Excitatory and inhibitory signaling in the rod pathway of the retina

Functional properties and activation of synaptic and extrasynaptic receptors

Pablo Beltrán Matas

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2021

UNIVERSITY OF BERGEN

© Copyright Pablo Beltrán Matas

The material in this publication is covered by the provisions of the Copyright Act.

Year: 2021

Title: Excitatory and inhibitory signaling in the rod pathway of the retina

Name: Pablo Beltrán Matas

Print: Skipnes Kommunikasjon / University of Bergen

Abstract

The visual system is arguably one of the most complex and studied sensory systems. The processing of visual information starts in the retina, where photoreceptors transduce light stimuli (photons) to electrochemical signals that are then transferred to bipolar cells grouped in different pathways. In the mammalian retina, vision under dim light conditions is mediated by the rod pathway. Glutamatergic rod bipolar cells are presynaptic to two inhibitory interneurons: the AII and A17 amacrine cells. The narrow-field glycinergic AII amacrine transfers rod signals to ON- and OFF-cone bipolar cells via electrical and chemical synapses, respectively. The wide-field GABAergic A17 amacrine provides inhibitory feedback to rod bipolar cells. The synaptic input to both amacrines is mediated by Ca^{2+}-permeable AMPA receptors. It has been recently demonstrated that these two cells express functional extrasynaptic NMDA receptors, with AII amacrines expressing GluN2B-containing receptors and A17 amacrines GluN2A-containing receptors. Ultrastructural studies reported the presence of inhibitory synaptic inputs from other amacrine cells to both the AII and the A17 amacrines. However, the activation and contribution to signal processing of the different receptors that mediate these excitatory and inhibitory inputs is not well understood.

In this thesis, we combined electrophysiological and pharmacological approaches to characterize these receptors and investigate the conditions required for their activation on AII and A17 amacrine cells. Pre-incubation of retinal tissue in either bafilomycin A1 (an inhibitor of neurotransmitter uptake into synaptic vesicles) or L-methionine-sulfoximine (a glutamine synthetase inhibitor) abolished NMDA receptor activation on AII amacrines, but not on A17 amacrines. This suggests a neuronal origin for the glutamate that activates NMDA receptors on AII amacrines and a glial source in the case of A17 amacrine cells. Degradation of endogenous D-serine by DAAO (the enzyme that breaks down D-serine) reduced NMDA activation on AII amacrines, but not on A17 amacrine cells, suggesting that D-serine could be the endogenous co-agonist at NMDA receptors on AII amacrines, but not on A17 amacrine cells.

We also performed a detailed analysis of the kinetics of synaptic and extrasynaptic GABA receptors on these two cells. We found spontaneous inhibitory postsynaptic currents on A17 amacrine cells that displayed fast decay kinetics (τ_w ~14 ms). Pharmacological investigations suggest that these events are mediated by $GABA_A$ receptors that are likely to be composed $\alpha_1\beta\gamma_2$ subunits. However, we did not observe GABAergic synaptic currents on AII amacrines. Application of brief pulses of GABA (3 mM) to outside-out and nucleated patches from A17 and AII amacrines, respectively, evoked $GABA_A$ receptor-mediated responses with relatively slow decay kinetics (τ_w 42 ms on A17; τ_w 163 on AII). The use of pharmacological agents suggest that these receptors are likely to be composed of $\alpha_{2,3}\beta\gamma_2$ subunits on both amacrine cells.

The major contribution of the studies outlined in this thesis are that (1) different sources of glutamate and potentially different endogenous co-agonists activate the different extrasynaptic NMDA receptor populations on AII and A17 amacrine cells. This suggests a differential contribution of glutamate inputs to excitability and signaling in these cells. (2) A17 amacrine cells receive synaptic GABAergic input mediated by receptors with fast kinetics that could be involved in shunting of excitatory transmission. (3) Both AII and A17 amacrine cells express somatic $GABA_A$ receptors with relatively slow kinetics that could mediate signals that allow for temporal summation of inhibitory inputs with a low frequency.

Scientific environment

This project was performed in the Neural Networks Research Group at the Department of Biomedicine, the University of Bergen, during the years 2017-2020 under the supervision of Professors Margaret L. Veruki and Espen Hartveit. Professor Arvid Lundervold served as an administrative co-advisor. The project received funding from the Research Council of Norway (261914 to MLV) and the University of Bergen. Additional financial support for courses and scientific meetings was provided by the Norwegian Research School in Neuroscience.

Acknowledgements

I would like to express my gratitude to my supervisor, Professor Margaret Lin Veruki and my co-supervisor, Professor Espen Hartveit. I cannot stress enough all the knowledge I have gained from two such wise researchers and how thankful I am for all the guidance and support you have given me all these years. Meg, I really value that your door has always been open for me to discuss about anything, scientific or not. I have learned many things from you during these three years, you have always motivated, inspired and pushed me to work harder, to be better at whatever I set my mind to and to become a better scientist. You always said that you would have done a good job if at the end of the PhD you had pushed me so hard that I didn't want to talk to you for a while. Well, I think you were wrong, I think you did an amazing job, but you failed at the last part because I have nothing but gratitude towards you. Espen, I am also really thankful for all our discussions in our Monday meetings, all the guidance in all the projects and all the knowledge you have shared with me. I am always astonished at your ability to remember and point out the smallest details of anything, and I firmly believe all your scrutiny of my work during this time has enormously help me to develop as a scientist. I am very thankful for the opportunity you both gave me in your group, Retinal Microcircuits, and all the work we have done together these three years.

I also want to give a special thanks to Dr. Áurea Castilho. I have also learned many things from you these three years Áurea, and your continuous help has been invaluable. I can only say thank you, you are the nicest person I ever had the luck to work with, and I wish you the very best, because you deserve every bit of it. To my PhD and postdoc colleagues as well, thank you so much for all these years, Rémi Fournel, Jian Hao Liu and Jeet Singh. Rémi, we have been together through bad and good, I would never be able to count the amount of coffees and discussions about everything we have had together, and all the nice times we have spent here in Bergen. You are a true friend. I have seen your worth through these years, and I have no doubts that you will become a great scientist. Jian, Jeet, we have also had many nice times with coffee breaks and discussions during lunches, and I am really thankful for our

time together. I wish you both the best in your future. I also want to thank Torhild Fjordheim Sunde for your excellent technical assistance and all your cheerful “god morgen”s every day in the lab (and your delicious salmon pie at Christmas).

The work during these three years wouldn’t have been possible without many people in the Department of Biomedicine. I would like specially to thank Margarethe Bittins, the PhD coordinator, Elisabeth Hove for helping me with all the administration stuff that has made my time here easier.

I also want to thank my MSc thesis supervisors, Professors Blas Torres, Luis Herrero and M. Ángeles Luque. You gave me my first opportunity in science, you initiated me in this path that has led to this PhD thesis, and you know I will never be able to thank you enough for that opportunity and for believing in me.

To my dear friends in Bergen (and those who have moved away), with whom I have had such nice hiking, skiing, and fun times. I will really miss those long nights playing board games or trying different gins and beers while crying of laughter, but I am really sure we will keep visiting each other. I long for that. To my friends in Spain, to you especially Alba and Brian, thank you so much for all the good times we had and we still have when I come back, for checking on me, for supporting me and being there for me, even when distance makes everything harder.

Ida, there are no words to express how much your support and love has helped me during these three years. Your enthusiasm has been always contagious, and no matter how difficult things sometimes look, I know you will get through because you always achieve what you propose to yourself. I am so happy we were at the right time at the right place to find each other. I am so happy we got through everything together… You know it, right?

And finally, my family, my parents and my brother. Your support and unconditional belief in me have helped to get through these years. I know distance is hard and videocalls are only band-aids on a bullet wound, but you are always with me. Papá, mamá, José, gracias por haber estado ahí siempre.

Table of Contents

LIST OF PAPERS ..... **XI**

ABBREVIATIONS ..... **XIII**

1. INTRODUCTION **1**

1.1 NEURAL NETWORKS AND MICROCIRCUITS 1

1.1.1 Role of inhibitory interneurons in excitation – inhibition balance 4

1.1.2 Synaptic and extrasynaptic receptors 5

1.2 MAMMALIAN VISION AND THE RETINA 10

1.2.1 The rod pathway 12

1.2.2 Amacrine cells in the rod pathway 14

1.3 ACTIVATION AND PHARMACOLOGICAL MANIPULATION OF NMDA AND GABA RECEPTORS 19

1.3.1 NMDA receptors 19

1.3.2 GABA receptors 22

2. AIMS OF THE THESIS **25**

3. METHODOLOGICAL CONSIDERATIONS **27**

3.1 ETHICS STATEMENT 27

3.2 RETINAL TISSUE PREPARATION AND TARGETING OF AMACRINE CELLS 27

3.3 ELECTROPHYSIOLOGICAL METHODS TO STUDY ION CHANNELS 29

3.3.1 Ion channels and Ohm's law 31

3.4 THE PATCH-CLAMP TECHNIQUE 34

3.4.1 Whole-cell recording 35

3.4.2 Outside-out and nucleated patch recording 37

3.4.3 Pharmacological tools 38

3.4.4 Ultrafast perfusion system 41

3.5 ELECTROPHYSIOLOGICAL DATA ACQUISITION 43

3.6 DATA ANALYSIS 44

3.6.1 Non-stationary noise analysis 45

3.6.2 Membrane noise analysis 46

4. OVERVIEW OF RESULTS 49

4.1 PAPER I 49

4.2 PAPER II 50

4.3 PAPER III 53

5. DISCUSSION 55

5.1 EXTRASYNAPTIC NMDA RECEPTORS ON AII AND A17 AMACRINE CELLS 56

5.1.1 Different sources of glutamate activate extrasynaptic NMDA receptors on AII and A17 amacrine cells 57

5.1.2 D-serine acts as a co-agonist at extrasynaptic NMDA receptors on AII but not on A17 amacrine cells 58

5.1.3 Functional role of extrasynaptic NMDA receptors on AII and A17 amacrine cells 61

5.2 $GABA_A$ RECEPTORS ON AII AND A17 AMACRINE CELLS 62

5.2.1 Molecular identity of synaptic $GABA_A$ receptors on AII and A17 amacrine cells 62

5.2.2 Molecular identity of extrasynaptic $GABA_A$ receptors on AII and A17 amacrine cells 64

5.2.3 Functional role of $GABA_A$ receptors on AII and A17 amacrine cells 67

6. CONCLUDING REMARKS 71

7. REFERENCES 73

List of papers

Paper I

Pablo Beltrán-Matas, Espen Hartveit, Margaret L. Veruki.

Different glutamate sources and and endogenous co-agonists activate extrasynaptic NMDA receptors on rod pathway amacrine cells in the rat retina (manuscript).

Paper II

Pablo Beltrán-Matas, Aurea Castilho, Barbora Tencerová, Margaret L. Veruki, Espen Hartveit.

Inhibitory inputs to an inhibitory interneuron: Spontaneous IPSCs and $GABA_A$ receptors on A17 amacrine cells in the rat retina (manuscript).

Paper III

Pablo Beltrán-Matas, Espen Hartveit, Margaret L. Veruki.

Functional properties of $GABA_A$ receptors on AII amacrine cells of the rat retina (manuscript).

Abbreviations

A	ampere, unit for electric current
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Bic	bicuculline
BZ	benzodiazepine
C	capacitance
C_{fast}	fast capacitance compensation of EPC circuitry (≈ pipette capacitance)
C_{slow}	slow capacitance compensation of EPC circuitry (≈ cell capacitance)
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPP	(RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid
Cx36	connexin 36
DAAO	D-amino acid oxidase
E_m	membrane potential
E_{rev}	reversal potential
GABA	γ-aminobutyric acid
$GABA_AR$	GABA receptor subtype A
GCL	ganglion cell layer
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
I	current
INL	inner nuclear layer

IPL	inner plexiform layer
IR-DIC	infrared differential interference contrast
LTD	long-term depression
LTP	long-term potentiation
MSO	L-methionine-sulfoximine
NMDA	*N*-methyl-D-aspartic acid
NSN	non-stationary noise analysis
ONL	outer nuclear layer
OPL	outer plexiform layer
P_{open}	channel open probability
PSC	postsynaptic current
R	electrical resistance / resistor
RBC	rod bipolar cell
spIPSC	spontaneous inhibitory postsynaptic current
spPSC	spontaneous postsynaptic current
TBOA	DL-*threo*-β-Benzyloxyaspartic acid
THIP	4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol
TTX	tetrodotoxin
UV	ultraviolet
V	volt, unit for voltage

1. Introduction

1.1 Neural networks and microcircuits

The central nervous system (CNS) is comprised of individual neurons, specialized cells that are involved in the processing and transmission of information. Neurons interact with each other and arrange to form a multitude of circuits (also referred to as neural networks). Each of these circuits computes precise and relevant information following a thorough analysis of the enormous number of complex signals they continually receive. They can be the result of a small group of neurons, involving a few cells to perform a relatively simple task. However, they can also be the product of the assembly of several smaller circuits to perform more complex analyses of a given signal. The subject of this thesis, the retina, is an excellent example of both cases: it is composed of many small circuits involving few neurons (Euler et al., 2002; Grimes et al., 2010) that analyze specific features of the visual information, such as light intensity, contrast, direction selectivity, etc. At the same time, all these small circuits also interact with each other and associate to refine the output of the visual information produced by the retina towards the visual cortex (reviewed in Wässle, 2004; Masland, 2012; Hoon et al., 2014; Franke & Baden, 2017). Circuits formed by few neurons that carry out specific functions are often referred to as microcircuits.

Neurons are specialized cells of the CNS with unique anatomical structures: they receive the information as inputs along their dendrites, which are specialized and branched structures. This information will lead to electrical and chemical events that are integrated along the dendrites, soma and the axon initial segment of the cell. The axon is the structure that conveys the electrical signals over relatively long distances. The information integrated in the cell will travel to the very end of the axon, the axon

terminal, that will transmit the information to the next cell. In some neurons, dendrites not only receive and integrate the information, but they also provide the output as an axon would do, like the well-studied amacrine cells in the retina (Masland, 1988; Kolb, 1997) or mitral and granule cells in the olfactory bulb (Price & Powell, 1970), but also in the thalamic reticular nucleus (Pinault et al., 1997) and the lateral geniculate nucleus (Cox et al., 1998).

