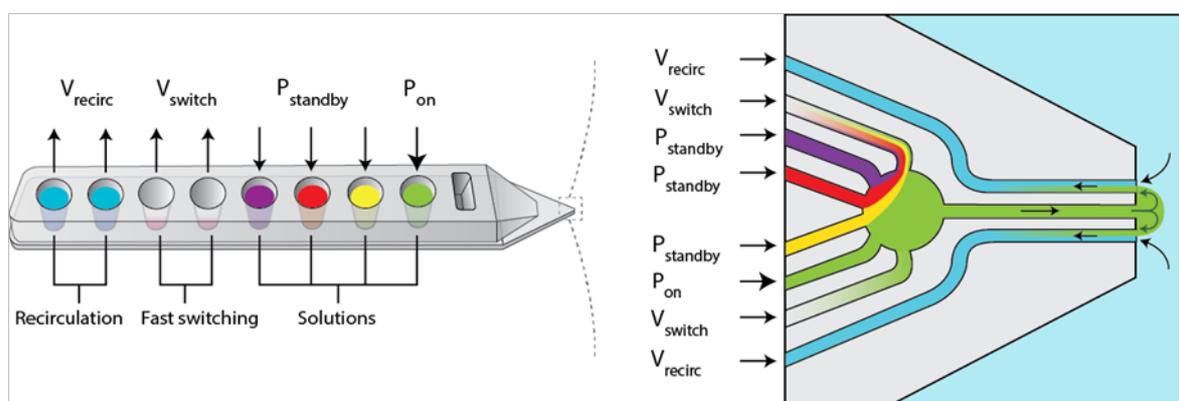


**Figure 12**. The multifunctional pipette. The MFP has 8 wells, 4 of which are for working solution and the others are used for fast valveless solution switching and solution recirculation. When one of the solution wells is applied with a working pressure ($P_{on}$), the solution flows through the connected microchannel towards the tip of the pipette. At the tip region of the pipette, the solution flows into a valveless solution switching chamber, where the liquid in the chamber is continuously aspirated back to the switching wells by applying a vacuum ($V_{switch}$). The working solution passes through the switching chamber and exits from the channel opening at the tip of the pipette, where two microchannels are closely located on both side of the solution channel. The two adjacent microchannels aspirate the working solution back to the recirculating wells via a vacuum ($V_{recir}$). Hydrodynamically confined flow (a virtual flow chamber) can be formed by this "push-pull" process on the tip of the pipette.

Four major parameters are used to control the flow of the multifunction pipette: standby pressure, working pressure, switching vacuum and recirculation vacuum. The standby pressure is a combination of comparatively low positive and negative pressures. It maintains a minimal HCF at

the tip of the pipette and prevents outside solution from entering it. The working pressure is the principle driving force for the working solutions in the pipette. The switching vacuum is used to enable switching of the solutions in the switching chamber, which is located in the tip, close to the channel exits. Recirculation vacuum, together with working pressure, are essential for controlling the HCF. They are required to ensure confinement of the solution upon entering the open volume. The two parameters are carefully balanced to adjust the inflow-to-outflow ration, *i.e.*, the extent and volume of the virtual flow chamber. Except for the recirculation wells, all the microchannels originating from the other 6 wells are connected to the switching microchamber, which is continuously operating. When working pressure is applied to one working solution, the others are under standby pressure and routed into the switching waste wells. The selected working solution passes through the switching chamber and enters the open volume through the outlet channel. When switching to a new solution, the previously active working solution is displaced in the switching chamber by adjusting working pressure and standby pressure for the old and new solution. The outflow is thus instantly replaced by the new active working solution. The solution switching time required to exchange the recirculation volume is < 100 ms.

# 6. Electrophysiology techniques

## 6.1 Patch clamp

Electrophysiology is the study of the electrical properties (membrane potential and electric current) of biological cells and tissues, which ranges from studying single ion channel properties to whole organs like the brain, including measurements of the electrical activity of neurons. The patch clamp technique is a useful electrophysiological tool for studying single or multiple ion channels in cells [146]. The activation of ion channels changes the membrane potential by allowing ions passing through the membrane. Therefore, recording the changes of the membrane potential is an important indicator of the ion channel activity. However, the changing of the membrane potential also affect the ion channel function since: i) changing the membrane potential causes changes in the electric force to the ions flowing through the membrane; ii) many ion channels are voltage gated (voltage dependent activation); iii) some ion channels have the ion flow restricted to a certain membrane potential. Therefore, it is often desirable to control the membrane potential and record the membrane current directly. This is called voltage clamp. In voltage clamp, the measured potential is continuously compared with a preset holding potential. Any deviation of the recorded potential from the holding potential is instantly corrected by compensatory current injection controlled by an electrical feedback system. This current is then an accurate representation of the ionic current over the membrane under investigation. In contrast, the current clamp configuration requires that a constant current is applied instead, and changes in potential generated by the cell are recorded. Most patch clamp experiments are voltage clamp experiments.

### 6.1.1 Patch clamp configuration

The patch clamp technique requires a fluid-filled micropipette, which is brought into contact with the cell membrane. The pipette contains an electrode, which is connected to a high performance amplifier (Figure 13). By applying a slight aspiration pressure to the pipette, a tight seal is formed between the pipette and the cell, which has an electrical resistance of several gigaohms (gigaseal). When the gigaseal has been obtained, increased aspiration can rupture the cell membrane in the pipette tip, and the solution in the pipette becomes connected with the intracellular fluid, which

enables the recording pipette to measure ion channel activities from the intracellular side of the plasma membrane. The "patched cell" is thus ready for *whole cell recording,* since it records the
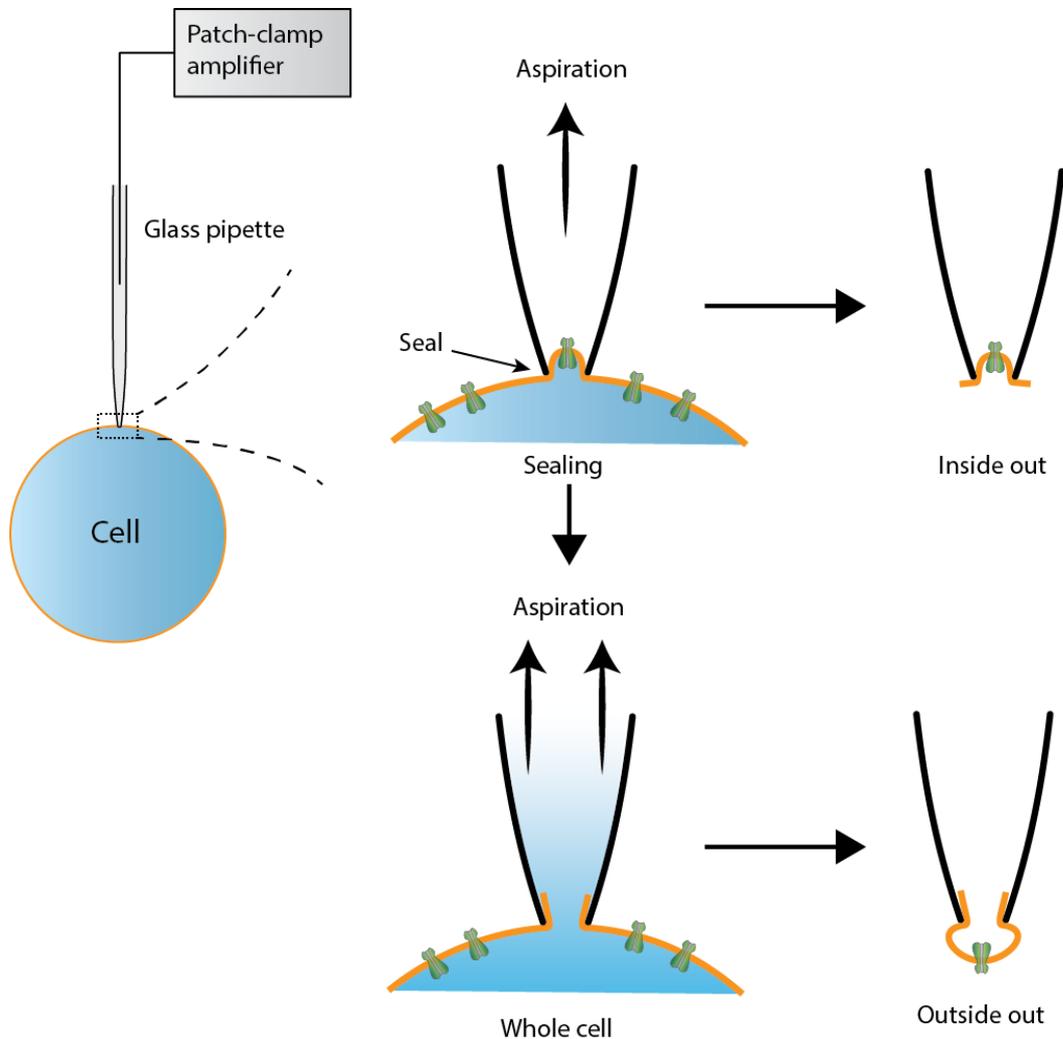


**Figure 13**. Patch clamp procedure. When the patch pipette contacts with the target cell, slight aspiration is applied to make a seal between cell membrane and the pipette tip. When a tight seal is achieved (seal resistance greater than 1GΩ), removing the pipette tears the membrane around the pipette tip. Residual membrane attached to the pipette tip reseals with the intracellular site of the membrane exposes to the environment ("inside out" configuration). Applying lower aspiration pressure (higher force on the membrane) instead of removing the pipette can rupture the cell membrane within the pipette tip and the whole cell patch clamp configuration is achieved. Removing the pipette at whole cell configuration results in an "outside out" configuration.

electrophysiological properties of the whole cell membrane. In addition to the whole cell recording, there are two configurations that can measure single channel activities. As shown in Figure 13, when the whole cell configuration is obtained, the pipette is gently pulled away from the cell. As a result, the membrane around the pipette breaks and reseals to form a new patch, which constitutes the *outside out configuration*. The i*nside out configuration*, where a piece of membrane is extracted from the membrane without inversion, is technically more difficult to establish. Pulling of the pipette can break the membrane around the pipette, but subsequent resealing creates a vesicle, which is not desirable. However, in this case the outward facing membrane can be ruptured by air exposure, which can be achieved by briefly lifting the pipette tip out of the bath solution. Outside out and inside out patching configurations provide access to single channel activity by exposing the extracellular and intracellular site of the single channel, respectively, to different stimuli.

### 6.1.2 Whole cell recording

The whole cell configuration is obtained by breaking the cell membrane under the pipette so that the electrode in the pipette has direct electrical contact with the cytoplasm. The equivalent circuit for the cell configuration is showed in Figure 14. The series circuit consists of the pipette resistance $R_{pipette}$, the access resistance $R_{access}$ and the membrane resistance $R_m$. The sum of $R_{access}$ and $R_{pipette}$ is sometimes referred to a series resistance ($R_{series} = R_{access} + R_{pipette}$). The membrane resistance is the largest current limiting resistor, so this configuration allows the observation of current through $R_m$. Because this current is the sum of currents through all activated ion channels in the membrane, it is named *whole-cell current* or *macro-current*.

The membrane capacitance imparts a delay on potential change of the membrane (stored charge), so the membrane capacitance is a limiting factor in action potential propagation speed. Similarly in whole-cell recording, the membrane capacitance affects the voltage clamp time characteristics, and any change in holding potential will be delayed. There are some methods to quantify and minimize the delaying effects. The quality criteria for whole cell recording are up to the experimenter, but typical criteria could be the following:

1.      *The seal resistance ($R_{leak}$) must be better than 1 GΩ (gigaseal)*. Seal resistance should be as high as possible to minimize short-circuiting caused by the leakage of the seal.

2.      *The series resistance must be lower than 20MΩ and stay that way throughout the recording.* Voltage clamp of the cell membrane is adversely affected by the series resistance.

3.      *Cell capacitance and resistance must be stable*. Membrane and seal leaking will increases the response current.
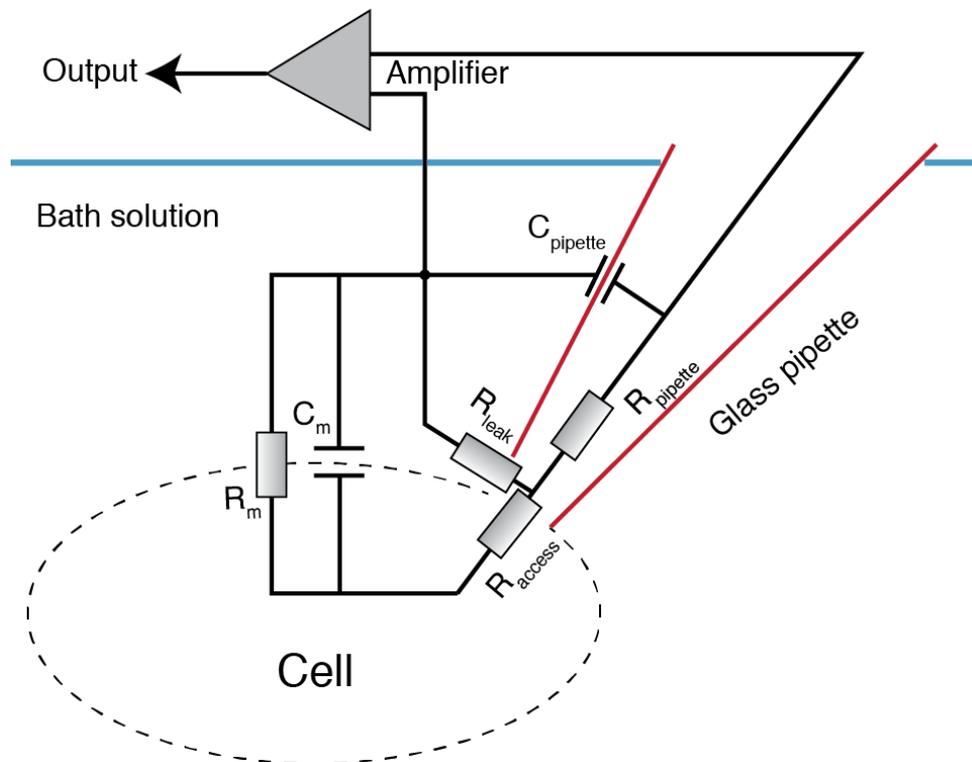


**Figure 14**. Equivalent circuit for the whole-cell recording configuration.

## 6.2 Extracellular recording

A far less demanding technique, extracellular recording, involves placing a sharp microelectrode (similar to the glass pipette in patch-clamp experiments) in close proximity to the excitable cell and the reference electrode at some location in the extracellular fluid. This arrangement records potential changes at the membrane surface rather than across the membrane, in contrast with patch clamp technique. Unlike intracellular recordings, individual EPSPs cannot be recorded by extracellular recording, since the current activity dramatically decreases at the distance away from the membrane, where the recording pipette is placed, and the size of the measured action potential will also be reduced significantly. Extracellular techniques are therefore better suited to record the activity of an entire population of cells, where the sum of the simultaneous activated individual EPSPs can easily be detected and measured by extracellular recording, which is a commonly used electrophysiological technique in the neurological studies.

The general electrical circuitry is illustrated in figure 15, where the microelectrode is placed in a neural tissue. A reference electrode is also present in the solution where the tissue is placed in. The local current generated by the activation of ion channels causes a large change in electrical potential between the extracellular medium and the interior. The induced current between the interior of the recording electrode and the extracellular medium, due to the potential change, changes the potential difference between recording electrode and reference electrode, which is then measured by the voltage meter. The measured signal from the voltage meter represents the sum of the synaptic activities close to the recording electrode. Since the recording electrode is placed in the extracellular space, the membrane activity of any adjacent neuron has the potential to be recorded. The tip size of the recording pipette determines the precision of the recording. When the tip size is about 1µm, the recoding pipette can detect activity of single neuron, which is called *single-unit recording*. A larger recording pipette is able to record simultaneous activities of several neurons, which is often referred to as *multi-unit recording*, which can involve up to thousands of cells. Even though the potential change due to single synaptic activation is too small to be detected, the *local field potential*, which is the synaptic activities within a volume of tissue, can easily be measured extracellularly and used for studying synaptic activity.

The extracellular recording technique is comparatively easy to apply, and causes much less damage to the cells. Due to the averaging over many individual signals, the extracellular recording technique can only be used to indicate the generation the action potential and activation of the synapses, but not for obtaining precise and detailed dynamics and kinetics. Moreover, the presence of a cell population which can be simultaneously activated is of importance, thus the extracellular recording is not suitable for single cell analysis.
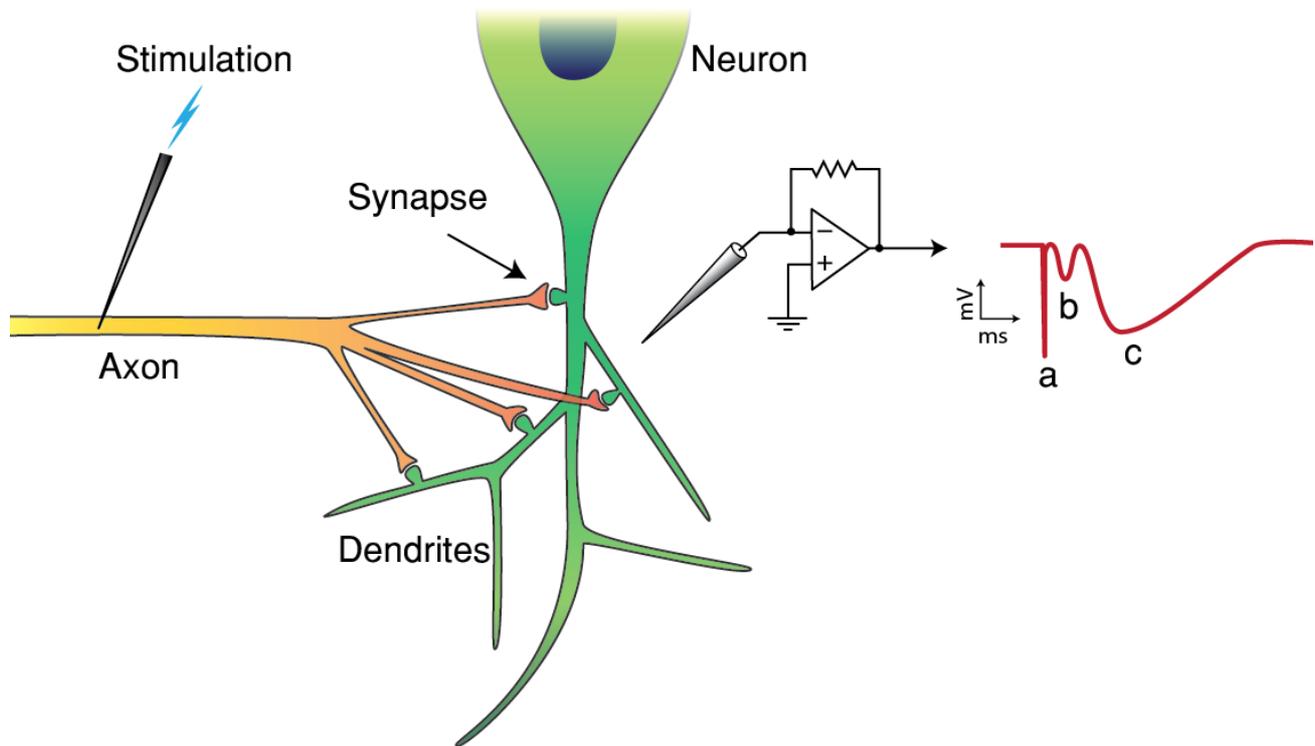
**Figure 15**. The extracellular recording. The synaptic activity due to the stimulated axon is measured with a recording pipette placed in the vicinity of the cell membrane. The recorded signal consists of three parts: (a) is the artifact due to the stimulation current; (b) is the presynaptic activation due to the stimulation current generated action potential; (c) is the excitatory postsynaptic potential.