The knowledge we have today about the morphology and function of different neurons is due to the intense work of a long list of exceptional scientists. However, I cannot miss the opportunity to mention the outstanding contribution of my most famous fellow countryman, Santiago Ramón y Cajal (Fig. 1C). During the 19th century, it was thought that the nervous system formed a continuous network, the so-called reticular theory. It was logical in that time to reach such a conclusion, as the microscopy and tissue preparation techniques were a considerable limitation. It was not until the development of the Golgi technique, by Camillo Golgi (initially termed the black reaction or *la reazione nera;* Swanson et al., 2017*;* Ramón y Cajal, 1989), that such a concept would be overturned. This technique allowed neurons and their processes to be visualized in their entirety. Paradoxically, Camillo Golgi stood by the established reticular theory and defended that the nervous system functioned as a network where neurons constituted a plexus of fused processes that formed the fundamental integrative structure. However, the young Spanish scientist, Ramón y Cajal, would propose something controversial and dramatically different: nerve cells were individual entities (Ramón y Cajal, 1989; López-Muñoz et al., 2006). They might touch one another, but they were independent cells and did not fuse. It is said that Ramón y Cajal always wanted to be an artist, though his father managed to interest his son in anatomy after taking him to a graveyard to draw bones of ancient burials (Ramón y Cajal, 1989). It is because of this parental decision and Cajal's brilliant skills that the neuron doctrine could be developed, together with exceptional drawings of neurons from several brain regions, including the subject of this thesis: the retina (Fig. 1A,B; see Swanson et al., 2017).

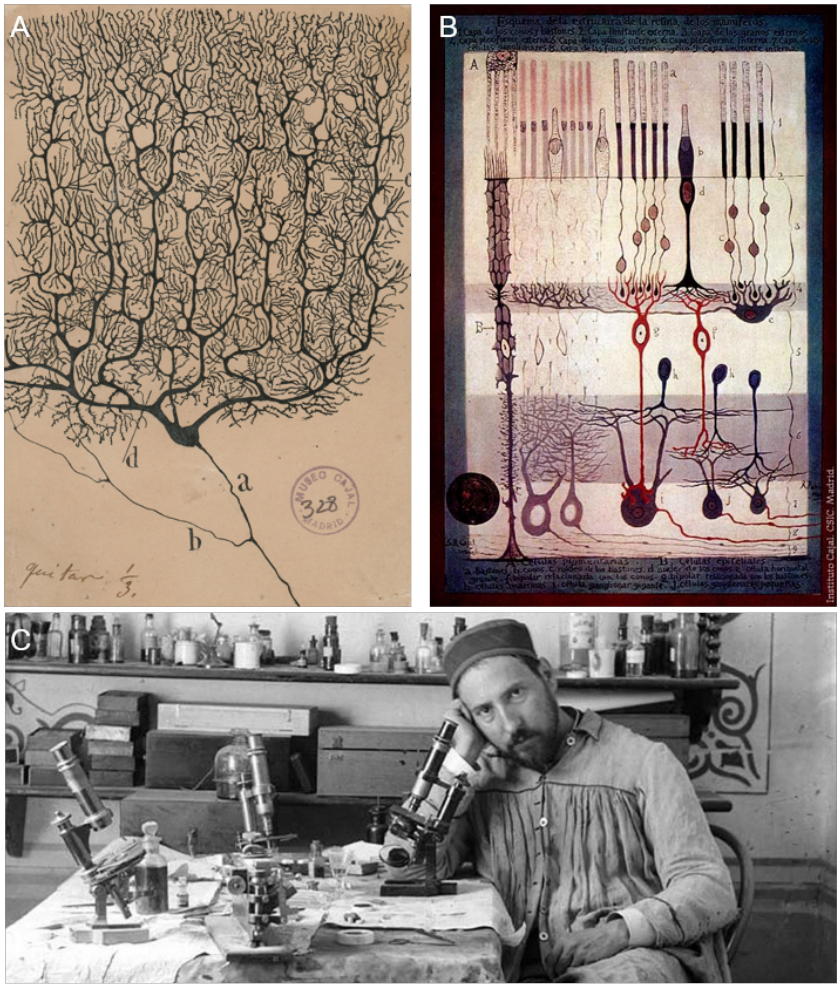

Figure 1. Santiago Ramón y Cajal and his drawings. A. Original drawing of a Purkinje cell, including many details of their highly branched dendrites. **B**. Drawing of the retina, including the layer pattern and the main cell types. **C**. Picture of Santiago Ramón y Cajal at his laboratory (1884 – 1887). Panels A and B are adapted from original drawings made by Santiago Ramón y Cajal on (1899) and (1900) respectively; *Cajal Institute*, Madrid, Spain.

Cajal's proposal, although supported by evidence obtained with Golgi's staining technique, could not conclude the debate between the reticular theory and the neuron doctrine. It is not a coincidence that Cajal ended the title of his famous book "Neuron Theory or Reticular Theory?" with a question mark, as we will see in the next paragraph

(López-Muñoz et al., 2006). The development of the electron microscopy technique in the 1950s would finally rule out the syncytial network proposal. The evidence provided with this technique was uncontestable: neurons were independent cells, and they did not touch each other. The history and relevance of Cajal's discoveries are wonderfully reviewed in López-Muñoz et al. (2006), and his most famous drawings are compiled in Swanson et al. (2017).

Although the membranes of neurons are physically separated, there are points where their membranes do approach within a distance of 20 - 50 nm: at the synapse (Sherrington, 1906; Fig. 2A,B). The space between the two membranes is called the synaptic cleft, where the chemical synapse, one of the two main mechanisms of neural communication, occurs. The presynaptic cell releases small packets of chemical messengers (neurotransmitters) across the narrow synaptic cleft that will bind to specific proteins (receptors) at the membrane of the postsynaptic neuron (Fig. 2A). The second mechanism is the electrical synapse. Here, neurons contact each other through small canals formed by proteins that directly connect the cytoplasm of two different cells forming specialized morphological junctions known as gap junctions. Although this mechanism resembles what was proposed in the reticular theory, it is worth noting that neurons do not form the meshwork of cytoplasm proposed in this theory, and gap junctions can be regulated and even closed (reviewed in Bennett, 2000).

We have more insights into neural communication today, but many aspects are still a mystery and will be addressed during the introduction chapters of this thesis. Understanding the fundamental mechanisms that underlie cell-to-cell transmission of information is essential to explain how thousands of cells work together in networks. In this sense, microcircuits composed of a smaller number of cells can provide an exceptional substrate for understanding brain function at the mechanistic level.

1.1.1 Role of inhibitory interneurons in excitation – inhibition balance

The neurotransmitters and receptors used to send and receive information determine the behavior and function of a given neuron. Thus, neurons that release

GABA (GABAergic neurons) classically carry out inhibitory tasks, whereas glutamatergic neurons will predominantly be excitatory cells. In neural circuits, several neurotransmitters are often involved; this means that neurons with different neurotransmitters will interact with each other to process the information at a cellular and circuit level. Thus, balance between excitation and inhibition will become a tool for circuits to shape information and produce a precise output as proven by neuronal models (Rubin et al., 2017) but also in brain areas like the visual cortex (Adesnik, 2018), the auditory cortex (Moore et al., 2018), the spinal cord (Guzulaitis & Hounsgaard, 2017) and the retina (Manookin et al., 2008). In this balance, inhibition will often be carried out by inhibitory interneurons. These interneurons can provide a downregulation of excitation in other neurons or inhibitory feedback, but they can also enable communication between different cells within the circuit, resulting in feedforward inhibition and providing parallel channels of information (Wässle, 2004; Pouille et al., 2009; Diamond, 2017).

The research interest in inhibitory interneurons has dramatically increased in the last two decades and it is becoming clear that the role of these interneurons is not merely restricted to inhibition at the synaptic level, but they are also involved in modulating the gain of circuits, timing, tuning and selective filtering of excitation in synapses (Pouille et al., 2009; Royer et al., 2012; Wilson et al., 2012). At a circuit or network level, inhibitory interneurons also allow the coordination of interactions between the "principal" cells to form cooperative assemblies that increase the efficacy of processing and transmission of information (Miles et al., 1996; Bikoff et al., 2016).

1.1.2 Synaptic and extrasynaptic receptors

All living cells are enveloped by a plasma membrane, forming a barrier between the intracellular and the extracellular environment. The inside of the cell is rich in proteins, and a good number of them are negatively charged. Negative ions in the inside are attracted by positive ions that accumulate on the outside. This creates a differential distribution of ions and the inside of the cell becomes more negatively charged than the outside. In a permeable system, this electrochemical gradient would induce ions to

diffuse from higher to lower concentration until they reach an equilibrium point, but the semipermeable lipidic bilayer prevents ions from freely flowing across the membrane.

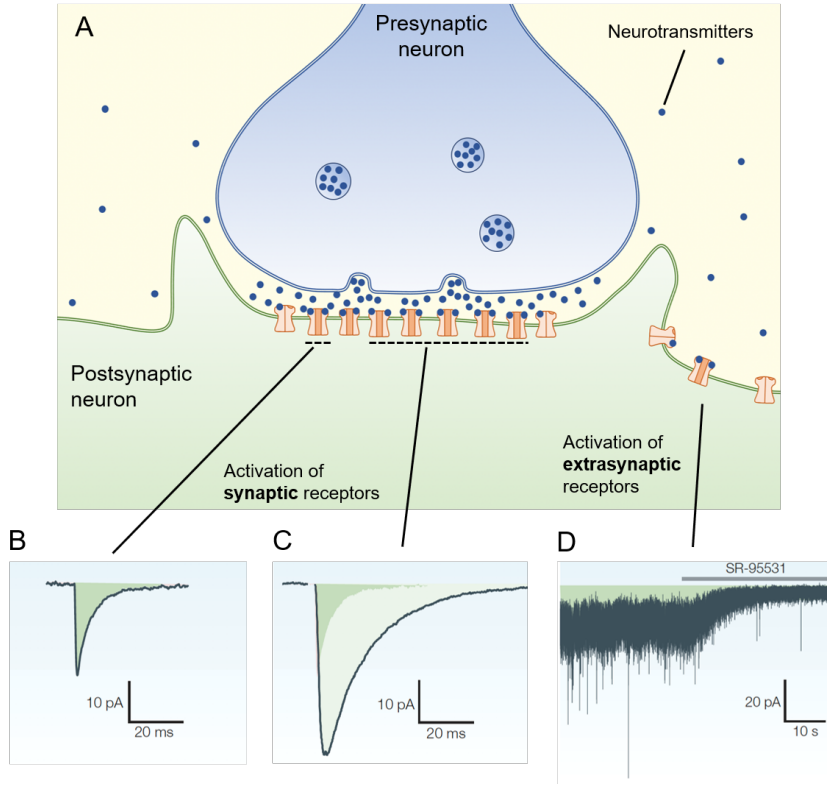

Figure 2. Synaptic and extrasynaptic transmission. A. Schematic drawing of a chemical synapse. **B**. Activation of a single postsynaptic receptor results in the opening of the receptor and the influx of ions measured as a postsynaptic current. **C**. Simultaneous activation of an increased number of postsynaptic receptors results in a larger postsynaptic current. **D**. Activation of extrasynaptic receptors leads to a tonic activation of these receptors (green area) rather than a phasic activation as in B and C. Panels B, C and D are adapted from Farrant & Nusser (2005).

The plasma membrane is embedded with specialized proteins that facilitate ion movements through pores or channels. These channels fluctuate between closed and open states, but the transition between these states is regulated by different mechanisms: some of them are physical, such as changes in the electrochemical gradient; others are chemical, involving the binding of specific molecules (ligand) to

induce the opening of the pore. Many of these ligands are common amino acids, like glycine or glutamate, although there are many types of molecules that can act as ligands, even gases like nitric oxide. Ligand-gated ion channels are a subtype of neurotransmitter receptors involved in chemical synapses and are often referred to as ionotropic receptors. Like other types of ion channels, ionotropic receptors are selectively permeable to specific ions. The nature of the ion determines the receptor function: The opening of receptors permeable to Na^+ (like glutamate receptors) will increase the concentration of positive charges in the inside of the cell (depolarization) and will excite it. On the other hand, receptors permeable to Cl^- (like GABA receptors) can increase the number of negative ions inside the cell and generally hyperpolarize and inhibit the cell. Thus, in classical chemical neurotransmission, the binding of neurotransmitter (ligand) to receptors will lead to a series of electrochemical events (Fig. 2A-C). The nature of these events will depend on the type of neurotransmitter released by the presynaptic neuron and the type of receptors present in the membrane of the postsynaptic neuron. Sometimes, the same neurotransmitter can lead to different events depending on the postsynaptic receptors.

At a synapse, the concentration of neurotransmitters released from presynaptic vesicles generally reaches the millimolar range (Mody et al., 1994). The rapid increase in neurotransmitter concentration triggers an almost simultaneous opening of receptors upon the binding of neurotransmitters (Fig. 2A-C). The duration that synaptic receptors are exposed to neurotransmitters can vary, but in some synapses, it has been calculated to be about 100 μs (Mozrzymas et al., 2003). The short time that neurotransmitters dwell in the synaptic cleft is due to both the rapid diffusion of neurotransmitters away from the synapse and the uptake of neurotransmitters by membrane transporters into neurons and glia. The short time that postsynaptic receptors are exposed to the neurotransmitter transient is a defining characteristic of the phasic mode of receptor activation that occurs in synapses (Fig. 2A-C). Following rapid removal of ligand, channels quickly transition from an open to a closed state, resulting in a fast decrease of the postsynaptic response amplitude, a phenomenon called deactivation. However, during high bursts of activity, more vesicles can be released, and the time course of neurotransmitter concentration in the synaptic cleft can be longer. In these cases, upon

continuous binding of neurotransmitter, receptors still transition to closed states and the amplitude of the response decays, a phenomenon called desensitization. The entry of synaptic receptors into a desensitized state affects the ability of these receptors to respond to repetitive high-frequency activation, which is important for shaping the time course of postsynaptic events (Jones & Westbrook, 1995; Bianchi & Macdonald, 2002). Activation, deactivation, desensitization and agonist affinity of neurotransmitter receptors are biophysical properties determined by the specific subunit composition of a given receptor (Gingrich et al., 1995; Lavoie et al., 1997; Paoletti et al., 2013). Therefore, it is of interest to investigate the subunits that form these receptors in order to better understand their different biophysical properties and how they contribute to signal processing in a given neuron.

Interestingly, neurotransmitters are not only found at the synaptic cleft, but also at lower concentrations in extracellular regions in the vicinity of the neural membrane that are outside the synapse. These regions are often referred to as the extrasynaptic compartment. Such low but constant presence of neurotransmitter can be due either to diffusion of molecules caused by synaptic activity or spillover, or an effect of glial cells that actively regulate the presence of neurotransmitter in the extracellular compartment by removing (uptake) or releasing neurotransmitters (Barbour & Häisser, 1997; Cavelier et al., 2005; Le Meur et al., 2007; Wu et al., 2012; Rusakov & Dityatev, 2014). These low levels of neurotransmitters can be sensed by specific receptors located outside the synapse (so-called extrasynaptic receptors), and their activation can modulate neuronal function (Rusakov & Dityatev, 2014).