# 7. Rat brain slice preparation

*In vitro* brain slices has become a powerful experimental model system for neurological studies due to its preserved original neural network. Using brain slices is especially valuable in studying specific mechanism of drugs, since the slice environment can easily be controlled and manipulated, *i.e.*, the content and the temperature of the extracellular solution. The preparation of rat brain slices is the first and also the most critical step for conducting a successful experiment. Since the brain obviously undergoes severe damage during the cutting of slices, the excellence of the preparation process directly determines the viability of the slices and so the experimental quality. Therefore, the slice preparation process must be well performed.

Figure 16 is a general illustration of the rat brain slice preparation process used in this thesis work. The rat is first anesthetized with inhalation anesthetics (*i.e.,* isoflurane) and decapitated. The brain is then quickly but carefully removed from the scull and placed in a cold, pre-oxygenated physiological buffer solution (pH 7.4) with low calcium (0.5 mM) and high magnesium content (6 mM). The combination of low calcium and high magnesium content together with decreased enzyme activity as a result of the low temperature, protect the tissue from further damage. After a short cooling time (30s to 1min), the brain is taken out of the buffer solution and placed in the slicing device, for example a tissue chopper or a vibrating microtome. In general, the basic structural outline of the brain slices is visible to the naked eyes. Therefore, the desired section of the brain can be observed referring to the rat brain atlas, while slicing, without the aid of microscope. The thickness of the slices is an important factor for the experiment. The thicker slices remain better cellular network, which has better correlation with the original structure in the brain. But the optical transparency of the thick slices (> 500 μm) is not optimal for many experiments (*i.e.,* electrophysiological studies). Additionally, the anaerobic glycolysis and the accumulation of lactate, due to the insufficient oxygen supply to the central layers of the slices, have potential injury to the slices and decrease the viability [147, 148]. Numbers of dual-superfusion chambers have been developed for improving the viability of thick brain slices, where the slices are effectively superfused with solution on both the top and bottom surfaces [10, 149, 150]. Since the chambers are closed, they are not compatible with electrophysiological techniques. Thin slices (< 200 μm), on the other hand, have better optical transparency, improved nutritional supply and waste removal, but the reduced cell content makes them less relevant to their original *in vivo* structure and function. Electrophysiological measurement of the slices also becomes difficult due to the reduced measurable cell population. The thickness of the slices used in this thesis work was 400 μm, which had good optical transparency, sufficient

oxygen supply. The thickness of about 10-15 cell layers (400 μm) also made it suitable for electrophysiological studies.
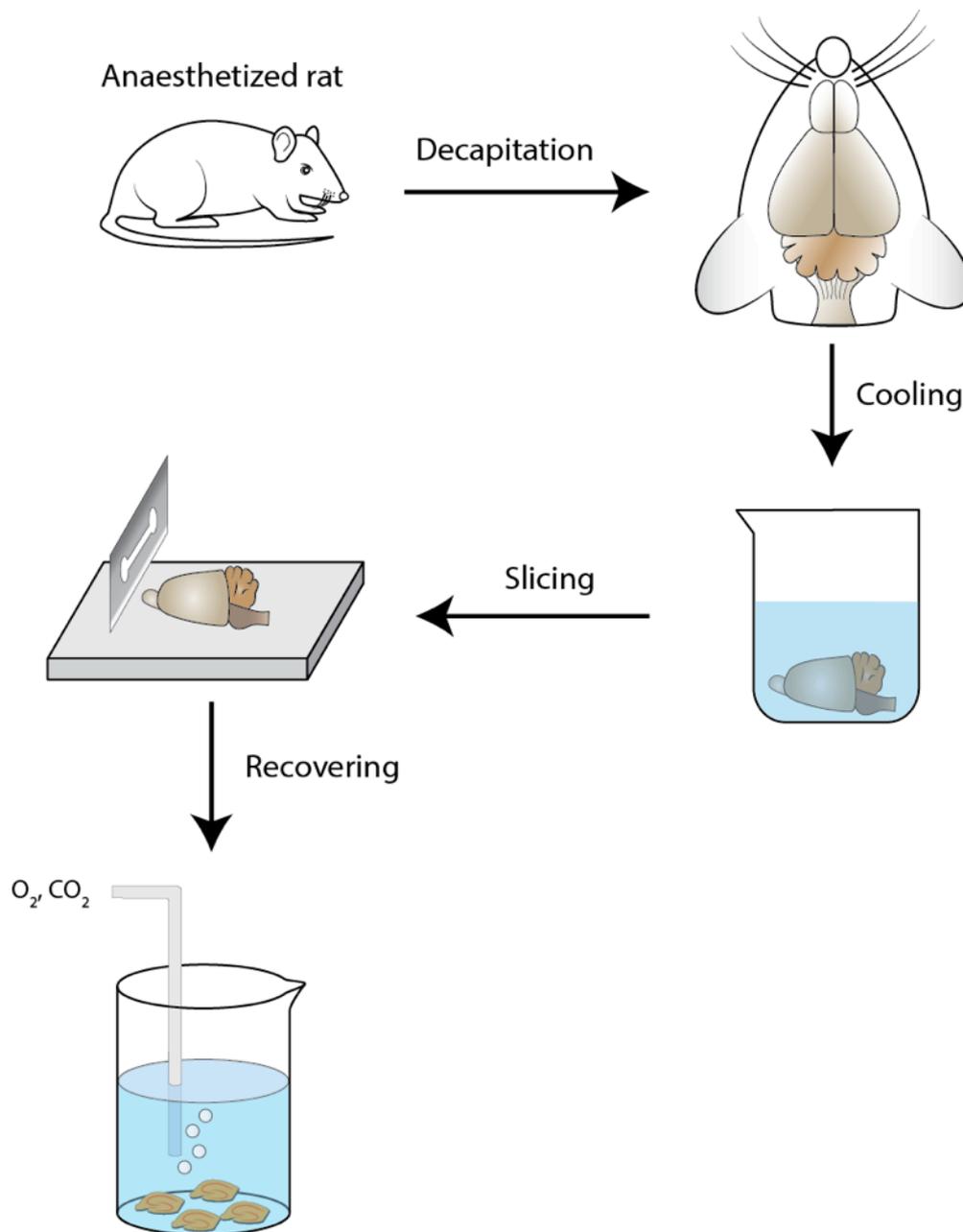


**Figure 16**. The brain slice preparation process. The rat is anaesthetized with inhalation anesthetics. The rat brain is immediately isolated form the skull and place into a cold and oxygen saturated buffer solution. The cooled brain is cut into slices and place in continuously oxygenated buffer solution for recovery.

The sliced tissue samples are placed in a holding chamber with continuously oxygenated physiological buffer solution at room temperature for recovery. The magnesium concentration is remained, while the calcium concentration is increased to 2mM, which is similar to the physiological calcium concentration in the brain [151]. After at least 90 min of recovery, the slices were transferred into a recording chamber where they were fixed by a net in a continuously superfusing physiological buffer solution saturated with oxygen. Since the buffer in the recording chamber is usually controlled at higher temperature than the recovery chamber to represent the physiological conditions, an adaptation period (15-30min) is needed for the slices before the experiments. Note that 1.8g/L (10 mM) glucose is added in all the physiological buffer solutions used above. The key role of glucose in the body is energy provision, and the brain depends on a sufficient glucose supply to operate normally [152].

In general, the basic steps of different brain slice preparation techniques are similar, including anesthetizing the animal, isolating the brain and preparing the slices. The major difference is in the composition of the cutting solutions. For example, replacing the NaCl in the cutting solution with an equimolar concentration of sucrose has showed great value of minimizing the excitotoxic damage during slice preparation [153]. Some laboratories have also tried choline-chloride [154] and different glutamate receptor antagonists [155, 156], with excellent results.

The viability of the slices during the experiment is mostly dependent on the maintenance of the physiological environment and the experimental stimulations. The lifespan of an acute brain slice is approximately 6–12 hours [157], while chemical stimulations can accelerate the cell death due to cell toxicity. Thus, the application of bioactive compounds via whole slice perfusion stresses the whole slice significantly, while localized superfusion via microfluidic devices stimulates only a small region and keeps all other parts of the slice unaffected, which introduces less stress on the viability of the slice, and increases reusability of the sample. Compared with the whole slice perfusion chamber, more consecutive experiments can be performed on a single slice, which reduces the number of laboratory animals to be sacrificed for a study.

# 8. Microscopy

Microscopy is a fundamental optical technique for magnifying and studying small objects, which are not visible by the naked eye. Microscopy can be used as either a visualizing tool for assisting the operation, e.g. patch clamp, or a direct measurement technique (fluorescent microscopy). In this thesis work, laser scanning confocal microscopy was used for determining the cholesterol content of the cell membrane with the fluorescent dye Filipin, which selectively binds to membrane-intrinsic cholesterol [158] and for measuring the TRPV1 ion channel permeability with the DNA stain YO-PRO, the uptake of which was controlled by the TPRV1 ion channel activity of the investigated cells.

## 8.1 Fluorescence

Fluorescence is the emission of light by certain molecules (fluorophores or fluorescent dyes) that has absorbed light. A simplified Jablonski diagram of the multi-stage fluorescence process is shown in figure 17. Stage 1 is the excitation event, where the fluorophore absorbs a photon of defined energy, supplied by an external light source source such as a laser, and creates an excited electronic singlet state ($S_1'$). Stage 2 is the excited state that typically lasts for 1–10 nanoseconds. During that ime the electron in the excited state ($S_1'$) relaxes to the lowest vibrational level ($S_1$), from which fluorescence emission originates. The emission event is the third stage of the process, where the fluorophore returns to its ground state $S_0$ by emitting a photon. Due to energy dissipation during the excited-state lifetime, the energy of the emitted photon is lower, and therefore of longer wavelength, than the excitation photon (Stokes shift). Fluorescence emission is not the only available means for the excited molecules to return to the ground state ($S_0$). There are several other processes, which are omitted in the figure for clarity. The fluorescence quantum yield, which is the ratio of the number of fluorescence photons emitted (Stage 3) to the number of photons absorbed (Stage 1), is a measure of the relative extent to which these competing processes occur [159].

Fluorescence has been widely used in biological studies, and a large number fluorophores has been developed, and are commercially available for specific purposes. For instance, fluorophores can be used as a reporter for gene expression [160], a specific ion detector [161], or a membrane probe [162]
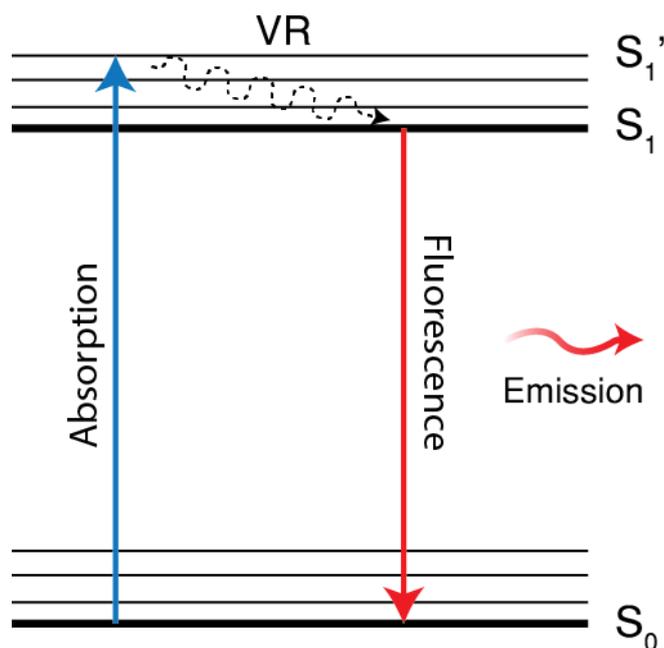
**Figure 17**. Simplified Jablonski diagram for the Fluorescence process. The electronic states S, depicted by horizontal lines, are energy levels, between which crossover is possible by and uptake (excitation) and release (emission) of photonic energy. Processes typically competing with the emission process are not shown.

## 8.2 Laser scanning confocal microscopy

Laser scanning confocal microscopy (LSCM) is an imaging technique widely used in biological research, *e.g.*, cell biology, genetic, microbiology and protein analysis. LSCM is a technique for obtaining high-resolution optical images with depth selectivity by limiting the field of view with apertures (pinholes). The sample is laterally scanned by using a point laser source and a point detector. The intensity values are acquired point-by-point and reconstructed with a computer to form an image. Figure 18 illustrates the basic principle of LSCM, where a laser beam passes through a light source aperture and is then focused by the objective lens onto a selected plane in the specimen to excite the fluorophores therein.
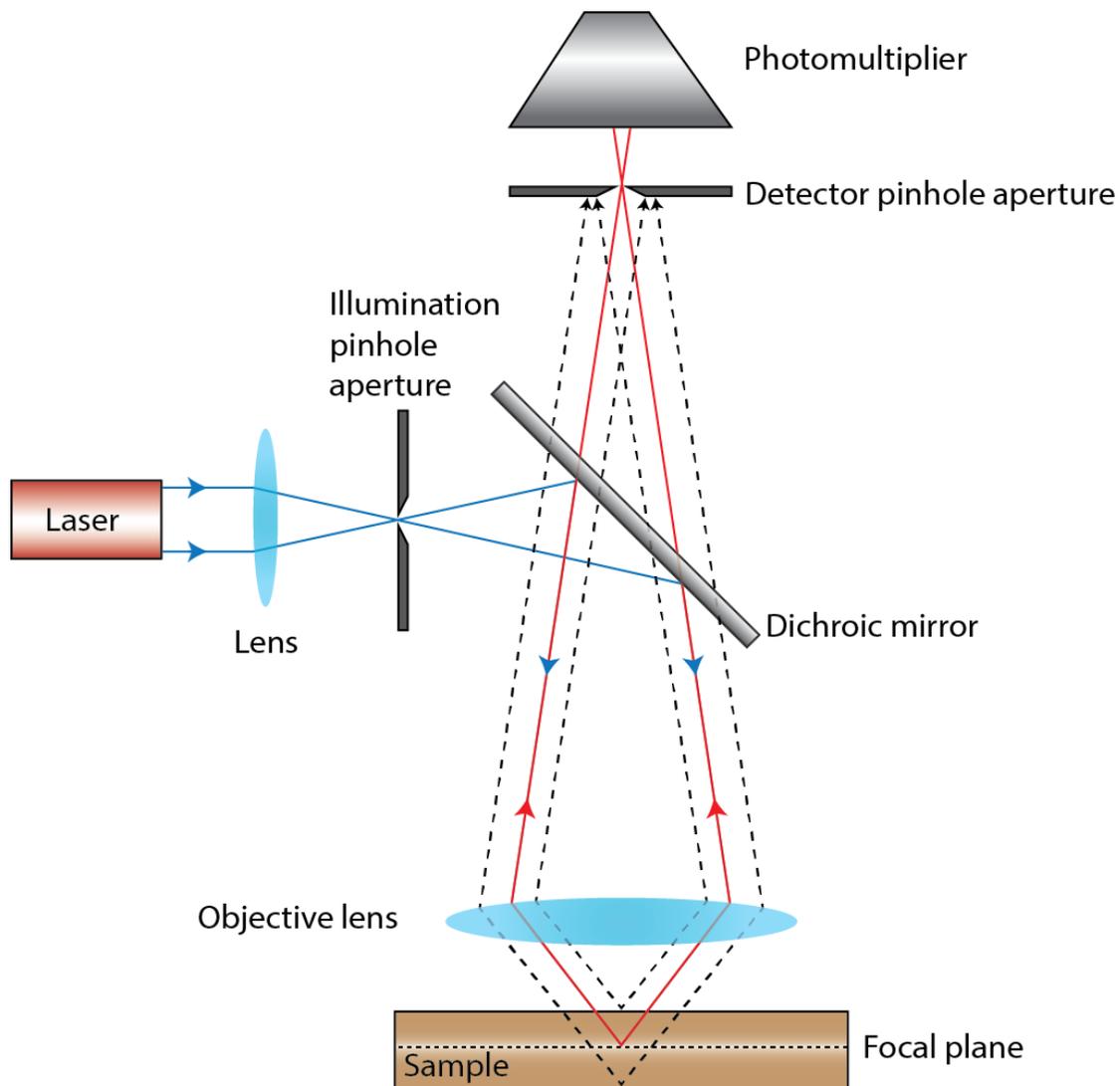
**Photomultiplier**

**Detector pinhole aperture**

**Illumination pinhole aperture**

**Laser**

**Lens**

**Dichroic mirror**

**Objective lens**

**Sample**

**Focal plane**

**Figure 18**. Fluorescence confocal microscopy. Only light form the focal plane can pass through the detector pinhole aperture and reaches the detector, while lights from other parts of the sample are scattered on the aperture wall. The red arrows represent light emitted from the focal plane, which reach the detector. Out-of-plane emission (dash lines) is rejected by the detector pinhole.

Fluorescent light originating from the illuminated spot together with any scattered and reflected laser light is then re-collected by the objective lens. A dicroic mirror separates incident light from emission, which passes through and reaches the detector through an exit pinhole, where only the light from the focused plane can pass through, while the light originating from out-of-plane emission from the sample is blocked by the aperture wall. The photodetection device is usually a photomultiplier tube (PMT) or an avalanche photodiode, which convert the detected photons into an intensity value of a pixel that is recorded by a computer. Three-dimensional image of the sample can

*8.2 Laser scanning confocal microscopy*

be obtained by LSCM, if the focal plane is systematically shifted along the axis of the objective lens, and the series of 2D recordings are finally combined.