Unlike synaptic receptors with phasic activation, characterized by a rapid synchronous opening of clusters of postsynaptic receptors, the activation of extrasynaptic receptors is characterized by long-lasting responses with slow kinetics. This is because extrasynaptic receptors usually have different affinities for ligands than receptors found at the synapses. This allows them to detect the lower concentrations of neurotransmitters that can oscillate between nanomolar and low micromolar ranges (Lerma et al., 1986; Yamashita et al., 2009). All this can lead to a long-lasting or rather a tonic activation of extrasynaptic receptors, as opposed to the phasic activation seen

in synapses (Fig. 2D). The immediate outcome of tonic activation is a constant input conductance into the cell. Tonic inhibition, for example, will narrow the temporal and spatial window over which signal integration can occur by accelerating the decrement of voltage of signals over distance, thus limiting the propagation and summation of excitatory signals (Chance et al., 2002; see section **3.3**). Therefore, the presence of a tonic inhibitory current can alter the input-output relationships in neurons since more excitatory inputs will be required to produce the same output. Tonic excitation, on the other hand, provides a constant input conductance that increases the level of excitability of the cell that can facilitate the propagation of postsynaptic potentials (Zhang et al., 2017). However, extrasynaptic receptors might play a more dynamic role than simply reduce or increase excitation because their low receptor occupancy, due to the lower subsaturating concentration of neurotransmitters to which they are exposed, offers a much larger dynamic range of modulation. The low but persistent concentration of neurotransmitters promotes receptors to entry into desensitized states, limiting the magnitude of tonic conductance and reducing the availability of receptors (Jones & Westbrook, 1995). However, the concentration of neurotransmitters in the extracellular environment can be changed and regulated. In this process, the role of glial cells cannot be overlooked.

Glial cells have been traditionally regarded as the support cells of the central nervous system and are known to regulate the concentration of neurotransmitters in the extracellular environment, thus limiting the diffusion and uptake of neurotransmitters (Henn et al., 1974; for review see also Bringmann et al., 2009). However, they can also be directly involved in synaptic communication by actively releasing transmitters (or rather gliotransmitters) that can bind to receptors located at neurons (reviewed in Araque et al., 1999). Some of the mechanisms via which glial cells regulate the concentration of neurotransmitters are probably also used to actively release them and include reverse uptake by amino acid transporters, amino acid exchange via antiporters, but also Ca^{2+}-dependent exocytosis (Malarkey & Parpura, 2008). Participation of glial cells in neurotransmission has been reported in many areas in the central nervous system, including the retina (Vijay, 1983; Pow & Robinson, 1994; Le Meur et al., 2007; Sullivan & Miller, 2010; Newman, 2015; Gómez-Gonzalo et al., 2018).

Receptors found outside of synapses often have different subunit composition than synaptic receptors and this gives them distinct biophysical properties (Clark et al., 1997; Farrant & Nusser, 2005; Papouin et al., 2012). Thus, the consequences of the activation of extrasynaptic receptors in neurons dramatically differ from synaptic receptors, and NMDA receptors (NMDARs) are a good example. Whereas activation of synaptic NMDARs promotes cell survival and long-term potentiation (LTP) in memory processes, activation of extrasynaptic NMDARs can lead to cell death and long-term depression (LTD) in memory formation (Hardingham et al., 2002; Liu et al., 2013). Extrasynaptic NMDARs have also been linked to several diseases, including ischemia, Parkinson's, Huntington's and Alzheimer's disease (reviewed in Paoletti et al., 2013; Parsons & Raymond, 2014; Pál, 2018). Others, like extrasynaptic GABA receptors (GABAR), have been linked to Down's syndrome, affective disorders, schizophrenia and autism disorders (Rudolph & Möhler, 2014).

The subunit composition and role of these receptors is of particular interest in disease treatments, since targeting receptors with general ion channel blockers can lead to unacceptable side effects since both synaptic and extrasynaptic receptors are blocked indistinctly (Hardingham & Bading, 2003). Thus, knowing the subunit composition and understanding the function of these receptors in the healthy CNS might help to develop more specialized and accurate therapeutic agents to treat these diseases.

1.2 Mammalian vision and the retina

An amazing and complex example of excitatory and inhibitory interaction, parallel processing and the implications of synaptic and extrasynaptic receptors in signal processing is the retina. The frequent use of the retina as a model tissue for CNS studies reflects the need for an accessible neural tissue that is well structured and whose connectivity is well known, such that we can control the inputs to this circuit and measure the outputs. By doing so, we can answer questions about how microcircuits work and what molecular and cellular elements they use to perform the specialized tasks they carry out. Since the neurotransmitters and receptors present in the rest of the

CNS are also in the retina, it becomes an ideal model tissue to study microcircuits and the role of inhibitory interneurons in the CNS.

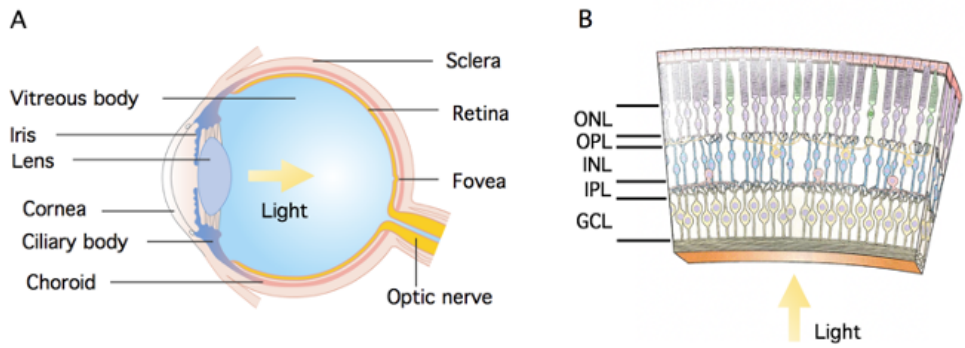

Figure 3. The retina. **A**. Schematic drawing of a human eye indicating its components and the position of the retina within the eye. **B**. Section of the retina showing the overall arrangement of retinal layers. Panel B is adapted from Neuroscience, 6th ed, Purves.

The retina is built up of ~30 parallel circuits, each of which processes different features of the visual signal such as color, direction selectivity, contrast, etc (Fig. 3A; Masland, 2012). Like many other areas of the CNS, the retina is a layered structure: Photoreceptors transduce light inputs into neural signals that are sent to ganglion cells via bipolar cells in the inner retina, thus creating two synaptic steps or synaptic layers (Fig. 3B, 4). Ganglion cells provide the only output of the retina, and the optic nerve formed by the axons of these cells will connect the retina to the rest of the CNS. The signals in this pathway are driven by glutamate, but there are two lateral inhibitory networks that will shape the signals at each synaptic step. First, synaptic transmission between photoreceptors and bipolar cells at the outer plexiform layer (OPL) is modulated by GABAergic horizontal cells, which provide feedback and feedforward inhibition to photoreceptors and bipolar cells, respectively, although the synaptic mechanisms remain unclear (for review see Diamond, 2017). So far, 3-4 different photoreceptors, ~15 different bipolar cells and 1-2 different types of horizontal cells are reported to be present in the mammalian retina (Helmstaedter, 2013). At the second step, in the inner plexiform layer (IPL) of the retina, bipolar cells contact ~30 different types of ganglion cells, and these synapses are modulated by ~45 different types of

amacrine cells (Fig. 3C, Fig. 4; Helmstaedter, 2013; Baden et al., 2016). The latest study has in fact identified more than 60 types of amacrine cells in mouse (Yan et al., 2020).

Amacrine cells were originally described as axonless interneurons by Ramón y Cajal, hence the name (*a-makrós-inos*, "without long fiber"). Today, we know that some do actually have axons or axon-like processes (Famiglietti, 1992; Wright & Vaney, 2004; Vaney, 2004). They are inhibitory interneurons that influence excitatory connections by providing feedback inhibition to bipolar cells and feedforward inhibitory synapses to ganglion cells, as well as lateral inhibition to other cells, including other types of amacrine cells (MacNeil & Masland, 1998, Gollisch & Meister, 2010). The diversity of amacrine cell types is of special interest to neuroscientists focused on microcircuit analysis and their functions within the CNS (Helmstaedter et al., 2013). The interaction between amacrine cells and different cell types enables parallel channels of communication between circuits that, for example, shape spatiotemporal processing of bipolar cell signals in the retina (MacNeil & Masland, 1998; Franke et al., 2017).

1.2.1 The rod pathway

Of the many different circuits in the retina, one of the most studied is the rod pathway circuit. The rod pathway processes visual signals in the dark (scotopic vision) and it is involved in cross-over inhibition between ON- and OFF-pathways during daylight (photopic vision; Manookin et al., 2008; Demb & Singer, 2012).

In nocturnal animals like rodents or hunters like humans, cats and owls, the photoreceptor layer of the retina contains ~95% of rods, which are much more sensitive to light than cones. They are so sensitive they can even respond to single photons (Baylor et al., 1979; Reingruber et al., 2015). Rod photoreceptors transfer this information to the rod bipolar cell (RBC; Fig. 4). The RBC then provides glutamatergic inputs to two postsynaptic partners at the same synapse: the AII and the A17 amacrine cells, that form a dyad synapse with the RBC (Fig. 4, 5; Famiglietti & Kolb, 1975). The RBC itself does not make synapses with ganglion cells. Instead, the AII amacrine cell

is the sole output of the circuit, acting as a relay between the rod pathway and OFF- and ON-pathways (Fig. 4, 5; Famiglietti & Kolb, 1975, Strettoi et al., 1990, 1992). On the other hand, A17 amacrine cells provide localized inhibitory feedback to RBCs. A17 amacrines receive glutamate from several RBC at distal varicosities, such that each varicosity will provide specific GABAergic feedback to one RBC (Nelson & Kolb, 1985). Thus, the RBC output is highly regulated by feedback from the A17 amacrine cell, and the output of the whole pathway is mediated by the AII amacrine cell.

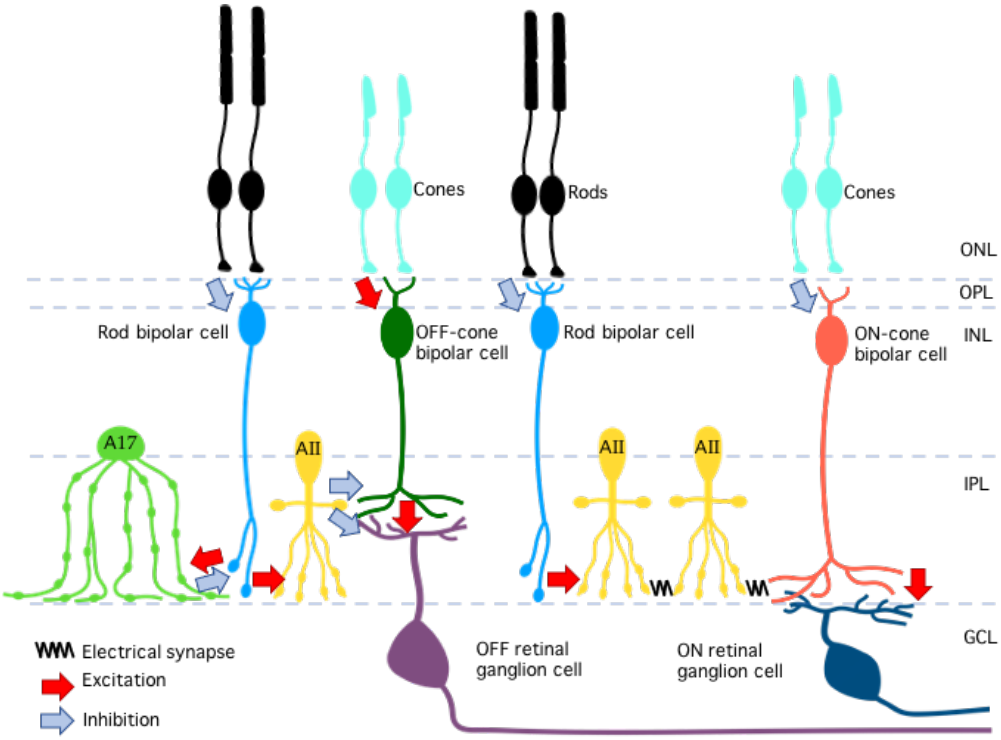

Figure 4. Pathways in the retina. **A**. Schematic drawing of the pathways of the retina, including the main cell types discussed in this thesis. Here, the main pathways of the retina are represented by the main cell types involved in each pathway. In the rod pathway, rod photoreceptors synapse upon the rod bipolar cell, which sends excitatory glutamatergic inputs to AII and A17 amacrine cells at the same synapse (dyad synapse). A17 amacrine cells provide inhibitory feedback to the rod bipolar, and AII amacrines provide feedforward inhibition to the OFF pathway and feedforward sign-conserving signals through gap junctions to on bipolar cells.

1.2.2 Amacrine cells in the rod pathway

Both AII and A17 amacrine cells play a central role in the rod pathway. Taking into account the importance and interactions of this pathway within the retinal circuitry (as outlined above), investigating how signals are processed in these two cells becomes essential to better understand their contribution to the processing of the visual information that takes place in the retina. Moreover, this can also increase our knowledge about how other interneurons in the CNS contribute to shape and refine the output of different circuits.

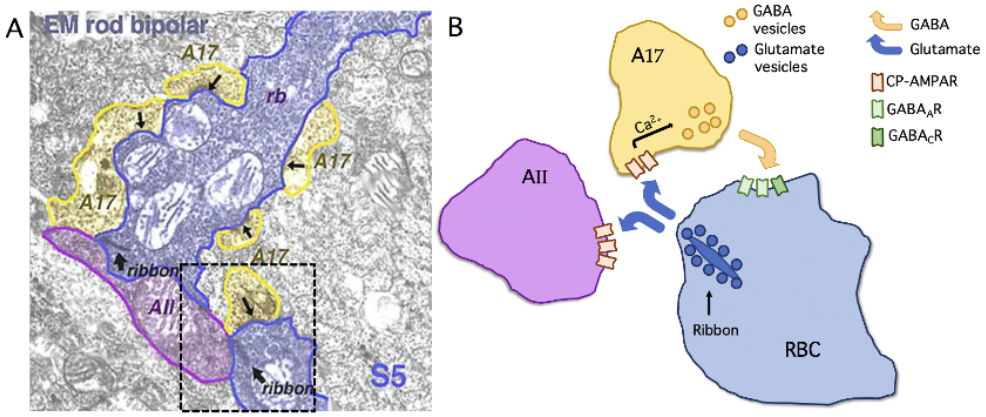

Figure 5. Ribbon synapse. **A**. Electron micrograph showing axons from a RBC (blue), A17 amacrine cells (yellow) and a process belonging to an AII amacrine cell (purple). **B**. Diagram of a magnified region of panel A, showing all basic elements of the dyad synapse formed by a RBC, an A17 and an AII amacrine cells. Panel A is adapted from webvision (http://webvision.org.es/part-iii-retinal-circuits/3-1-circuitry-for-rod-signals).