# 9. Summary of results

The three papers that comprise this thesis have a common theme: the multifunctional pipette, a new microfluidic device, was employed to administer various active chemical compounds to biological tissue. This includes cell lines with artificially expressed human nociceptive ion channels (Paper 1) as well as acutely prepared rat brain slices, either from the prefrontal cortex (Paper 2) or the hippocampus (Papers 2 and 3). Intracellular recording was performed by the whole cell patch clamp method (Paper 1) or by means of conventional sharp microelectrodes (Paper 2). Extracellular field potentials were used to monitor glutamatergic synaptic transmission in brain slices and its blockade by a specific antagonist (Papers 2 and 3). In addition to the electrophysiological recording, high resolution imaging was employed via fluorescence confocal microscopy to study uptake of a specific marker in single cells (Paper 1).

**Paper I**

The TRPV1 is a nociceptive ion channel, which is widely expressed in both peripheral and central nervous system. Its importance in inflammatory pain sensing (heat hyperalgesia) and involvement in mediation of LTD indicates that pharmacological modulation of its function may offer valuable therapeutic treatment not only of pain but also for psychological conditions such as anxiety. The aim of this study was to investigate the effect of cholesterol depletion of plasma membrane on ion-permeability of the TRPV1 ion channel. The ion-permeability properties of TRPV1 were investigated with whole-cell patch-clamp and YO-PRO uptake rate studies using laser scanning confocal microscopy on a Chinese hamster ovary (CHO) cell line expressing this ion channel. The TRPV1 exhibits dynamic ion selectivity, which is time and concentration dependent permeability of the ion channel to large cations. The dynamic permeability was used as the indicator for the pore dilation of TRPV1. The depletion of the cholesterol in the plasma membrane was found to impair the dynamic permeability to large cations N-methyl-D-glucamine (NMDG) and fluorescent dye YO-PRO during sustained activation with capsaicin and protons (pH 5.5) under hypocalcemic conditions. We found that the depletion level of cholesterol required for decreasing the permeability of TRPV1 to large cations is depended on the type of the agonist, as depletion of cholesterol by 35% was sufficient to significantly decrease the permeability under activation with $1\mu M$ capsaicin, whereas, when protons (pH5.5) was used as agonist, 54% depletion of cholesterol was needed for inhibiting

the dynamic permeability of TRPV1. These results propose a novel mechanism of cellular cholesterol-depletion depended modulation of TRPV1 permeability, which may provide a novel pharmacological target for alleviating neuropathic pain and improving cognitive behavior.

**Paper II**

*In vitro* brain slices constitute a powerful experimental model system for exploring neuronal function at the network, cellular, synaptic, and single channel levels, and studying pharmacological effects on neurons and neuronal networks related to symptoms in diseases of the brain in an environment which preserves the cellular network, such as the organization in cell layers. Among the various fluidic control technologies, microfluidic devices are becoming powerful tools for pharmacological studies using brain slices, since these devices overcome traditional limitations of conventional submerged slice chambers, and provide better spatiotemporal control over drug delivery to specific regions in the slices. The main focus of this paper is to introduce a multifunctional pipette (MFP), which we have recently developed, to brain slice studies. The MFP, which is a novel free-standing hydrodynamically confined flow (HFC) based microfluidic device, allows diverse superfusion experiments, such as testing the effects of different concentrations of drugs or drug candidates on neurons in different cell layers with high positional accuracy, affecting only a small number of cells. We have demonstrated the use of the method with electrophysiological recordings of pyramidal cells in prefrontal cortex brain slices from rats and determined the spatial and temporal characteristics of the superfusion, which included a three-fold gain in solution exchange time, a well-defined superfusion region on the slice specimen, and greatly reduced reagent consumption.

**Paper III**

The advantages in using the MFP for brain slice studies have been successful demonstrated in paper II. In paper III, we have characterized the ability of the MFP for highly localized perfusion of drugs, using hippocampal slices from rats. The hippocampus, with its important role in memory and learning and special anatomical structure (*i.e.,* distinctly separated layers), has not only been intensely used as a model for pharmacological studies, but has also become a useful platform for

developing new technologies in neuroscience research. The MFP demonstrated the ability to selectively perfuse one dendritic layer in the CA1 region of hippocampus with a competitive AMPA receptor antagonist, CNQX, while not affecting the other layers in this region. The spatial profile measurement showed the benefits of the hydrodynamic confinement in sharpening the spatial selectivity in brain slice experiments. The concentration-response curve revealed the efficient drug delivery capability of the new microfluidic device, which is comparable with that of whole slice perfusion. However, the required drug amounts per experiment were, in comparison to conventional bath superfusion, smaller by several orders of magnitude.

## 10. Concluding remarks

This thesis work investigates the application of microfluidic devices on the electrophysiological studies of cell communication, which is conducted by the activation of ion channels. The work has been mainly focused on studying the effect of cholesterol on dynamic ion permeability of the TRPV1 ion channel and the application of the multifunctional pipette on the brain slice studies.

Cholesterol content in the plasma membrane has showed important roles in the functioning of the TRPV1 ion channel, which is a nociceptor ion channel and involved in the transmission and modulation of pain, as well as the integration of diverse painful stimuli [49]. In this work, the effect of the membrane cholesterol on pore dilation of the TRPV1 ion channel has been studied for contributing valuable data on better understanding of its role in pain, which may provide therapeutic values. We have discovered that depletion of the membrane cholesterol reduced the dynamic ion permeability of the ion channel activated by different agonists, which implies that cholesterol can be a potential pharmaceutical target for pain.

In the brain slice studies, highly localized delivery of active solutions has become one of the major goals of designing microfluidic devices for the field. Localized drug administration on brain slices may reveal finer details of the mechanism of drug action, since the neural networks have different connections and functions in different regions of the slice. For example, one pyramidal neuron cell in the CA1 region of the hippocampus receives axonic projections at different parts of the dendrites from totally distinct brain regions [163]. In this context, the multifunctional pipette has been introduced as superfusion device in brain slice studies. The multifunctional pipette is a freestanding, microfluidic device, which utilized hydrodynamically confined flow to achieve well-controlled and highly localized solution exchange. We developed an experimental setup, performed feasibility studies, characterized device performance and compared it with common superfusion techniques. With the advantages of a high-resolution spatiotemporal profile, efficient drug delivery capability and reduced reagent consumption, the multifunctional pipette has showed great potential and value in advanced pharmacological studies on brain slices. The strictly controlled localized drug administration ability of the multifunctional pipette improves the usability of the slice by allowing more data to be collected from a single tissue. As a result, the tissue culture expenses can be reduced significantly, which reduces the number of laboratory animals to be sacrificed for a study. Our research has opened pathways to more advanced experiments in future studies, where we will use the

multifunctional pipette in various pharmacological studies not only on brain slices, but on any tissues where localized delivery of the drugs is desirable.

As a newly introduced concept, improving of the device and optimizing of the method is necessary. For example, the fabrication material PDMS is less compatible with organic solvents, and the absorption to certain drugs happens. The PDMS can be replace by some other materials with different physical and chemical properties such as silicon, glass and various types of plastics, which may introduces more complicated fabrication process, but the pipette will benefit with better solvent compatibility, reduced drug absorption. Other improvements can also be achieved by changing the fabrication material, including reduced channel size, which introduces smaller footprint and further improve the spatial resolution of the multifunctional pipette. Additionally, the quality of the slices can be improved by using more advanced slicing techniques. For example, comparing with slice chopper and vibrating microtome used in this work, the laser microtome has reduced interaction zone of the cut and introduces much less damage to the tissue during the slicing process, resulting in smoother cutting surface and better slice viability.

Open volume microfluidics, and particularly the HCF variant of it, is a promising technological development, which has the potential to greatly benefit neuropharmacological studies on brain slices. In particular freestanding devices, which can be easily moved to target selective regions on a specimen, allow for new creative approaches, which are not possible in conventional perfusion chambers. The multifunctional pipette has the ability to provide the neurosciences with extended experimental opportunities, which are greatly needed to address the increasing occurrence of neurodegenerative diseases in our aging society.

# 11. Acknowledgment

# 12. References

1. Nicolson, G.L., *The Fluid-Mosaic Model of Membrane Structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more than 40years.* Biochim Biophys Acta, 2014. **1838**(6): p. 1451-1466.

2. Almen, M.S., et al., *Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin.* BMC Biol, 2009. **7**: p. 50.

3. Alberts, B., J.H. Wilson, and T. Hunt, *Molecular biology of the cell.* 5th ed. 2008, New York: Garland Science. 1601, 90 p.

4. Shepherd, G.M., *Neurobiology.* 3rd ed. 1994, New York: Oxford University Press. 760 p.

5. Tamamaki, N., K. Abe, and Y. Nojyo, *Columnar organization in the subiculum formed by axon branches originating from single CA1 pyramidal neurons in the rat hippocampus.* Brain Res, 1987. **412**(1): p. 156-60.

6. Andersen, P., *The hippocampus book.* 2007, Oxford ; New York: Oxford University Press. xx, 832 p.

7. Huang, Y., J.C. Williams, and S.M. Johnson, *Brain slice on a chip: opportunities and challenges of applying microfluidic technology to intact tissues.* Lab Chip, 2012. **12**(12): p. 2103-17.

8. Tabeling, P., *Introduction to microfluidics.* 2005, Oxford, U.K. ; New York: Oxford University Press. 301p.

9. Holmes, D. and S. Gawad, *The application of microfluidics in biology.* Methods Mol Biol, 2010. **583**: p. 55-80.

10. Blake, A.J., et al., *Multilayer PDMS microfluidic chamber for controlling brain slice microenvironment.* Lab Chip, 2007. **7**(7): p. 842-9.

11. Christ, K.V. and K.T. Turner, *Design of hydrodynamically confined microfluidics: controlling flow envelope and pressure.* Lab Chip, 2011. **11**(8): p. 1491-501.

12. Kaigala, G.V., et al., *A vertical microfluidic probe.* Langmuir, 2011. **27**(9): p. 5686-93.

13. Litovsky, B. and S. Arancibia, *["Push-pull" perfusion technic, a new approach applied to the study of transdentinal fluid].* Actual Odontostomatol (Paris), 1990. **44**(169): p. 153-64.

14. Nge, P.N., C.I. Rogers, and A.T. Woolley, *Advances in microfluidic materials, functions, integration, and applications.* Chem Rev, 2013. **113**(4): p. 2550-83.

15. Ainla, A., et al., *Single-cell electroporation using a multifunctional pipette.* Lab Chip, 2012. **12**(22): p. 4605-9.

16. Ainla, A., et al., *A multifunctional pipette.* Lab Chip, 2012. **12**(7): p. 1255-61.

17. Ahemaiti, A., et al., *A multifunctional pipette for localized drug administration to brain slices.* J Neurosci Methods, 2013. **219**(2): p. 292-6.

18. Molleman, A., *Patch clamping : an introductory guide to patch clamp electrophysiology : Areles Molleman*. 2003, Chichester: J. Wiley. x, 175 p.

19. Yeagle, P.L., *Lipid regulation of cell membrane structure and function*. FASEB J, 1989. **3**(7): p. 1833-42.

20. Goldstein, S.A., et al., *International Union of Pharmacology. LV. Nomenclature and molecular relationships of two-P potassium channels*. Pharmacol Rev, 2005. **57**(4): p. 527-40.

21. Ren, D., *Sodium leak channels in neuronal excitability and rhythmic behaviors*. Neuron, 2011. **72**(6): p. 899-911.

22. Aidley, D.J. and P.R. Stanfield, *Ion channels : molecules in action*. 1996, Cambridge: Cambridge University Press. 307p.

23. Ashcroft, F.M., *Ion channels and disease : channelopathies*. 2000, San Diego, Calif. ; London: Academic. 481p.

24. Jozwiak, Z. and A. Marczak, *[The role of ion channels in apoptosis]*. Postepy Biochem, 2006. **52**(4): p. 373-82.

25. Ye, S., et al., *Crystal structures of a ligand-free MthK gating ring: insights into the ligand gating mechanism of K+ channels*. Cell, 2006. **126**(6): p. 1161-73.

26. Tosteson, M.T., et al., *Primary structure of peptides and ion channels. Role of amino acid side chains in voltage gating of melittin channels*. Biophys J, 1990. **58**(6): p. 1367-75.

27. Kurata, H.T., et al., *Voltage-dependent gating in a "voltage sensor-less" ion channel*. PLoS Biol. **8**(2): p. e1000315.

28. Ruffert, S., et al., *Identification of mechanosensitive ion channels in the cytoplasmic membrane of Corynebacterium glutamicum*. J Bacteriol, 1999. **181**(5): p. 1673-6.

29. Gu, L., W. Liu, and B. Martinac, *Electromechanical coupling model of gating the large mechanosensitive ion channel (MscL) of Escherichia coli by mechanical force*. Biophys J, 1998. **74**(6): p. 2889-902.

30. Montell, C., *Physiology, phylogeny, and functions of the TRP superfamily of cation channels*. Sci STKE, 2001. **2001**(90): p. re1.

31. Clapham, D.E., *TRP channels as cellular sensors*. Nature, 2003. **426**(6966): p. 517-24.

32. Montell, C., *The TRP superfamily of cation channels*. Sci STKE, 2005. **2005**(272): p. re3.

33. Nilius, B. and T. Voets, *TRP channels: a TR(I)P through a world of multifunctional cation channels*. Pflugers Arch, 2005. **451**(1): p. 1-10.

34. Harteneck, C., *Function and pharmacology of TRPM cation channels*. Naunyn Schmiedebergs Arch Pharmacol, 2005. **371**(4): p. 307-14.

35. Nagata, K., et al., *Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing*. J Neurosci, 2005. **25**(16): p. 4052-61.

36. Dhaka, A., V. Viswanath, and A. Patapoutian, *Trp ion channels and temperature sensation*. Annu Rev Neurosci, 2006. **29**: p. 135-61.

37. Sokabe, T. and M. Tominaga, *A temperature-sensitive TRP ion channel, Painless, functions as a noxious heat sensor in fruit flies*. Commun Integr Biol, 2009. **2**(2): p. 170-3.

38. Patapoutian, A., et al., *ThermoTRP channels and beyond: mechanisms of temperature sensation.* Nat Rev Neurosci, 2003. **4**(7): p. 529-39.

39. Ro, J.Y., J.S. Lee, and Y. Zhang, *Activation of TRPV1 and TRPA1 leads to muscle nociception and mechanical hyperalgesia.* Pain, 2009. **144**(3): p. 270-7.

40. Caterina, M.J., et al., *The capsaicin receptor: a heat-activated ion channel in the pain pathway.* Nature, 1997. **389**(6653): p. 816-24.

41. Welch, J.M., S.A. Simon, and P.H. Reinhart, *The activation mechanism of rat vanilloid receptor 1 by capsaicin involves the pore domain and differs from the activation by either acid or heat.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13889-94.

42. Tominaga, M., et al., *The cloned capsaicin receptor integrates multiple pain-producing stimuli.* Neuron, 1998. **21**(3): p. 531-43.

43. Hellwig, N., et al., *TRPV1 acts as proton channel to induce acidification in nociceptive neurons.* J Biol Chem, 2004. **279**(33): p. 34553-61.

44. Chung, M.K., A.D. Guler, and M.J. Caterina, *TRPV1 shows dynamic ionic selectivity during agonist stimulation.* Nat Neurosci, 2008. **11**(5): p. 555-64.

45. Virginio, C., et al., *Pore dilation of neuronal P2X receptor channels.* Nat Neurosci, 1999. **2**(4): p. 315-21.

46. Chen, J., et al., *Pore dilation occurs in TRPA1 but not in TRPM8 channels.* Mol Pain, 2009. **5**: p. 3.

47. Clapham, D.E., L.W. Runnels, and C. Strubing, *The TRP ion channel family.* Nat Rev Neurosci, 2001. **2**(6): p. 387-96.

48. Long, S.B., E.B. Campbell, and R. Mackinnon, *Crystal structure of a mammalian voltage-dependent Shaker family K+ channel.* Science, 2005. **309**(5736): p. 897-903.

49. Cui, M., et al., *TRPV1 receptors in the CNS play a key role in broad-spectrum analgesia of TRPV1 antagonists.* J Neurosci, 2006. **26**(37): p. 9385-93.

50. Jara-Oseguera, A., S.A. Simon, and T. Rosenbaum, *TRPV1: On the Road to Pain Relief.* Curr Mol Pharmacol, 2008. **1**(3): p. 255-69.

51. Martins, D., I. Tavares, and C. Morgado, *"Hotheaded": The role OF TRPV1 in brain functions.* Neuropharmacology, 2014. **85C**: p. 151-157.

52. Herculano-Houzel, S., *The human brain in numbers: a linearly scaled-up primate brain.* Front Hum Neurosci, 2009. **3**: p. 31.

53. Thompson, R.F., *The brain : a neuroscience primer.* 2nd ed. A Series of books in psychology. 1993, New York: W.H. Freeman & Co. 475 p.

54. Siegel, A., H.N. Sapru, and H. Siegel, *Essential neuroscience.* Third edition. ed. p.

55. Kuffler, S.W. and J.G. Nicholls, *From neuron to brain : a cellular approach to the function of the nervous system.* 1976, Sunderland, Mass.: Sinauer Associates. 486 p.

56. Bear, M.F., B.W. Connors, and M.A. Paradiso, *Neuroscience : exploring the brain.* 3rd ed. 2007, Philadelphia, PA: Lippincott Williams & Wilkins. 857 p.

57. Schneggenburger, R. and E. Neher, *Presynaptic calcium and control of vesicle fusion.* Curr Opin Neurobiol, 2005. **15**(3): p. 266-74.

58. Tigges, M. and J. Tigges, *Presynaptic dendrite cells and two other classes of neurons in the superficial layers of the superior colliculus of the chimpanzee.* Cell Tissue Res, 1975. **162**(3): p. 279-95.

59. Bennett, M.V., et al., *Physiology and ultrastructure of electrotonic junctions. IV. Medullary electromotor nuclei in gymnotid fish.* J Neurophysiol, 1967. **30**(2): p. 236-300.

60. Auerbach, A.A. and M.V. Bennett, *A rectifying electrotonic synapse in the central nervous system of a vertebrate.* J Gen Physiol, 1969. **53**(2): p. 211-37.

61. Stanley, E.F., *The calcium channel and the organization of the presynaptic transmitter release face.* Trends Neurosci, 1997. **20**(9): p. 404-9.

62. Bliss, T.V. and T. Lomo, *Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path.* J Physiol, 1973. **232**(2): p. 331-56.

63. Landfield, P.W. and S.A. Deadwyler, *Long-term potentiation from biophysics to behavior.* Neurology and neurobiology. 1988, New York: Liss. 548 p.