It has been demonstrated that upon synaptic release of glutamate by the RBC, calcium-permeable AMPA receptors (CP-AMPAR) are activated and carry out the immediate postsynaptic response in both AII and A17 amacrine cells (Fig, 5A,B; Singer & Diamond 2003; Veruki et al., 2003; Chávez et al., 2006). However, the presence of functional non-synaptic NMDAR conductance in both types of amacrine cells was recently reported (Zhou et al., 2016; Veruki et al., 2019). This suggests that the processing of glutamatergic inputs in these cells might not be as straightforward as previously thought. It is known from ultrastructural studies that A17 amacrine cells

receive synapses from other amacrine cells at the proximal parts of their dendrites (Nelson & Kolb, 1985) that could be GABAergic, glycinergic, or both, and AII amacrine cells receive contacts at the origin of their dendritic tree from dopaminergic neurons that could co-release GABA (Contini & Raviola, 2003). A recent study has demonstrated synaptic GABA input to AII amacrine cells from another type of amacrine cell in mice (Park et al., 2020). Yet very little is known about these inputs and how they could shape the output of these cells.

1.2.2.1 AII amacrine cells

The AII amacrine cell is a narrow-field glycinergic amacrine cell (Kolb & Famiglietti, 1974; Famiglietti & Kolb, 1975). Its cell body lays at the border between the inner nuclear layer (INL) and the IPL (Fig. 6A). The dendritic tree starts with a thick apical dendrite that descends into the IPL and shows a bi-stratified morphology (Fig. 6B): a first proximal arborization (lobular appendages) and a second more distal, closer to the ganglion cell layer (GCL; arboreal dendrites). AII amacrine cells receive their main synaptic inputs from the RBC at the arboreal dendrites (Kolb, 1979; Strettoi et al., 1990, 1992). The AII amacrine cell is the sole output of the rod pathway and provides feedforward sign-conserving signals via gap junctions to ON-cone bipolar cells (Fig. 4; Kolb & Famglietti, 1974; Strettoi et al., 1992, 1994) and sign-inverting inputs to OFF-cone bipolar cells, respectively (Kolb, 1979; Strettoi et al., 1992; Murphy & Rieke, 2006). AII amacrine cells also receive glutamate inputs from OFF-cone bipolar cells at the lobular appendages (Strettoi et al., 1992) and inhibitory glycinergic inputs, presumably from other amacrine cells (Gill et al., 2006). Moreover, AII amacrines cells are interconnected by gap junctions (Kolb & Famiglietti, 1974), forming a vast network of electrically coupled neurons (Veruki & Hartveit, 2002).

In addition to glutamate receptors (Singer & Diamond, 2003; Veruki et al., 2003), AII amacrines also express glycine, GABA and NMDA receptors (Boos et al., 1993; Zhou & Dacheux, 2004; Gill et al., 2006; Zhou et al., 2016; Veruki et al., 2019), but so far only glycine and AMPA receptors have been reported to mediate spontaneous synaptic inputs. It has been proposed that AII amacrine cells could receive GABAergic inputs from dopaminergic amacrine cells that could co-release GABA and dopamine

(Contini & Raviola, 2003; Völgyi et al., 2014). Recent research in mouse successfully identified evoked synaptic GABAergic inputs to AII amacrine cells from another interneuron that contributes to the receptive field of the AII amacrine and could potentially be a presynaptic candidate in the rat: the NOS-1 amacrine cell (Zhu et al., 2014; Park et al., 2020). There are several open questions about GABAR on AII amacrines: Are they synaptically activated? Where are they located? Are GABA receptors activated by ambient GABA and function as extrasynaptic receptors? What is the subunit composition of these receptors? What are the kinetic properties of these receptors?

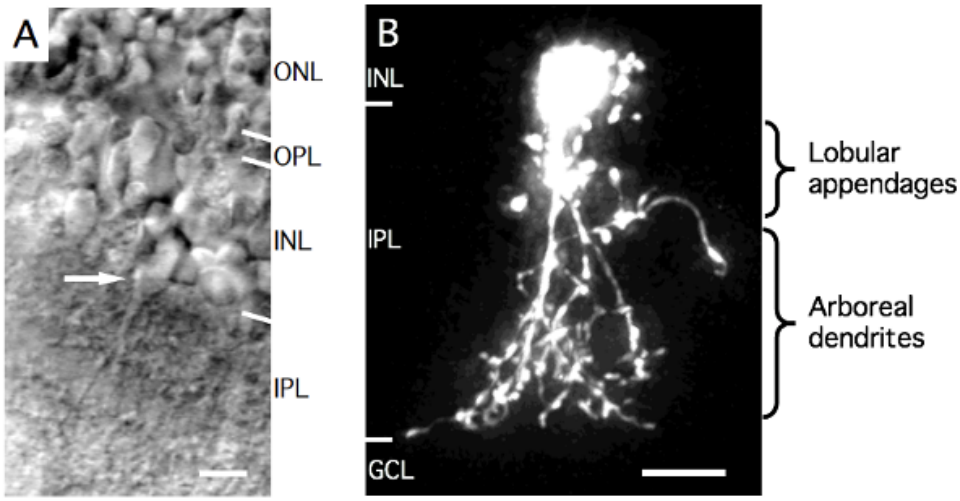

Figure 6. The AII amacrine cell. **A**. Infrared differential interference contrast (IR-DIC) videomicrograph of an AII amacrine cell (arrow) in an acutely isolated retinal slice. **B**. A fluorescence image of an AII amacrine cell filled with Alexa Fluor 594 via a patch pipette. Scale bars, 10 μm. Both panels are adapted from **Paper I**.

AII amacrines also express GluN2B-containing extrasynaptic NMDARs. These receptors are activated by ambient glutamate in AII amacrines and are located close to gap junctions (Veruki et al., 2019). There is evidence that NMDARs on AII amacrines modulate some aspects of gap junctions coupling between these cells (Kothmann et al., 2012). However, the conditions required for the activation of these receptors are still unknown.

Given the role of the AII amacrine cell in the retina as the output of the rod pathway and the connection with ON and OFF pathways, it is of interest to investigate the subunit composition and biophysical properties of GABARs and the activation of NMDARs in order to understand their contribution to signal processing and function on AII amacrine cells.

1.2.2.2 A17 amacrine cells

The A17 amacrine was initially named AI when first identified as it was one of the two postsynaptic profiles of amacrine cells that contact the RBC (Famiglietti & Kolb, 1975; Sandell et al., 1989; Strettoi et al., 1990). Similar cells were found in the rabbit (S1 and S2) and in cat (A17) (Nelson & Kolb, 1985; Sandell & Masland 1986; Vaney 1986; Wässle et al., 1987). These cells would later be referred to as A17 amacrine cells for simplicity.

The A17 is a GABAergic wide-field amacrine cell. Its dome-shaped cell body lies at the border between the INL and the IPL (Fig. 7A). Its dendrites carry distinct varicosities and terminate close to the GCL and its dendritic field covers around 400 μm (Fig. 7B). There are numerous small varicosities (about 1 μm in diameter) located along the dendrites, with ~ 20 μm separation. At the distal varicosities, located close to the GCL, the A17 amacrine cell receives the synaptic input from the RBC and produces a reciprocal inhibitory output to the same RBC (Nelson & Kolb, 1985). The varicosities of the A17 are particularly interesting, not only because of the reciprocal synapse, but also because it is thought that these varicosities are electrotonically isolated, such that signals produced in a single varicosity do not propagate along the dendrite (Grimes et al., 2010). Thus, it is possible that each varicosity can form an independent microcircuit.

The excitatory input to the A17 amacrine from RBC is predominantly mediated by CP-AMPAR (Singer & Diamond, 2003; Chávez et al., 2006). The AMPAR-mediated Ca^{2+} influx directly induces GABA vesicles release, but it also triggers calcium-induced calcium release via ryanodine receptors that contribute to vesicle release (Chávez et al., 2006). This was a surprising finding back in 2006, as it is

commonly voltage-gated calcium channels activation that couple postsynaptic depolarization and neurotransmitter release in most neurons (Katz & Miledi, 1967). At the same time, these findings are in accordance with subsequent research that suggests that the depolarization is confined and compartmentalized, allowing the A17 amacrine cell to form independent and electrotonically isolated microcircuits at their varicosities (Grimes et al., 2010). As pointed out above, A17 amacrine cells also receive synaptic (presumably inhibitory) input from other amacrine cells to their proximal dendrites, both at and between varicosities (at intervaricosity segments; Nelson & Kolb, 1985). Thus, the idea that signal processing is confined to independent microcircuits in the varicosities of A17 amacrines (Grimes et al., 2010) is challenged by this apparent segregation of distal excitatory and proximal inhibitory inputs to the A17 amacrine cell. Very little is known about the nature of these inputs or the biophysical properties of the receptors mediating such inputs.

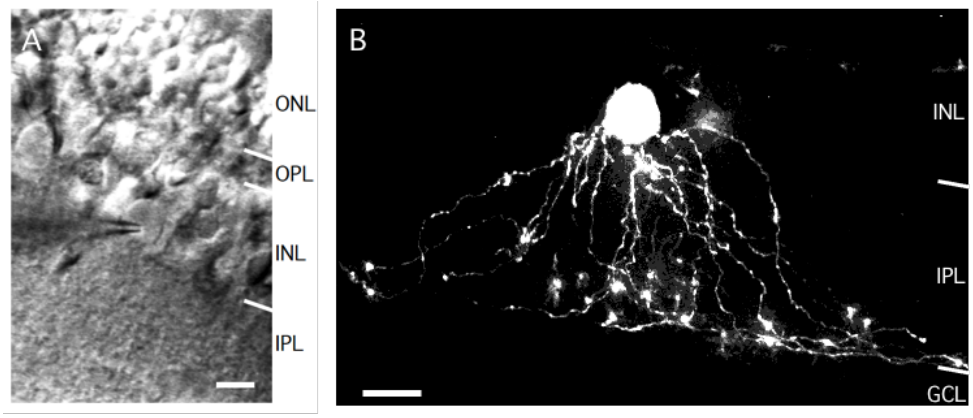

Figure 7. The A17 amacrine cell. **A**. IR-DIC videomicrograph of an A17 amacrine cell (with pipette attached) in a retinal slice. **B**. A fluorescence image of an AII amacrine cell filled with Alexa Fluor 594 via a patch pipette. Scale bars, 10 μm. Panel A is modified from **Paper I**.

A17 amacrines express GluN2A-containing NMDARs (Zhou et al., 2016; Veruki et al., 2019). The presence of non-synaptic NMDARs in A17 amacrine cells also raises some questions about their functional role. The activation of extrasynaptic NMDARs on A17 amacrines increases the baseline membrane noise (as in AII

amacrines; Veruki et al., 2019). This has also been observed in hippocampal pyramidal cells and in retinal ganglion cells before (Sah et al., 1989; Gottesman & Miller, 2003; Le Meur et al., 2007). In A17 amacrine cells, extrasynaptic NMDAR activation has been shown to contribute to the inhibitory feedback provided to the RBC (Veruki et a., 2019). However, the physiological conditions required for the activation of these receptors as well as the identity of co-agonists are not known. Investigating this will provide more insights into the functional role extrasynaptic NMDARs have in signal processing in A17 amacrine cells.

1.3 Activation and pharmacological manipulation of NMDA and GABA receptors

Understanding the structure and function of neurotransmitter receptors and the sources and spatiotemporal dynamics of the neurotransmitters that activate them will help us understand their contribution to signal processing. Providing new insights into the synaptic mechanisms that neurons use to communicate and transfer information in the retina can be further used to develop new strategies and treatments in the diseases that disrupt or affect neural communication in the retina, but also in the rest of the CNS. The presence and relevance of GABA and NMDA receptors on AII and A17 amacrines have been highlighted in previous sections.

1.3.1 NMDA receptors

NMDARs are a particular subtype of ionotropic glutamate receptor: they require the binding of a co-agonist (glycine or D-serine) in addition to glutamate, as well as a coincident depolarization of the membrane to expel the Mg^{2+} ion that blocks the channel at a resting membrane potential (Fig. 8; Johnson & Ascher, 1987; Kleckner & Dingledine, 1988; Lerma et al., 1990; Schell et al., 1995; Mothet et al., 2000). Moreover, they are highly permeable to Ca^{2+}, so the activation of NMDARs is related to a wide range of intracellular signals that can lead to completely opposite events that will depend on the subunit composition of the receptor. This includes LTP and LTD, cell death and excitotoxicity, but also cell survival, etc. (Papouin & Oliet, 2014).

Structurally, NMDARs are heterotetrameric proteins, and they are assembled by two mandatory GluN1 subunits together with two GluN2 subunits or a combination of GluN2 and GluN3 subunits (Fig. 8; Schorge & Colquhoun, 2003; Ulbrich & Isacoff, 2008). There are four subtypes of GluN2 subunits (GluN2A-D) that determine the biophysical properties of these receptors (kinetics, affinity for agonists and co-agonists, sensitivity to Mg^{2+}, Ca^{2+} permeability, etc.; Monyer et al., 1994; Cull-Candy et al., 2001; Neyton & Paoletti, 2006; Paoletti & Neyton, 2007; Paoletti et al., 2013). An often-proposed idea is that GluN2A-containing receptors are located at synapses, whereas GluN2B-containing receptors would be mainly extrasynaptic (Dalby & Mody, 2003). Although this is the case in some brain areas (Tovar & Westbrook, 1999; Groc et al., 2006; Martel et al., 2009), there are other examples where this distribution is different (Scimemi et al., 2004: Harris & Pettit, 2007; Petralia et al., 2010; reviewed in Paoletti et al., 2013). Previous investigations indeed found that in AII and A17 amacrine cells in the rat retina, GluN2A and GluN2B subunits expressed by these cells were exclusively extrasynaptic (Veruki et al., 2019).

The different NMDAR subunits expressed in AII (GluN2B subunit) and A17 (GluN2A subunit) amacrines suggest a different spatiotemporal processing of glutamate inputs. NMDARs have been proposed to modulate the strength of gap junctions coupling in AII amacrines (Kothmann et al., 2012). In A17 amacrines, they seem to contribute to GABA release (Veruki et al., 2019), although the mechanisms via which they do so are unclear. Evidence of ambient glutamate activating these receptors also raises some questions about the source of glutamate and the co-agonists that activate them. This information could help us to understand the conditions required for the activation of these receptors and provide more insights into their physiological role.

The presence of neurotransmitters in the extracellular environment has been discussed above (see section **1.1.2**). Both synaptic spillover and/or glial release of glutamate could activate NMDARs on AII and A17 amacrine cells. In the retina, spillover of glutamate at the rod bipolar cell terminal has been previously reported (Veruki et al., 2006), and activation of NMDARs by synaptic spillover of glutamate

has also been found in retinal ganglion cells (Chen & Diamond, 2002). There is also evidence that Müller cells (the main glial cell type in the retina) actively release neurotransmitters (Vijay, 1983; Loiola & Ventura, 2011), including glutamate (Zhang et al., 2019).