64. Wigstrom, H., F. Asztely, and M.Y. Xiao, *Associative Properties of Long-Term Potentiation.* Memory Concepts - 1993, 1993. **1036**: p. 185-202.

65. Dunwiddie, T. and G. Lynch, *Long-Term Potentiation and Depression of Synaptic Responses in Rat Hippocampus - Localization and Frequency Dependency.* Journal of Physiology-London, 1978. **276**(Mar): p. 353-367.

66. Levy, W.B. and O. Steward, *Synapses as Associative Memory Elements in the Hippocampal-Formation.* Brain Research, 1979. **175**(2): p. 233-245.

67. Artola, A., et al., *Homosynaptic Long-Term Depression and Its Relationship to Long-Term Potentiation in the Rat Neocortex Invitro.* Structural and Functional Organization of the Neocortex, 1994. **24**: p. 80-91.

68. Linden, D.J., *Long-term synaptic depression in the mammalian brain.* Neuron, 1994. **12**(3): p. 457-72.

69. Baenziger, J.E. and P.J. Corringer, *3D structure and allosteric modulation of the transmembrane domain of pentameric ligand-gated ion channels.* Neuropharmacology, 2011. **60**(1): p. 116-25.

70. Masson, J., et al., *Neurotransmitter transporters in the central nervous system.* Pharmacol Rev, 1999. **51**(3): p. 439-64.

71. Shigeri, Y., R.P. Seal, and K. Shimamoto, *Molecular pharmacology of glutamate transporters, EAATs and VGLUTs.* Brain Res Brain Res Rev, 2004. **45**(3): p. 250-65.

72. Petroff, O.A., *GABA and glutamate in the human brain.* Neuroscientist, 2002. **8**(6): p. 562-73.

73. Lynch, G., L. McGaugh James, and N.M. Weinberger, *Neurobiology of learning and memory.* 1984, New York: Guilford Press. 528 p.

74. Mukherjee, S. and D. Manahan-Vaughan, *Role of metabotropic glutamate receptors in persistent forms of hippocampal plasticity and learning.* Neuropharmacology, 2013. **66**: p. 65-81.

75.    Antonelli, T., et al., *Neurotensin receptor mechanisms and its modulation of glutamate transmission in the brain: relevance for neurodegenerative diseases and their treatment.* Prog Neurobiol, 2007. **83**(2): p. 92-109.

76.    Dorval, K.M., et al., *Association of the glutamate receptor subunit gene GRIN2B with attention-deficit/hyperactivity disorder.* Genes Brain Behav, 2007. **6**(5): p. 444-52.

77.    Bitanihirwe, B.K., et al., *Glutamatergic deficits and parvalbumin-containing inhibitory neurons in the prefrontal cortex in schizophrenia.* BMC Psychiatry, 2009. **9**: p. 71.

78.    Woo, T.U., et al., *N-methyl-D-aspartate receptor and calbindin-containing neurons in the anterior cingulate cortex in schizophrenia and bipolar disorder.* Biol Psychiatry, 2008. **64**(9): p. 803-9.

79.    Gereau, R.W. and G. Swanson, *The glutamate receptors.* The receptors. 2008, Totowa, N.J.: Humana Press. 576 p.

80.    Traynelis, S.F., et al., *Glutamate receptor ion channels: structure, regulation, and function.* Pharmacol Rev, 2010. **62**(3): p. 405-96.

81.    Stawski, P., H. Janovjak, and D. Trauner, *Pharmacology of ionotropic glutamate receptors: A structural perspective.* Bioorg Med Chem, 2010. **18**(22): p. 7759-72.

82.    Yamazaki, M., et al., *Molecular cloning of a cDNA encoding a novel member of the mouse glutamate receptor channel family.* Biochem Biophys Res Commun, 1992. **183**(2): p. 886-92.

83.    Lomeli, H., et al., *The rat delta-1 and delta-2 subunits extend the excitatory amino acid receptor family.* FEBS Lett, 1993. **315**(3): p. 318-22.

84.    Safieddine, S. and R.J. Wenthold, *The glutamate receptor subunit delta1 is highly expressed in hair cells of the auditory and vestibular systems.* J Neurosci, 1997. **17**(19): p. 7523-31.

85.    Yuzaki, M., *The delta2 glutamate receptor: 10 years later.* Neurosci Res, 2003. **46**(1): p. 11-22.

86.    Tzschentke, T.M., *Glutamatergic mechanisms in different disease states: overview and therapeutical implications -- an introduction.* Amino Acids, 2002. **23**(1-3): p. 147-52.

87.    Platt, S.R., *The role of glutamate in central nervous system health and disease--a review.* Vet J, 2007. **173**(2): p. 278-86.

88.    Dingledine, R., et al., *The glutamate receptor ion channels.* Pharmacol Rev, 1999. **51**(1): p. 7-61.

89.    Mahajan, S.S. and E.B. Ziff, *Novel toxicity of the unedited GluR2 AMPA receptor subunit dependent on surface trafficking and increased Ca2+-permeability.* Mol Cell Neurosci, 2007. **35**(3): p. 470-81.

90.    Makino, H. and R. Malinow, *AMPA receptor incorporation into synapses during LTP: the role of lateral movement and exocytosis.* Neuron, 2009. **64**(3): p. 381-90.

91.    Song, I. and R.L. Huganir, *Regulation of AMPA receptors during synaptic plasticity.* Trends Neurosci, 2002. **25**(11): p. 578-88.

92.    Gressens, P., et al., *The effects of AMPA receptor antagonists in models of stroke and neurodegeneration.* Eur J Pharmacol, 2005. **519**(1-2): p. 58-67.

93.    Li, X., et al., *Antidepressant-like actions of an AMPA receptor potentiator (LY392098).* Neuropharmacology, 2001. **40**(8): p. 1028-33.

94.    Chang, P.K., D. Verbich, and R.A. McKinney, *AMPA receptors as drug targets in neurological disease--advantages, caveats, and future outlook.* Eur J Neurosci, 2012. **35**(12): p. 1908-16.

95.    Swanson, G.T., *Targeting AMPA and kainate receptors in neurological disease: therapies on the horizon?* Neuropsychopharmacology, 2009. **34**(1): p. 249-50.

96.    Lee, K.H., J.Y. Park, and K. Kim, *NMDA receptor-mediated calcium influx plays an essential role in myoblast fusion.* FEBS Lett, 2004. **578**(1-2): p. 47-52.

97.    Lisman, J., *A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory.* Proc Natl Acad Sci U S A, 1989. **86**(23): p. 9574-8.

98.    Dalton, G.L., et al., *NMDA GluN2A and GluN2B receptors play separate roles in the induction of LTP and LTD in the amygdala and in the acquisition and extinction of conditioned fear.* Neuropharmacology, 2012. **62**(2): p. 797-806.

99.    Rammes, G., et al., *Isoflurane anaesthesia reversibly improves cognitive function and long-term potentiation (LTP) via an up-regulation in NMDA receptor 2B subunit expression.* Neuropharmacology, 2009. **56**(3): p. 626-36.

100.   Bartlett, T.E., et al., *Differential roles of NR2A and NR2B-containing NMDA receptors in LTP and LTD in the CA1 region of two-week old rat hippocampus.* Neuropharmacology, 2007. **52**(1): p. 60-70.

101.   Cain, D.P., *LTP, NMDA, genes and learning.* Curr Opin Neurobiol, 1997. **7**(2): p. 235-42.

102.   Laube, B., et al., *Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit.* Neuron, 1997. **18**(3): p. 493-503.

103.   Hardingham, G.E. and H. Bading, *The Yin and Yang of NMDA receptor signalling.* Trends Neurosci, 2003. **26**(2): p. 81-9.

104.   Alex, A.B., et al., *CGX-1007 prevents excitotoxic cell death via actions at multiple types of NMDA receptors.* Neurotoxicology, 2011. **32**(4): p. 392-9.

105.   Jane, D.E., D. Lodge, and G.L. Collingridge, *Kainate receptors: pharmacology, function and therapeutic potential.* Neuropharmacology, 2009. **56**(1): p. 90-113.

106.   Chittajallu, R., et al., *Regulation of glutamate release by presynaptic kainate receptors in the hippocampus.* Nature, 1996. **379**(6560): p. 78-81.

107.   Rodriguez-Moreno, A., *Kainate receptors. Their function in the regulation of GABAergic synaptic transmission in the hippocampus.* Rev Neurol, 2003. **36**(9): p. 852-9.

108.   Lerma, J. and J.M. Marques, *Kainate receptors in health and disease.* Neuron, 2013. **80**(2): p. 292-311.

109.   Semyanov, A. and D.M. Kullmann, *Kainate receptor-dependent axonal depolarization and action potential initiation in interneurons.* Nat Neurosci, 2001. **4**(7): p. 718-23.

110.   Contractor, A., C. Mulle, and G.T. Swanson, *Kainate receptors coming of age: milestones of two decades of research.* Trends Neurosci, 2011. **34**(3): p. 154-63.

111. Smolders, I., et al., *Antagonists of GLU(K5)-containing kainate receptors prevent pilocarpine-induced limbic seizures.* Nat Neurosci, 2002. **5**(8): p. 796-804.

112. Nicoletti, F., et al., *Metabotropic glutamate receptors: from the workbench to the bedside.* Neuropharmacology, 2011. **60**(7-8): p. 1017-41.

113. Viaene, A.N., I. Petrof, and S.M. Sherman, *Activation requirements for metabotropic glutamate receptors.* Neurosci Lett, 2013. **541**: p. 67-72.