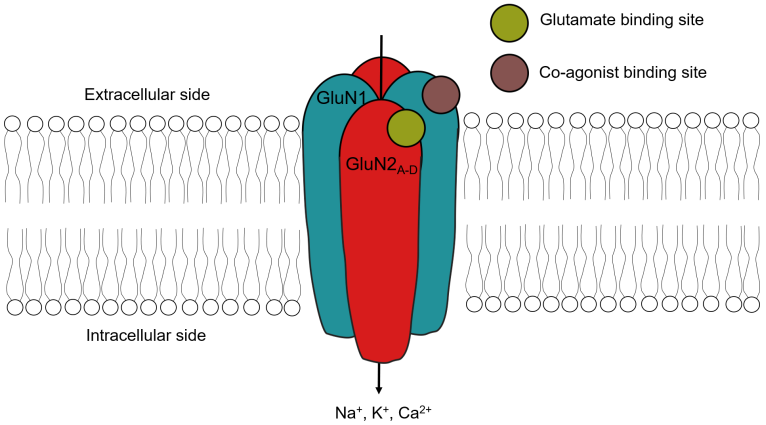

Figure 8. NMDA receptor. Schematic diagram of an NMDAR in the membrane, including the main subunits discussed in this thesis and the binding sites for glutamate and the co-agonist.

To investigate the source(s) of glutamate that activate extrasynaptic NMDARs in both AII and A17 amacrine cells, we used two pharmacological agents that block the glutamate cycle at different points. We used Bafilomycin A1, a vacuolar-type H^+-ATPase inhibitor, that prevents the loading of neurotransmitters into synaptic vesicles (Dröse & Altendorf, 1997). Eliminating the possibility of synaptic release of neurotransmitters should lead to a decrease in NMDA activation if the source of glutamate is neural. We also used L-methionine-sulfoximine (MSO), an inhibitor of the glutamine synthetase (Ronzio et al., 1969), the enzyme that converts glutamate into glutamine in glial cells (see Hamberger et al., 1979). This enzyme is essential in the glutamate cycle, as it allows neurons to obtain glutamine from glial cells that will then be transformed into glutamate in neurons again. Thus, MSO should deplete glutamate from neurons, while glial cells should not be affected. This could be reflected as a

decrease in NMDA activation if the glutamate source is neural, but no changes in activity if the glutamate source is glial (Le Meur et al., 2007).

1.3.2 GABA receptors

GABARs are classified into two categories: ionotropic receptors ($GABA_A$) and metabotropic ($GABA_B$), being $GABA_A$ the major receptor type in the brain. $GABA_A$Rs are chloride ion channels composed of five subunits, with most of these receptors composed of two α, two β and one auxiliary subunit (γ, δ, ε, θ and π; Fig. 9; Farrant & Nusser, 2005). There is another subtype of ionotropic GABAR formed by the ρ subunit that has been traditionally considered a different subtype, the $GABA_C$R (Drew et al., 1984; Feigenspan et al., 1993; for review see Bormann & Feigenspan, 1995). However, ρ subunit expression seems to be restricted to bipolar and horizontal cells in the retina (Feigenspan et al., 1993; Enz et al., 1996), and therefore I will not further discuss them in this section. The most abundant $GABA_A$R subunit combination found in synapses is $\alpha_1\beta_x\gamma_2$ (Sieghart & Sperk, 2002), although assemblies with $\alpha_{2,3}$ are also common in some brain areas (McKernan & Whiting, 1996). The wide presence of γ subunit in synapses is due to their role in anchoring the receptor to the proteins at the postsynaptic density (Lüscher & Keller, 2004) whereas other subunits, like δ, are only found extrasynaptically (Nusser et al., 1998; Farrant & Nusser, 2005).

GABA binding site is located at the interface between α and β subunits (Fig. 9; Bauman et al., 2003). Because $GABA_A$Rs are composed of two α and two β subunits, they have two functional agonist sites and efficient gating of this receptor requires both sites to be occupied by GABA (Bauman et al., 2003). Additionally, $GABA_A$Rs can be modulated by a variety of pharmacologically and clinically relevant drugs, such as benzodiazepines, steroids, anesthetics, barbiturates and convulsants that bind to the receptor via different binding sites (reviewed in Sieghart, 2015). Of the many binding sites for different drugs at $GABA_A$Rs, perhaps the most studied is the benzodiazepine (BZ) binding site for its relevance in the past century. BZs bind to the interface between α and γ subunits (Sigel & Buhr, 1997), therefore the presence of γ subunit, specifically the γ_2 subunit, is a requirement for the correct interaction between BZs and the

$GABA_AR$ (Pritchett et al., 1989). BZs and other drugs that interact with $GABA_ARs$ are very relevant for the work outlined in this thesis and are further described in section **3.4.3**.

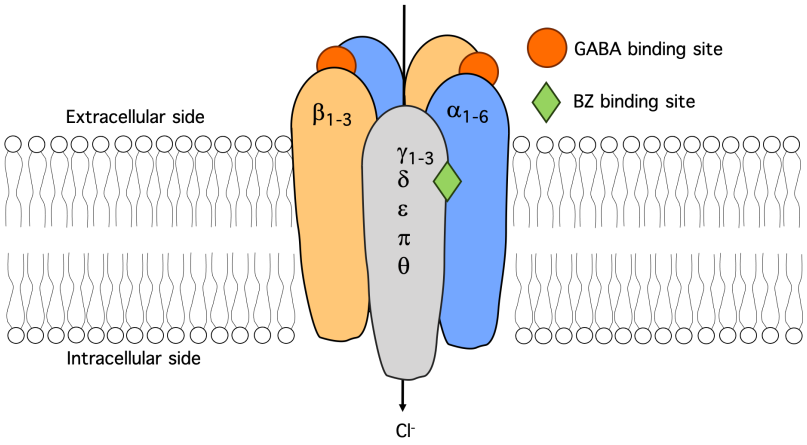

Figure 9. $GABA_A$ receptor. Schematic diagram of a $GABA_AR$ and its subunits in the membrane. Both agonist and BZ binding sites are highlighted.

In the retina, there is evidence for the presence of $\alpha_{1\text{-}4,6}$, $\beta_{1\text{-}3}$, $\gamma_{1,2}$, and δ subunits (Greferath et al., 1995; Gutiérrez et al., 1996; Khan et al., 1996; Wässle et al., 1998; Gustincich et al., 1999). However, there is very little evidence of precise $GABA_ARs$ subunit expression on specific neurons, with the exceptions of dopaminergic cells (Feigenspan et al., 2000) and horizontal cells (Feigenspan & Weiler, 2004). Little is known about the nature of the GABAergic inputs and the molecular identity, subcellular localization and function of the receptors mediating these signals in AII and A17 amacrine cells.

Understanding the biophysical properties, subunit composition and the conditions required for the activation of GABA and NMDA receptors will help us to understand the role these receptors play in signal processing in AII and A17 amacrine cells. In this thesis, we have investigated these scientific questions combining both electrophysiological and pharmacological approaches.

2. Aims of the thesis

The aim of this thesis is to increase our understanding of the contribution of synaptic and extrasynaptic inputs to signal processing in the two main interneurons of the rod pathway of the mammalian retina, the AII and the A17 amacrine cells. Specifically, we wanted to study the biophysical and pharmacological properties of synaptic and extrasynaptic receptors present on these amacrine cells. We wanted to determine the conditions required for the activation of extrasynaptic NMDARs in both AII and A17 amacrine cells by identifying the sources of glutamate and the identity of the co-agonist that activate these receptors. We also aimed to characterize potential synaptic and extrasynaptic inhibitory inputs to these two inhibitory interneurons, and to determine the kinetic properties and subunit compositions of the receptors that mediate them. The results outlined in this thesis are important to increase our knowledge of signal processing in AII and A17 amacrine cells of the retina, but they will also be useful to better understand synaptic and extrasynaptic neurotransmission elsewhere in the central nervous system.

3. Methodological considerations

The primary methods used in this thesis are based on cellular electrophysiology, more specifically patch-clamp recordings from visually identified neurons in rat retinal slices. Here I will discuss this technique and explain why it is the most relevant and powerful technique for my work.

3.1 Ethics statement

The use of animals in this study was carried out under the approval of and in accordance with the regulations of the Animal Laboratory Facility at the Faculty of Medicine at the University of Bergen (accredited by AAALAC International).

3.2 Retinal tissue preparation and targeting of amacrine cells

Retinas were dissected from female and male albino rats (Wistar HanTac), aged between 4 and 7 weeks old. First, rats were exposed to oxygen 100% for 10 minutes, followed by deep anesthesia with isoflurane. Animals were sacrificed by cervical dislocation. Afterward, eyeballs were quickly removed and placed in a dish that had been previously filled with cold 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffered solution (145 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Na-HEPES, 10 mM Glucose; pH adjusted to 7.4 with HCl). Using a low magnification microscope for dissection, we cut around the eyeball, right below the *ora serrata*, and the lens and cornea were removed. We then removed the vitreous with forceps, and carefully separated the retina from the sclera. The retina was cut into 4 quadrants, and each quadrant was placed in a thin piece of lens paper and transferred

to an interface chamber containing Ames' solution which was being constantly bubbled with 95% O_2 and 5% CO_2 gas. Retinal quadrants remained viable for at least 12h.

Before patch-clamp recordings, retinal quadrants were cut into thin slices (150 – 200 μm) by hand and then transferred to the recording chamber where they were held in place using a platinum iridium grid (Fig. 10A). Because targeting the right cells in our preparations is critical, the slices were visualized using an Axioskop 2 FS (Zeiss) with a 40x water-immersion objective and infrared differential interference contrast (IR-DIC) videomicroscopy. This type of microscopy allows enhancing the contrast of unstained and transparent samples such as ours (Fig. 10B). A17 amacrine cells were identified by their dome-shaped soma laying at the border of the INL with the IPL (Fig. 10C). AII amacrine cells have a thick primary dendrite that descends into the IPL, while the body remains positioned at the border between INL and the IPL (Fig. 10C). Each cell type was visually confirmed after the recording by imaging the cells under UV light and the fluorescent dye Alexa 594 (40 μM, Invitrogen), which had been added to the pipette solution. All recordings where performed at room temperature (22 – 25 °C).

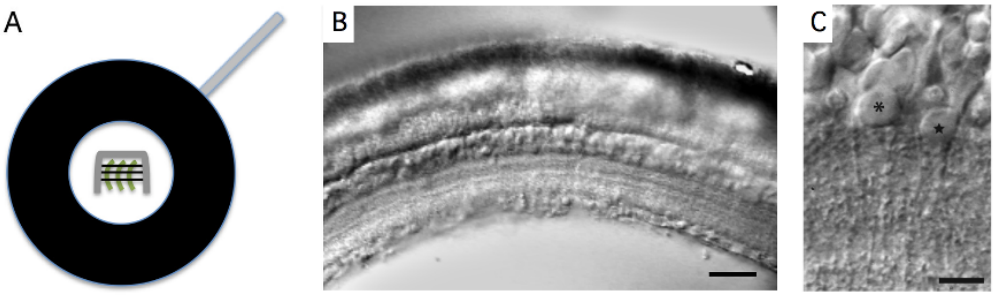

Figure 10. The recording chamber. **A** Schematic diagram of the recording chamber used during the experiments carried out in this thesis. Notice that the slices (green lines) are fixed in the chamber by placing on top a U-shaped grid (grey) with nylon threads. **B**. IR-DIC videomicrogaph of a retinal slice. Scale bar, 50 μm. **C.** IR-DIC videmicrograph of a retinal slice with cell bodies of an A17 (asterisk) and an AII (star) amacrine cell. Scale bar, 10 μm. Panels A and B are adapted from Hartveit & Veruki, (2019). Panel C is adapted from Castilho et al. (2015).

There are many reasons why we chose slices over other preparations for patch-clamp experiments. It is easier to access the cells and manipulate both the extracellular

and the intracellular fluids, as opposed to *in vivo* experiments. Slices also preserve cell and circuitry structure, and synaptic connections are not disrupted as opposed to cell cultures. On the other hand, the slicing process involves cutting some processes or dendrites, and cells that have a wide field arborization such as A17 amacrine cells can be affected. To ensure the integrity of the cell, we always aimed at cells located deeper in the tissue.

3.3 Electrophysiological methods to study ion channels

The scope of this thesis, the results obtained during the years I have worked on it and the expertise I have acquired during these years involve the use of electrophysiological methods to study cells, their signals and synaptic and extrasynaptic receptors. Therefore, I consider it important to introduce a few fundamental aspects of electrophysiology and the primary technique used in this thesis, the patch-clamp technique.

The bilayer of phospholipids that compose the membrane insulates the cytoplasm from the extracellular solution and creates an effective barrier to charged molecules: it is sufficiently thin (3 – 5 nm) that it allows positively charged particles (cations) from the outside to attract negative ions (anions) in the cytoplasm, but at the same time thick enough such that the particles do not interact directly. Charges are accumulated or stored at each side of the membrane, which is the definition of a capacitor.

The capacitance (C) is a measure of the capacity of the cell membrane to store charges, and it is defined by the following equation:

$$C = \frac{A\,\varepsilon_r}{d}$$

The more membrane surface area (A) a cell has, the more charges it can store. The dielectric constant (ε_r) refers to the ability of the components of the membrane to store charges, and it is similar throughout cells. Finally, the thickness of the membrane (d) turns out not to be much of a variable at all, as changes in thickness are negligible

for all animal cells. Thus, the capacitance of a given cell provides a good estimation of the only variable value of this equation: the membrane area under investigation.

Because of the differential distribution of charges, the outside of the cell is more positively charged than the inside due to the large number of anions present in proteins, among other things. The difference of charges between the inside with respect to the outside of the cell is called membrane potential (E_m), and the outside of the cell is always used as the reference point. C can also be described then as:

$$C = \frac{g}{V}$$

Where g is the number of charges and V is the potential measured in volts. This means that the higher the C is, the more charges can be stored by the capacitor at a given V, or stated conversely, the more charges are required to attain a given V. If we send a current through a capacitor (I_C) it will result in a change of the potential over the capacitor (Fig. 11A) that follows this equation:

$$V\,(t) = \frac{I_C}{C}t$$

Changes in V at a given time (t) depend on the relationship between the I_C and the capacity to store charges or C over time.

The concentration gradients generated by the differential distribution of ions induce the diffusion of particles from higher to lower concentration because of the tendency of particles to spread equally over a given space, reaching an equilibrium point. The equilibrium point of a given ion can be defined by the Nernst equation:

$$E = \frac{RT}{zF}\ln\frac{[ion]_o}{[ion]_i}$$

Where E is the equilibrium potential for the ion under consideration, R is the universal gas constant (8.31 J mol^{-1} K^{-1}), T is the temperature in Kelvin, z is the oxidation state of the ion under consideration, F is the Faraday constant (9.65 x 10^4 C

mol^{-1}) and $[ion]_o$ and $[ion]_i$ are extracellular and intracellular concentrations of the ion being considered, respectively.

Because there is an uneven distribution of charges, ions are not in balance on either side of the membrane, and the inside is more negatively charged than the outside at rest, generating the E_m described above. Under these circumstances, if the plasma membrane were only permeable to potassium, the E_m would follow the E for potassium (E_K), typically -80 to -90 mV. But in physiological conditions, the plasma membrane is permeable to a lesser extent to more ions that have a more positive E than E_K. At rest, E_m is typically around -60 mV.

3.3.1 Ion channels and Ohm's law

Because the lipidic bilayer is semipermeable (see section **1.1.2**), ions cannot freely flow across the membrane. Instead, specialized proteins embedded in the membrane facilitate ion movements through pores or channels. These ion channels hold several properties that make them highly effective in this task: (1) an aqueous pore that connects both the intra- and extracellular environments through which ions will diffuse and (2) a gating mechanism that can open and close the pore. This mechanism can be voltage-dependent, and channels will open or close depending on the E_m, or ligand-gated channels, where channels open and close upon binding of specific molecules such as neurotransmitters. (3) A selectivity to specific ions that make ion channels more permeable to some ions than others. This last property is based on the size and charge of the ion that interacts with specific protein residues in the channel pore.

Ions flowing through protein channels in the membrane are the equivalent to current through a resistor: ions do not diffuse freely but with a certain degree of resistance (R) because of the intrachannel environment. The change in the distribution of charges results in a change in E_m. Charge movement across the membrane results in a current (I) that is inversely proportional to R: if there are more channels open (low R), more ions (more I) can flow through the membrane at a given E_m. This may sound rather strange, but "resistance" is a misleading term. It helps to think of resistors as

leakages of current (conductance): the more leakages, the more conductance, the lower the total resistance. In summary, the current *I* through a resistor is proportional to the potential (*E*) across it, and inversely proportional to the resistance *R*. This is Ohm's law:

$$I = \frac{E}{R}$$

Following Ohm's law, if we send a current through a resistor (I_R), the relationship between I_R and voltage (V) would look like in Fig. 11B. The higher the *R*, the less current generated by a given potential and the result is a square pulse that follows this equation:

$$V(t) = I_R\, R$$

Cell membranes function as a capacitor and ion channels as resistors. In this case, the response in *V* to a current injected through the membrane (I_m) is the result of the combination of the two curves described before as seen in Fig. 11C that follows this equation:

$$I_m = I_R + I_C$$

$$I_R = \frac{V(t)}{R} \qquad I_C = C\frac{V(t)}{t}$$

$$I_m = \frac{V(t)}{R} + \frac{V(t)}{t}C$$

This equation can be solved as

$$V(t) = I_m R\,(1 - e^{-t/RC})$$

At $t = 0$, $e^0 = 1$, so $V(t) = 0$.

At $t = \infty$, $e^{\infty} = e$ and $e^{-t/RC} = e^{-\infty} = 1/e^{\infty} = 0$, so $V(t) = I_m R = V_{max}$

At t = RC, $e^{1} = 2.72$ and $e^{-1} = 1/2.72 = 0.37$, so $V(t) = 0.63\ I_m\ R$

RC is the time when 63% of V_{max} is reached, and this is called membrane time constant (Fig. 11D). The concept of time constant (τ) can be applied in other situations, and it is typically used to measure other biophysical properties such as decay kinetics of ion channels (see section **3.6**), which provides valuable information on neurotransmitters receptors kinetics.

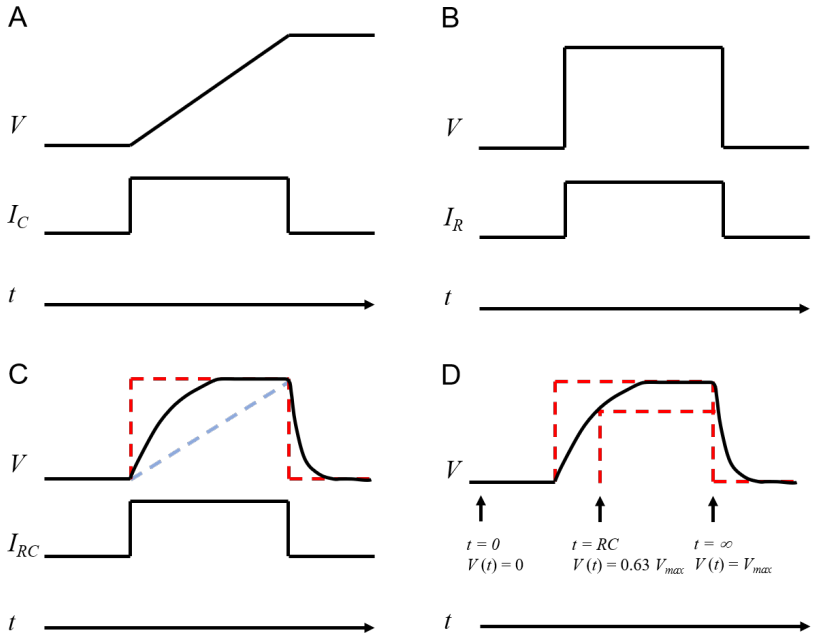

Figure 11. Passive membrane properties of a patch of a membrane. **A**. Voltage response (top) of current sent through a capacitor (middle) over time (bottom). **B**. Voltage response (top) to a current sent through a resistor (middle) over time (bottom). **C**. Voltage response (top) to a current sent through an RC-circuit (middle) over time (bottom). Dashed blue lines indicate the response if only C had been taken into account (as in panel A), and dashed red lines indicate the response if only R had been into account (as in panel B). **D**. Voltage response (top) to a current sent through an RC circuit (as in panel C) over time (bottom) where the equation represented in panel C has been derived.

Studying electrical properties of neurons is of great interest in neuroscience, as changes in the E_m of neurons encode information in the CNS. Early techniques such as voltage-clamp recordings led to the discovery of the ionic basis of action potentials

(Hodgkin & Huxley, 1952; reviewed in Schwiening, 2012). However, the high-resistance electrodes required to perform this technique provided a poor electrical access to the intracellular environment and prevented the study of currents generated by individual ion channels. The development of the patch-clamp technique (see **section 3.4**; Hamill et al., 1981; Sakmann & Neher, 1984) refined the voltage-clamp technique. Here, the recording electrodes are used to seal onto the cell membrane. This results in a high resistance seal (gigaohm seal or gigaseal; Hamill et al., 1981) that ensures that any current generated by any ion channel flows into the recording pipette and thus, recorded by the electrode. The electrode is connected to a patch-clamp amplifier that digitalizes the analog physiological signal. The patch-clamp technique and the various recording modes used in this thesis are further discussed in the next sections.

3.4 The patch-clamp technique

The patch-clamp technique was developed about 40 years ago by Erwin Neher, Bert Sakmann and colleagues (Hamill et al., 1981; Sakmann & Neher, 1984), and as of today, no other technique has been able to substitute it. The advantage of this technique is that the larger opening at the tip of the recording electrode provides a lower resistance, and thus better electrical access to the inside of the cell than the one obtained by high-resistance electrodes.

Other techniques have been developed in the past years that allow us to study the excitability of cells, such as calcium imaging and voltage-sensitive dye imaging. As important and useful as these techniques are, neither of them are able to achieve the temporal resolution of patch-clamp: whereas calcium and voltage-sensitive dye imaging can achieve a maximum time resolution of a few milliseconds (ms), patch-clamp amplifiers can record signals with a frequency of tens of microseconds (μs). This is important because many relevant features of synaptic signals happen within the μs range. The peak response of AMPA receptors can take place in about 300 μs following agonist exposure (Veruki et al., 2003). Although these receptors have particularly fast kinetics, this is also valid for receptors with slower kinetics such as GABA or NMDA

receptors, making patch-clamp the only technique developed so far that is able to detect the full range of activity of these signals.

There are different patch-clamp configurations that allow scientists to study different properties of excitable cells and ion channels, making this technique very versatile. In the next sections, I will discuss the different recording configurations I have used.

3.4.1 Whole-cell recording

Establishing a whole-cell configuration of the patch-clamp technique will be essential for all electrophysiological experiments performed during this thesis, as it will be used as a recording mode but also as a first step towards the other recording mode used in this thesis: the outside-out configuration.

The patch pipette containing the intracellular solution is lowered to the surface of the cell while applying positive pressure (~10 mBar). This positive pressure has two functions: prevent dirt from the tissue from obstructing the tip of the pipette and gently squeeze the membrane of the cell as the pipette is lowered and placed onto the membrane. There are several clues that can be used as a signal to release the pressure: some researchers use the visual input from the TV monitor to see when the pipette is close enough and it is creating and invagination of the membrane by squeezing the cell. Some others use the electrophysiological input, which is an increase in the resistance reading on the amplifier monitor. Once the pressure is released, the membrane goes back to its natural place and if the pipette is in the correct position, the membrane will contact the tip of the patch pipette and will form a seal (see Fig. 12).

Having the pipette positioned correctly, the membrane will stick to the glass walls of the pipette forming a tight seal, but if this is not the case, light suction can be applied to gently suck the membrane into the pipette tip. This tight seal between the lipid bilayer and the glass pipette tip is called the “gigaseal” or the “gigaohm (GΩ) seal” because of the omega shape the membrane acquires with the tip of the pipette, which at the same time, creates a 1-100 GΩ resistance that can be observed in the

amplifier monitor window. The tighter this seal is the better, so it is often recommended to wait between 30 seconds and 1 minute to make sure the membrane of the cell and the pipette formed a proper gigaseal.

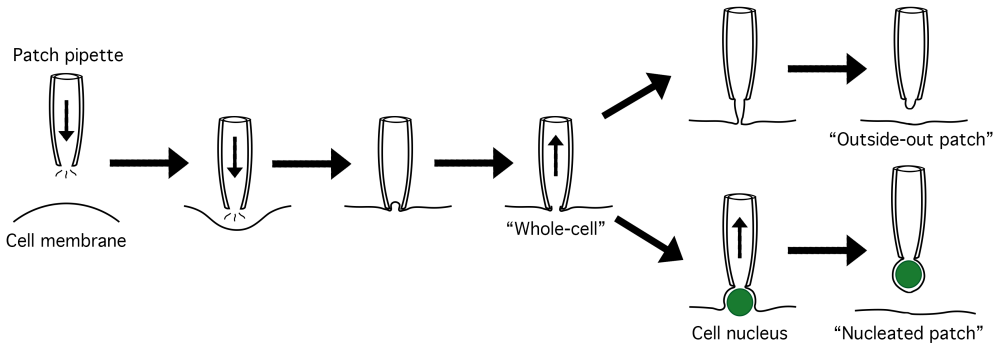

Figure 12. Different recording configurations used during this thesis and how to obtain them. Diagram showing step by step how to establish a whole-cell recording and obtain conventional or nucleated outside-out patches.

The next step is the rupture of the membrane, which can be achieved by applying suction pulses or by applying brief depolarizing pulses called ZAP. As described in Molleman (2002), suction must be carefully applied without excessive force at the beginning. If the membrane does not break, a combination of mild ZAP stimulus together with suction pulses can be used. As a last resource, should the membrane still resist our efforts, Molleman (2002) recommends "incrementing ZAP pulses while applying strong suction until the patch breaks, the seal breaks, the amplifier starts to smoke, or the scientist's fillings come loose." Hopefully, the membrane breaks at some point, and thus the whole-cell patch-clamp configuration can be established (Fig. 12). The intracellular solution will slowly diffuse into the inside of the cell and replace the cytoplasm, thus allowing us to modify and control the intracellular composition, one of the many more reasons this technique is so versatile.

Turning our focus on the biophysical aspects, two recording modes can be used following the previously described Ohm's law (**section 3.3**) where voltage (V) equals the amplitude of the current (I) multiplied by the resistance (R) of the circuit ($V = I \times R$). First, with current-clamp mode voltage changes are measured upon current

injections into the cell. Second, voltage-clamp mode, where the voltage of the cell membrane is held at a constant value and the magnitude of the current flowing across the membrane via ion channels is measured. In this thesis, only voltage-clamp has been used.

3.4.2 Outside-out and nucleated patch recording

To understand the physiological and functional properties of a given synapse, it is important to understand the functional properties of the ion channels activated in it. Most of the synapses are located far away from the soma, which is the part of the cell where we usually place our recording electrode. Investigating receptors that are being activated far away from our electrode might be challenging due to two phenomena: (1) signals that occur far away from a synapse can be electrotonically filtered, meaning that signals recorded at the soma will be slowed and reduced in amplitude relative to the signals generated locally at the synaptic points; and (2) the degree of voltage-clamp at distant areas might be poor, resulting in a phenomenon referred to as space-clamping problem (Molleman, 2002).

One solution would be to perform the recording closer to synaptic contacts, like axonal or dendritic recordings. This is particularly difficult in the retina due to the densely packed layers where dendrites and axons from cells are very thin (~0.25 μm) and indistinguishable from each other. Even if it were feasible, there are still other limitations that include the spatial restriction for other synaptic inputs generated elsewhere, a worse voltage-clamp condition due to the high resistance electrodes that would be needed to patch and record thin processes like dendrites, and the lack of control over intrinsically spontaneous synaptic events.

Another approach is to use small portions of excised membrane from the cell or outside-out patches containing receptors (Hamill et al., 1981), such that activation conditions are now controlled, and the space-clamp problem is removed from the equation. Agonists can be applied to patches with different techniques to study channel responses and obtain biophysical and functional information from ion channels. Modulators of those channels can also be applied using the same systems to modify

channel behavior and study the impact of drugs on ion channels. This can be useful to study their subunit composition as described later in this thesis.

As mentioned in the section before, obtaining an outside-out patch begins with the whole-cell configuration. Once this configuration is established, the recording pipette is slowly withdrawn while the cell is still in whole-cell configuration until the membrane is excised, with the outside part of the membrane facing outward from the pipette and the intracellular solution acting as cytoplasm (Fig. 12). In cases where the density of receptors that are going to be studied is low, outside-out patches might not contain enough receptors and responses might rapidly fade due to the rundown of receptors. There is a variant of the outside-out patch that has also been used in this thesis: the nucleated-patch. The process is roughly identical, except that while withdrawing the pipette, light negative suction (~ - 50 mBar) is applied to attract the soma of the cell to the pipette tip. Thus, the nucleus of the neuron surrounded by cell membrane will be isolated from the rest of the cell, forming a giant nucleated outside-out patch or nucleated patch (Fig. 12). The advantage of this patch is the larger membrane surface that can be exposed to agonists and drugs, together with the greater stability due to the structural support provided by the enclosed nucleus (Sather et al., 1992). Previous studies have not found significant differences in kinetics between outside-out and nucleated patches in AMPA receptors (Vandenberghe et al., 2000). However, we have restricted the use of nucleated patches to pharmacology experiments and to those situations where we wanted to maximize stability and duration of responses (see **Paper III**). In my experience, this is because recordings from outside-out patches tend to be less noisy and therefore more suitable for kinetic analysis, but also because the agonist cannot be applied to all receptors simultaneously in a nucleated patch (see section **3.4.4**).