114. Dubey, G.P. and S. Ben-Yehuda, *Intercellular nanotubes mediate bacterial communication.* Cell, 2011. **144**(4): p. 590-600.

115. Selmeczy, Z., et al., *Role of nonsynaptic communication in regulating the immune response.* Neurochem Int, 2008. **52**(1-2): p. 52-9.

116. Corkin, S., et al., *H. M.'s medial temporal lobe lesion: findings from magnetic resonance imaging.* J Neurosci, 1997. **17**(10): p. 3964-79.

117. Kathirvelu, B., et al., *Lentivirus-mediated chronic expression of dominant-negative CREB in the dorsal hippocampus impairs memory for place learning and contextual fear conditioning.* Neurobiol Learn Mem, 2013. **99**: p. 10-6.

118. Yang, F.C. and K.C. Liang, *Interactions of the dorsal hippocampus, medial prefrontal cortex and nucleus accumbens in formation of fear memory: Difference in inhibitory avoidance learning and contextual fear conditioning.* Neurobiol Learn Mem, 2014. **112**: p. 186-94.

119. Sun, Y., et al., *Cell-Type-Specific Circuit Connectivity of Hippocampal CA1 Revealed through Cre-Dependent Rabies Tracing.* Cell Rep, 2014. **7**(1): p. 269-80.

120. Lushnikova, I., et al., *Excitatory synaptic activity is associated with a rapid structural plasticity of inhibitory synapses on hippocampal CA1 pyramidal cells.* Neuropharmacology, 2011. **60**(5): p. 757-64.

121. Freund, T.F. and G. Buzsaki, *Interneurons of the hippocampus.* Hippocampus, 1996. **6**(4): p. 347-470.

122. Loy, R., et al., *Noradrenergic innervation of the adult rat hippocampal formation.* J Comp Neurol, 1980. **189**(4): p. 699-710.

123. Conrad, L.C., C.M. Leonard, and D.W. Pfaff, *Connections of the median and dorsal raphe nuclei in the rat: an autoradiographic and degeneration study.* J Comp Neurol, 1974. **156**(2): p. 179-205.

124. Shimamura, A., *The role of the prefrontal cortex in dynamic filtering.* Psychobiology, 2000. **28**(2): p. 207-218.

125. Changeux, J.-P., *Neuronal man : the biology of mind.* Princeton science library. 1997, Princeton, N.J.: Princeton University Press. 348 p.

126. Cruikshank, S.J., et al., *Thalamic control of layer 1 circuits in prefrontal cortex.* J Neurosci, 2012. **32**(49): p. 17813-23.

127. Little, J.P. and A.G. Carter, *Subcellular synaptic connectivity of layer 2 pyramidal neurons in the medial prefrontal cortex.* J Neurosci, 2012. **32**(37): p. 12808-19.

128. Song, D., et al., *Functional connectivity between Layer 2/3 and Layer 5 neurons in prefrontal cortex of nonhuman primates during a delayed match-to-sample task.* Conf Proc IEEE Eng Med Biol Soc, 2012. **2012**: p. 2555-8.

129. Miller, B.L. and J.L. Cummings, *The human frontal lobes : functions and disorders.* 2nd ed. The science and practice of neuropsychology. 2007, New York, NY: Guilford Press. xx, 666 p.

130. Shipp, S., *Structure and function of the cerebral cortex.* Curr Biol, 2007. **17**(12): p. R443-9.

131. Jones, E.G., *Viewpoint: the core and matrix of thalamic organization.* Neuroscience, 1998. **85**(2): p. 331-345.

132. Rubio-Garrido, P., et al., *Thalamic input to distal apical dendrites in neocortical layer 1 is massive and highly convergent.* Cereb Cortex, 2009. **19**(10): p. 2380-95.

133. Funahashi, S. and J.M. Andreau, *Prefrontal cortex and neural mechanisms of executive function.* Journal of Physiology-Paris, 2013. **107**(6): p. 471-482.

134. Uylings, H.B., H.J. Groenewegen, and B. Kolb, *Do rats have a prefrontal cortex?* Behav Brain Res, 2003. **146**(1-2): p. 3-17.

135. Ongur, D. and J.L. Price, *The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans.* Cereb Cortex, 2000. **10**(3): p. 206-19.

136. Sinclair, J., et al., *A cell-based bar code reader for high-throughput screening of ion channel-ligand interactions.* Anal Chem, 2002. **74**(24): p. 6133-8.

137. Brett, R.S., et al., *A method for the rapid exchange of solutions bathing excised membrane patches.* Biophys J, 1986. **50**(5): p. 987-92.

138. Colquhoun, D., P. Jonas, and B. Sakmann, *Action of brief pulses of glutamate on AMPA/kainate receptors in patches from different neurones of rat hippocampal slices.* J Physiol, 1992. **458**: p. 261-87.

139. Brody, J.P., et al., *Biotechnology at low Reynolds numbers.* Biophys J, 1996. **71**(6): p. 3430-41.

140. Squires, T.M. and S.R. Quake, *Microfluidics: Fluid physics at the nanoliter scale.* Reviews of Modern Physics, 2005. **77**(3): p. 977-1026.

141. Grunberger, A., W. Wiechert, and D. Kohlheyer, *Single-cell microfluidics: opportunity for bioprocess development.* Curr Opin Biotechnol, 2014. **29C**: p. 15-23.

142. Delamarche, E., et al., *Pharmacology on microfluidics: multimodal analysis for studying cell-cell interaction.* Curr Opin Pharmacol, 2013. **13**(5): p. 821-8.

143. Chung, B.G., et al., *Human neural stem cell growth and differentiation in a gradient-generating microfluidic device.* Lab Chip, 2005. **5**(4): p. 401-6.

144. Shelby, J.P., et al., *A microfluidic model for single-cell capillary obstruction by Plasmodium falciparum-infected erythrocytes.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 14618-22.

145. Duffy, D.C., et al., *Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane).* Anal Chem, 1998. **70**(23): p. 4974-84.

146. Hamill, O.P., et al., *Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches.* Pflugers Arch, 1981. **391**(2): p. 85-100.

147.    Newman, G.C., F.E. Hospod, and S.L. Schissel, *Ischemic brain slice glucose utilization: effects of slice thickness, acidosis, and K+.* J Cereb Blood Flow Metab, 1991. **11**(3): p. 398-406.

148.    Lipinski, H.G., *Brain tissue slice thickness monitored by ion-profile measurement.* J Neurosci Methods, 1992. **42**(1-2): p. 37-43.

149.    Kirschner, D.L., et al., *Simultaneous efflux of endogenous D-ser and L-glu from single acute hippocampus slices during oxygen glucose deprivation.* J Neurosci Res, 2009. **87**(12): p. 2812-20.

150.    Paizs, C., A. Katona, and J. Retey, *The interaction of heteroaryl-acrylates and alanines with phenylalanine ammonia-lyase from parsley.* Chemistry, 2006. **12**(10): p. 2739-44.

151.    Egelman, D.M. and P.R. Montague, *Calcium dynamics in the extracellular space of mammalian neural tissue.* Biophys J, 1999. **76**(4): p. 1856-67.

152.    Mergenthaler, P., et al., *Sugar for the brain: the role of glucose in physiological and pathological brain function.* Trends Neurosci, 2013. **36**(10): p. 587-97.

153.    Aghajanian, G.K. and K. Rasmussen, *Intracellular studies in the facial nucleus illustrating a simple new method for obtaining viable motoneurons in adult rat brain slices.* Synapse, 1989. **3**(4): p. 331-8.

154.    Hoffman, D.A. and D. Johnston, *Downregulation of transient K+ channels in dendrites of hippocampal CA1 pyramidal neurons by activation of PKA and PKC.* J Neurosci, 1998. **18**(10): p. 3521-8.

155.    Christie, B.R., et al., *Different Ca2+ channels in soma and dendrites of hippocampal pyramidal neurons mediate spike-induced Ca2+ influx.* J Neurophysiol, 1995. **73**(6): p. 2553-7.

156.    Magee, J.C., et al., *Dihydropyridine-sensitive, voltage-gated Ca2+ channels contribute to the resting intracellular Ca2+ concentration of hippocampal CA1 pyramidal neurons.* J Neurophysiol, 1996. **76**(5): p. 3460-70.

157.    Buskila, Y., et al., *Extending the viability of acute brain slices.* Sci Rep, 2014. **4**: p. 5309.

158.    Blau, L., et al., *Organization of unesterified cholesterol in high density lipoproteins probed by filipin.* Biochim Biophys Acta, 1982. **712**(3): p. 437-43.

159.    Lakowicz, J.R., *Principles of fluorescence spectroscopy.* 3rd ed. 2006, New York: Springer. 954 p.

160.    Phillips, G.J., *Green fluorescent protein--a bright idea for the study of bacterial protein localization.* FEMS Microbiol Lett, 2001. **204**(1): p. 9-18.

161.    Li, Q., et al., *Novel enantioselective fluorescent sensors for malate anion based on acridine.* Dyes and Pigments, 2014. **109**: p. 169-174.

162.    Bouvrais, H., et al., *Impact of membrane-anchored fluorescent probes on the mechanical properties of lipid bilayers.* Biochim Biophys Acta, 2010. **1798**(7): p. 1333-7.

163.    Igarashi, K.M., et al., *Functional diversity along the transverse axis of hippocampal area CA1.* FEBS Lett, 2014. **588**(15): p. 2470-2476.