3.4.3 Pharmacological tools

To investigate the physiology of receptors it is often needed to isolate their activity, which can be achieved by pharmacological blockade of other receptors that might be activated at the same time in the cell. In other situations where the aim is to

study the biophysical properties and subunit composition of those receptors, it is important to control the moment when the agonist is being released, which can be achieved by using advanced perfusion systems. It is also of interest during these studies to apply drugs that modify the activity and/or that are selective for specific receptor subunits. In this thesis, we will often use a combination of agonists and antagonists of different receptors to study their properties, and in this section, I will explain the choice of drugs we made and the methods we used to apply them. The use of pharmacological agents is one of the most reliable methods to modify ion-channel activity and/or to characterize their subunit composition using electrophysiology. Whereas other methods like immunostaining of receptor subunits have been widely used for subunit characterization, it has been often argued the lack of control over immunoreactivity with the protein of interest as a disadvantage and this could result in a false positive signal. Used in electrophysiology, pharmacological agents that modify ion channels activity can be very precise and provide more accurate results. These experiments also have a disadvantage: most of the drugs are initially tested on heterologous expression systems such as oocytes or HEK cells, and results depend on the correct expression of the specific subunit combinations, which can be challenging. Most likely, a combination of these methods would yield the best results.

For pharmacological experiments where the exchange time between solutions was not critical, drugs were applied directly to the extracellular perfused solution.

3.4.3.1 General pharmacological

In this thesis, we have used pharmacological agents during electrophysiological recordings to investigate the biophysical properties of ion channels and their subunit composition.

To study synaptic receptors, it is a requirement to isolate their activity. To do so, we used a combination of drugs to block synaptic receptors and voltage-gated channel's activity, including (in μM) 10 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block AMPA receptors, 1 tetrodotoxin (TTX) to block Na_v^+ channels, 0.3 strychnine to block glycine receptors, 10 bicuculline or 3, or 10 SR95531 to block

$GABA_A$Rs and 20 (RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) to block NMDARs.

3.4.3.2 Drugs that modulate $GABA_A$ receptors

To study the properties of $GABA_A$Rs, we used specific drugs aimed at different $GABA_A$ subunits that modify the receptor's activity in a differential manner. Zn^{2+} is a potent inhibitor of $GABA_A$Rs (Draguhn et al., 1990; Smart et al., 1991) and sensitivity to Zn^{2+} inhibition (IC_{50}) is modulated by the subunit composition: 88 nM at αβ receptors, 1-20 μM at αβδ and >50 μM at αβγ (Draguhn et al., 1990; Saxena & Macdonald, 1996; Mortensen & Smart, 2006). At αβγ $GABA_A$Rs, sensitivity to Zn^{2+} inhibition also varies depending on the α subunit present, being α_1 subunit less sensitive (IC_{50} = 245 μM) compared to other α subunits (IC_{50} = 50 – 80 μM) (Draguhn et al., 1990, Smart et al., 1991). Thus, Zn^{2+} can be used to obtain information about the presence of α and γ subunits, and it can help to distinguish between α_1 and other α subunits.

Zolpidem is a $GABA_A$Rs agonist and it potentiates $GABA_A$ responses at low concentration only on α_1,γ_2-containing receptors (Pritchett & Seeburg, 1990; Wafford et al., 1993; Criswell et al., 1997; Cope et al., 1999; Dämgen & Lüddens, 1999). As a BZ, zolpidem potentiates $GABA_A$Rs responses at nanomolar concentrations if γ_2 subunit is present (Puia et al., 1991; Dämgen & Lüddens, 1999), but the sensitivity to zolpidem also varies depending on the α subunit expressed, being α_1-,γ_2-containing receptors more sensitive (EC_{50} ~ 19 – 57 nM) than any other subunit combination (Pritchett & Seeburg, 1990; Wafford et al., 1993). Other subunits combination including γ and α subunits are less sensitive to zolpidem (EC_{50} at $\alpha_2\gamma_2$ ~ 450 nM, EC_{50} at $\alpha_3\gamma_2$ ~ 400 nM; Pritchett & Seeburg, 1990; Wafford et al., 1992; Perrais & Roper, 1999; Sieghart, 2015).

4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol (THIP, gaboxadol) was used during this thesis to test the presence of δ-containing receptors. This subunit is only found extrasynaptically and it tends to co-assembly with $\alpha_{4,6}$ subunits (Laurie et al., 1992; Jones et al., 1997; Korpi et al., 2002). The particularity of THIP is that is a full agonist

at $GABA_ARs$ by itself, it does not require the binding of GABA. Moreover, it is a super agonist at $\alpha_{4,6}\beta\delta$ receptors (Stórustovu & Ebert, 2003, 2006), and THIP application at concentrations $\leq 1\ \mu M$ can unequivocally activate δ-containing receptors (Jia et al., 2005; Marowsky & Vogt, 2014).

3.4.3.3 Manipulation of neurotransmitter release and sources

In **Paper I**, we wanted to investigate the activation of extrasynaptic NMDARs. We used drugs that disrupt the glutamate cycle at different points and NMDA co-agonists so that we could investigate how these receptors are being activated. Bafilomycin A1 is a toxin that inhibits the Vacuolar-type H^+-ATPase that mediates the loading of neurotransmitters into synaptic vesicles, thus preventing glutamate from being released (Dröse & Altendorf, 1997). MSO inhibits the glutamine synthetase, the enzyme that transforms glutamate into glutamine in glial cells (Ronzio et al., 1969). These two drugs have been used to identify the sources of glutamate that activate NMDARs (Le Meur et al., 2007; Wu et al., 2012). We also used DL-*threo*-β-Benzyloxyaspartic acid (TBOA), a glutamate-transporter inhibitor (Shimamoto et al., 1998). D-Aminoacid oxidase (DAAO) was used to remove D-serine from our preparations (Dixon & Kleppe, 1965; Molla et al., 2006) and test if D-serine acted as an endogenous co-agonist at these receptors.

In **Paper III**, we attempted to evoke presynaptic release of neurotransmitters under different conditions. We stimulated presynaptic terminals by local application of sucrose (0.5 M; Bekkers & Stevens, 1995; Yu & Miller, 1995) or high-K^+ (Hartveit, 1996; Mørkve & Hartveit, 2009) solutions. We applied these solutions via a puffer pipette or a theta tube pipette, as indicated.

3.4.4 Ultrafast perfusion system

Studying biophysical properties of receptors using outside-out patches necessarily implies the need for a system that mimics the temporal profile of neurotransmitters in a given synapse. At a synapse, neurotransmitters are released into the synaptic cleft in a concentration within the mM range and the fastest

neurotransmitters, the amino-type like glutamate, GABA or glycine, can travel from the pre- to the postsynaptic membrane in less than 1 ms (Clements, 1996; Veruki et al., 2003; Scimemi & Beato, 2009). Neurotransmitters are then removed from the synapses so that receptors are phasically but not tonically activated. Mimicking these conditions represents a challenge because it is necessary to apply the transmitters as transient pulses as rapidly as at synapses. Such a fast exchange of solutions can only be achieved on excised membrane patches and not in whole cells.

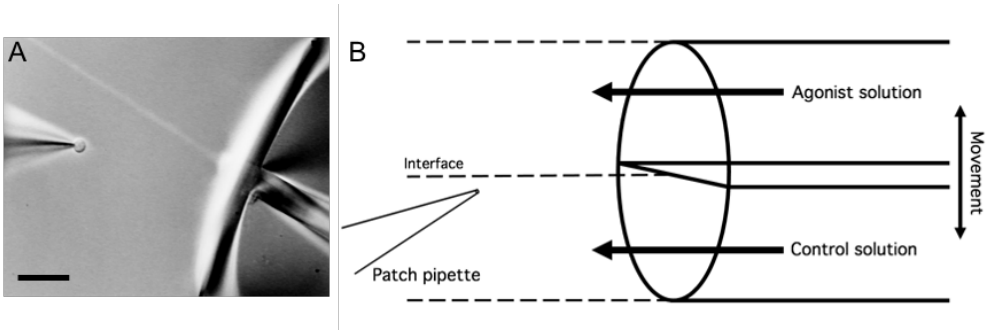

Figure 13. Ultrafast perfusion system. A. IR-DIC videomicrograph of a nucleated patch positioned below the interface formed by the two solutions flowing out of a theta-tube application pipette. Scale bar is 30 μm. **B.** Diagram of the theta-tube application pipette used to apply agonists and other chemicals to excised membrane patches from AII and A17 amacrine cells. Panel A is adapted from **Paper III**.

One application system that fulfills such characteristics is the ultrafast perfusion system (Jonas, 1995). A double-barreled application glass pipette or theta tube (Fig. 13A,B; named after the resemblance with the Greek letter θ) is continuously perfused with control and test (agonist) solution. The thick wall inside the pipette that divides the tube into the two chambers creates an interface between the two solutions that can be moved across the patch by a piezo element to which the theta tube is attached to. Fast movements of the piezoelectric element where the theta tube is mounted are achieved by high voltage applications controlled from the recording amplifier. In our system, we can use up to 6 different solutions and exchange them by using a manifold designed to rotate and allow one test solution to flow through the theta tube at a time. The control solution flows independently.

Reliability of solution exchange was tested before and after every experiment by measuring the liquid junction potential change of open-tip responses to the control solution and a 10% dilution of the control solution. Such different solutions have different liquid junction potentials that can be measured by the amplifier as a change of the pipette current in the voltage-clamp mode. This is used to test for the optimal exchange time between the two solutions and to avoid oscillations in the responses that can lead to several applications of the test solution. Because the two solutions also have different refractory indices, the interface between them becomes visible in the microscope (Fig, 13A), which can be used as a guide to correctly position the theta tube.

It should be mentioned that when using nucleated patches, we cannot consider the solutions exchange to be "ultrafast". That is because the size of the nucleated patch prevents the application of agonist to the whole patch simultaneously. This is especially relevant for kinetic analysis in **Paper III**, and even though in some previous research for some specific receptors there have not been found any differences between conventional and nucleated outside-out recordings (Vandenberghe et al., 2000), word of caution is advised when interpreting these results.

3.5 Electrophysiological data acquisition

General aspects of data acquisition are detailed in the methods section of each of the papers included in this thesis.

It is important to remark that we corrected all holding potentials for theoretical liquid junction potentials online. Liquid junction potential happens when two solutions with different concentrations of ions are in contact with each other, and due to the uneven rates of diffusion of cations and anions, more mobile ions diffuse faster across the concentration gradient at the interface. Because the composition of the intracellular solution we use is different from the extracellular, the liquid junction potential generated at the tip of the recording pipette will interfere with the correct measurement of the membrane potential. Knowing the molecular composition of our solutions, this

potential can be calculated as the potential of the bath solution with respect to the pipette solution and can be corrected in PatchMaster. We calculated the liquid junction potential with JPCalcW software (Molecular Devices).

3.6 Data analysis

Data were analyzed with FitMaster (HEKA Elektronik), IGOR Pro (WaveMetrics, Lake Oswego, OR, USA), Excel (Microsoft, Redmond, WA, USA), MiniAnalysis (Synaptosoft, Decatur, CA, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Spontaneous synaptic currents were detected using a threshold of 9-12 pA (MiniAnalysis) and confirmed by eye. Since only spontaneous inhibitory postsynaptic currents (spIPSCs) mediated by GABA were recorded in this thesis, I will often be referring to them only as spIPSCs.

Quantifying and comparing decay kinetics of responses is useful not only to build models of neurons, but also to understand the role a given receptor might play in signal processing in neurons. For example, a slower deactivation of receptors leads to a higher charge transfer (Nusser et al., 2001; Potapenko et al., 2011). Previous studies have shown that, when only one type of receptor is activated, the decay phase of synaptic currents can be described by a single exponential equation or by a sum of exponentials (Major, 1993). However, the decay time constant obtained by a single exponential equation could underestimate underlying components that could contribute to the decay of responses in some cases. Increasing the number of exponential components in this equation (double or triple exponential instead of single) will then yield a better fit. The decay phase of individual spIPSCs could be well fitted by a single-exponential equation, whereas averaged spIPSCs and GABA-evoked responses in outside-out and/or nucleated patches often required a double- or a triple-exponential equation.

Before averaging, individual spIPSCs and agonist-evoked currents were aligned at 50% of the rise time. The decay time-course of individual and averaged spIPSCs and evoked responses was estimated by curve fitting with exponential functions. General aspects and equations for decay fitting are detailed in **Papers II** and **III**. To avoid

artificial overestimation of time constants, fitting was started a short time after the peak, typically 400 μs (range 300 – 600 μs). Setting time 0 correctly is relevant since the relative amplitude of the fitted exponential function depends on this. For both spIPSCs and agonist-evoked responses in patches, time 0 was defined as the start of the response by eye at the time-point where the current rose from the baseline noise. Rise-time of responses was calculated using IGOR Pro, and both 10-90% and 20-80% rise-times were measured and reported to allow a more complete comparison with other studies.

3.6.1 Non-stationary noise analysis

In this thesis, we wanted to investigate and understand the properties of transmitter-gated ion channels that are responsible for synaptic or extrasynaptic signals. There are three properties of these signals that are of high interest: the number and density of receptors at the postsynaptic membrane, the single-channel conductance and the number of receptors that are open at the peak of the postsynaptic response.

In our preparations, we cannot access the synapses and record directly from them. One approach would be to isolate membrane patches from areas as close as possible to the synaptic point to study ion channel properties. For some cell types, somatic receptors are very similar to the synaptic ones, and can be used to investigate the biophysical properties of these receptors (Gill et al., 2006). Outside-out patches are used to directly resolve single-channel activity, but it is technically challenging and not possible for all types of receptors (Hartveit & Veruki, 2007). However, recording responses to saturating concentrations of neurotransmitter (macroscopic responses) where channels are activated by ultrafast perfusion of agonists can be used to investigate single-channel conductance and the number of receptors in a synapse by using an alternative method: the non-stationary noise analysis (also named non-stationary fluctuation analysis; Hartveit & Veruki, 2007). When we studied $GABA_AR$ responses to GABA (3 mM) application, we found that after the decay, it could often be seen spontaneous single-channel openings. We decided to use non-stationary noise analysis because single-channel openings have fast kinetics and several conductance levels, and the level of background noise, although reduced in outside-out patches

when compared to whole-cell recordings, might mask some of the smaller levels of conductances.

General aspects of non-stationary noise analysis are detailed in **Papers II** and **III**, and thoroughly described in Hartveit & Veruki (2007). Conventional non-stationary noise analysis can only be applied when the current variance arises from stochastic ion channel gating and/or any time-invariant background noise. However, the number and/or the identity of release sites can vary in spontaneous postsynaptic events (spPSCs), and the application of conventional non-stationary noise analysis becomes problematic (Traynelis et al., 1993; Silver et al., 1996). During synaptic events, responses are generated following the release of vesicles from different release sites of the same synaptic entity and will not be identical to each other. This will introduce variability in the amplitude of the postsynaptic current (PSC). To perform non-stationary noise analysis, we need then to isolate this variability from the variations arising from the stochastic gating of ion channels. In conventional non-stationary noise analysis, the variability of responses around the mean is solved by subtracting the mean response from each individual event (see details in **Papers II** and **III**; Hartveit & Veruki, 2007). To analyze PSCs arising from more than one release site, we need to scale the averaged PSC wave to the peak amplitude of each individual event before the subtraction is performed (peak-scaled non-stationary noise analysis). This should isolate fluctuations around the mean from stochastic ion channel gating (Traynelis et al., 1993).

3.6.2 Membrane noise analysis

NMDARs on AII and A17 amacrine cells are extrasynaptic, and their activation does not result in phasic events with fast kinetic properties. Instead, there seem to be fluctuations or variations in the membrane holding current caused by NMDA activation, as these fluctuations are reduced in the presence of NMDAR blockers. Activity of NMDARs resulting in current variations (membrane noise) can be measured as membrane variance (Veruki et al., 2019).

We examined membrane variance before and after applying NMDAR antagonist (CPP 20 μM) and measured NMDA noise as the variance of the membrane current recorded in the whole-cell voltage-clamp configuration (Veruki et al., 2019). For each condition, the current variance was calculated for traces of 20 s of duration, and measurements of at least 4 traces (80 s) were averaged. These measurements were performed after digital low-pass filtering of signals at 1 kHz, - 3dB. For membrane variance experiments, we recorded with a Butterworth filter, as opposed to the Bessel filter used in the rest of the experiments included in this thesis. Both types of filters are commonly used in electrophysiology, albeit for different purposes. The Butterworth filter has a sharper cut-off frequency due to the higher number of poles in its transfer function, but at the same time, this increases the internal filter delay, which makes it a less ideal filter to study fast transients. Thus, this filter is useful when applied to slow currents (especially noise recordings and noise analysis). On the other hand, the Bessel filter has a less sharp cut-off frequency but a smaller delay, which is especially good for macroscopic currents (such as postsynaptic currents; Horowitz & Hill, 1980; Moran, 1996).

4. Overview of results

4.1 Paper I

The aim of the project included in **Paper I** was to investigate the sources of glutamate and endogenous co-agonists that contribute to the activation of the extrasynaptic NMDARs on AII and A17 amacrine cells of the rat retina. We used whole-cell voltage-clamp recordings to monitor NMDA activity in these two amacrine cells. We combined this with a series of pharmacological agents aimed at blocking the glutamate cycle at different points and at altering the level of co-agonists that potentially activate these receptors.

Activation of extrasynaptic NMDARs evokes an increase in membrane noise measured as current variance (**Paper I, Fig. 2A-D**) on AII and A17 amacrine cells as it has been previously reported (Veruki et al., 2019). To investigate the source of glutamate that activates these receptors, we first used Bafilomycin A1, which prevents neurons from releasing neurotransmitters (see sections **1.3.1** and **3.4.3.3**). Following incubation of retinal tissue in Bafilomycin A1, both AII and A17 amacrine cells did not display spontaneous events (**Paper I, Fig. 3A,B**), suggesting that presynaptic release of neurotransmitters had been blocked. Under these conditions, AII amacrine cells did not display a significant level of extrasynaptic NMDA activation (**Paper I, Fig. 3C,D**), whereas A17 amacrines still showed extrasynaptic NMDA activity (**Paper I, Fig. 3E,F**). We next used L-methionine-sulfoximine (MSO) that blocks the enzymatic transformation of glutamate into glutamine in glial cells (Müller cells in the retina). Because glutamatergic transmission depends on neurons obtaining glutamine from glial cells, incubation in MSO disrupts the ability of neurons to release glutamate. Incubating retinal tissue in MSO substantially reduced gating of NMDARs on AII

amacrine cells, but not on A17 amacrine cells (**Paper I, Fig. 4**). These experiments suggest that glutamate that activates extrasynaptic NMDARs on AII amacrines is likely to arise from a neuronal source (spillover of neurotransmitters). In contrast, it is likely to be of non-neuronal origin in the case of A17 amacrine cells (most likely Müller cells). Following incubation in MSO, glutamate is redistributed to Müller cells (Pow & Robinson, 1994; Winkler et al., 1999; Barnett et al., 2000). Therefore, if the source of glutamate is glial in A17 amacrines as suggested in previous experiments, we could expect an increase in NMDA activation following incubation in MSO. However, a complicating factor is the ability of glutamate transporters to buffer changes in glutamate concentration in the extracellular compartment. We then applied the glutamate transporter blocker TBOA after incubating retinal slices in MSO. Under these conditions, A17 amacrines did show a significant increase of NMDA activation during TBOA application (**Paper I, Fig. 5**). These experiments strengthened the previous results, demonstrating that the glutamate that activates extrasynaptic NMDARs on A17 amacrine cells is from glial origin.

Next, we investigated the identity of the endogenous co-agonist that activates extrasynaptic NMDARs on these two amacrine cells. First, application of a saturating concentration of D-serine to the bath recording increased NMDAR activation in both AII and A17 amacrine cells (**Paper I, Fig. 6A-D**), which suggests that the co-agonist binding site is not saturated under physiological conditions. We then used D-amino acid oxidase (DAAO) to deplete the levels of endogenous D-serine. We observed a slow decrease in NMDA activation in AII amacrine cells but not in A17 amacrine cells (**Paper I, Fig. 7A-F**). These results demonstrate that D-serine acts as a co-agonist at extrasynaptic NMDARs on AII amacrines but not on A17 amacrine cells.

4.2 Paper II

The study in **Paper II** was undertaken to characterize $GABA_AR$s on A17 amacrine cells to better understand GABAergic inputs to A17 amacrines and how they influence signal processing on this cell. The biophysical and functional properties of these receptors were examined using a combination of electrophysiological,

pharmacological and biophysical methods in voltage-clamp recordings using the whole-cell and outside-out patch configurations of the patch-clamp technique (see section **3.4.1** and **3.4.2**). The main findings of this study were that A17 amacrine cells express synaptically active $GABA_AR$s but also extrasynaptic $GABA_AR$s on the soma.

We performed whole-cell recordings of spontaneous activity in these retinal interneurons. When we blocked excitatory glutamatergic inputs and presynaptic action potentials with CNQX and TTX, respectively, these cells displayed spontaneous postsynaptic currents (**Paper II, Fig. 1D-F**). These postsynaptic events were not blocked by strychnine (0.3 μM; **Paper II, Fig. 2A, C**). However, the $GABA_AR$ antagonist SR95531 (3 μM; **Paper II, Fig. 2D-G**) completely (and reversibly) blocked the inward postsynaptic currents. This strongly suggests that the observed spontaneous postsynaptic currents where inhibitory (spIPSCs) and mediated by $GABA_AR$s.

Analysis of the kinetics of well-separated spIPSCs revealed an averaged peak amplitude of 21.6 pA and a 20-80% rise time of 471 μs (**Paper II, Fig. 3A-D**). The decay of averaged spIPSCs was well fitted by a double-exponential function, with an amplitude-weighted τ_{decay} of 14.5 ms (**Paper II, Fig. 3E,F**). We also looked at the correlations between peak amplitude, 10-90% rise time and τ_{decay} (**Paper II, Fig. 3G-I**) and found out that the spIPSCs recorded might be, to some extent, electrotonically filtered. The investigation of the *I-V* relationship of spIPSCs under these conditions yielded E_{rev} that followed E_{Cl} (**Paper II, Fig. 4**). These results suggest that these channels have a high selectivity for chloride. Non-stationary noise analysis performed on individual well-separated events yielded an averaged single-channel conductance of ~ 21 pS and an average number of channels open at the peak of 17 (**Paper II, Fig. 5**). Next, we performed pharmacological experiments to investigate the subunit composition of these receptors (see section **3.4.3.2**). Use of Zn^{2+} (10 μM, 100 μM, 500 μM and 1 mM) showed no blockade of spIPSCs at the lowest concentration (10 μM), but a significant reduction of spIPSCs amplitude at 500 μM and 1 mM, suggesting the presence of $\alpha\beta\gamma$ subunits and indicating that α_1 could be present (**Paper II, Fig. 6A,B**). Zolpidem at low concentrations slowed down the deactivation kinetics in all cells

studied under these conditions (**Paper II, Fig. 6C-G**). These results suggest the presence of α_1 and γ_2 subunits in synaptic $GABA_A$Rs on A17 amacrine cells.

We isolated outside-out patches from the somas of A17 amacrines and analyzed responses evoked by GABA applied via ultrafast perfusion (see sections **3.4.3.2** and **3.4.4**). Application of GABA (3 mM) to nucleated patches evoked large responses that were unequivocally blocked by SR95531 (3 μM; **Paper II, Fig. 7A**), suggesting that the receptors contributing to these evoked responses are $GABA_A$Rs. We then examined the kinetic properties of these receptors. Application of brief pulses (2 - 3 ms) of GABA (3 mM) evoked responses with an average peak amplitude of 154 pA and a 20-80% rise time of 299 μs (**Paper II, Fig. 7B**). The decay of these responses was well fitted with a triple-exponential function, with an weighted τ_{decay} of ~ 42 ms (**Paper II, Fig. 7B**). Long pulses (1 s) of GABA were used to study the desensitization of $GABA_A$Rs in somatic outside-out patches. The desensitization phase was well fitted by a double-exponential function, and the average weighted τ_{decay} was 164 ms (**Paper II, Fig. 7C**). The deactivation phase following removal of GABA was well fitted with a single-exponential function with an average τ_{decay} of 367 ms (**Paper II, Fig. 7C**). To investigate the extent of desensitization, we applied longer pulses (5 s) of GABA that yielded an average weighted τ_{decay} of 563 ms (**Paper II, Fig. 7D**). Differences in the time course of desensitization were obvious when overlaying short (3 ms) and longer (500 ms and 1 s) pulses (**Paper II, Fig. 7E**). Non-stationary noise analysis of evoked responses by brief (3 ms) pulses of GABA yielded an averaged maximum P_{open} at the peak of the response of 0.57, an averaged single-channel conductance of ~ 25 pS and an average number of available channels of 134 (**Paper II, Fig. 8A-E**). Conductance values did not significantly differ from spIPSCs (**Paper II, Fig. 5, 8F**) and were no different from analysis of direct observations of single-channel gating at the end of the decay of some responses in the patches analyzed (**Paper II, Fig. 8F,G**).

The differences observed in the kinetics of extrasynaptic receptors compared to synaptic receptors suggests a different subunit composition. We investigated this by applying the pharmacological agents used before for spIPSCs. Application of Zn^{2+} (10, 100 and 500 μM) significantly blocked responses, even at low concentrations (10 μM;

Paper II, Fig. 9A-C). We then tested the potentiation effect of zolpidem (100 nM and 1 μM) on GABA responses. The decay of these responses did not change under the presence of zolpidem 100 nM (**Paper II, Fig. 9D-G**), as opposed to spIPSCs (**Paper II, Fig. 6C-G**). However, zolpidem 1 μM did significantly slow down the deactivation kinetics of the responses (**Paper II, Fig. 9H-K**). Together, these results suggest a different subunit composition than the synaptic receptors, probably $\alpha_{2,3}$ and γ_2 subunits. We excluded the presence of δ-containing receptors by testing of THIP (1, 5 and 10 μM). The lack of responses evoked by THIP 1 μM suggests the absence of δ-containing $GABA_A$Rs (**Paper II, Fig. 10**).

4.3 Paper III

The project outlined in **Paper III** was carried out to investigate the activation, biophysical properties and subunit composition of $GABA_A$Rs on AII amacrine cells of the rat retia. Similar to what was done in **Paper II**, we used a combination of electrophysiological and pharmacological approaches in voltage-clamp recordings in whole-cell and outside-out (nucleated patches, see section **3.4.2**) configurations of the patch-clamp technique.

We performed whole-cell recordings in the presence of TTX, CNQX and strychnine to block Na_v channels, glutamate and glycine receptors, respectively. Under these conditions, we did not observe any spontaneous activity in AII amacrines (**Paper III, Fig. 1**). Next, we investigated the presence of evoked synaptic GABAergic currents by activating potential presynaptic partners as demonstrated in mice (Park et al., 2020). We stimulated the area around the cell by applying 0.5 M sucrose (**Paper III, Fig. 2A-D**) or high-K^+ solutions (**Paper III, Fig. 2E,F**) under the same blockade conditions as before. We could not observe any evoked event in AII amacrines, as opposed to A17 amacrines (**Paper III, Fig. 2**) used as a control for these methods.

We then used nucleated patches from AII amacrine cells to study the kinetics of these receptors. Application of GABA (3 mM) through a theta tube system (see section **3.4.4**) evoked macroscopic currents that were completely blocked by the $GABA_A$R

SR95531 (3 μM; **Paper III, Fig. 3A-C**), providing solid evidence that these responses were mediated by $GABA_A$Rs. We then examined the kinetics of these receptors (**Paper III, Fig. 3D-F**). Application of short (2 ms) pulses of GABA (3 mM) evoked responses with an average peak amplitude of 47 pA and a 20-80% rise time of 913 μs. The deactivation phase of these responses was well fitted with a triple-exponential function that yielded a weighted τ_{decay} of 163 ms (**Paper III, Fig, 3E**). We also used long (1 s) pulses of GABA (3 mM) to study the time course of desensitization of these receptors. The double-exponential function required to fit desensitization phase of the decay yielded a weighted τ_{decay} of desensitization of 465 ms, which was followed by a deactivation phase that was well fitted with a single-exponential function with a τ_{decay} of 564 ms (**Paper III, Fig. 3F**). Non-stationary analysis of evoked responses by brief (2 ms) pulses of GABA yielded an averaged maximum P_{open} at the peak of the response of 0.56, an averaged single-channel conductance of ~ 23 pS and an average number of available channels of 68 (**Paper III, Fig. 4A-E**). Single-channel conductance values were very similar to directly resolved single-channel openings (**Paper III, Fig. 4F-H**).

We investigated the subunit composition of these receptors by using a combination of pharmacological agents (similar to **Paper II**). Application of Zn^{2+} (10 and 100 μM) significantly blocked the GABA-evoked responses (**Paper III, Fig. 5A-C**). We next examined the potentiation effect of the benzodiazepine zolpidem (100 nM and 1 μM). Although zolpidem 100 nM failed to potentiate the decay or amplitude of the responses (**Paper III, Fig. 5D-F**), we did observe an increase in the amplitude of responses during application of zolpidem 1 μM (**Paper III, Fig. 5G-I**). We investigated the presence of δ-containing receptors by applying THIP (1 and 10 μM). THIP failed to evoke a measurable response at the lowest concentration (**Paper III, Fig. 5J**), suggesting the absence of receptors containing δ subunit. Altogether, these experiments suggest that these receptors lack α_1 subunit and could be composed of $\alpha_{2,3}$ and γ_2 subunits.

5. Discussion

The AII and the A17 amacrine cells are two inhibitory interneurons with a crucial role in the rod pathway: The A17 amacrine cell provides inhibitory feedback to the RBC, thus regulating RBC output, and the AII amacrine cell is the sole output of the rod pathway. As discussed in the introduction, AII amacrines synapse with ON- and OFF-Cone bipolar cells, and therefore, the rod and cone pathways are highly interconnected. Thus, the way these two amacrine cells process the signals they receive will be crucial for the output of the whole retina. It has been shown that CP-AMPARs are activated on both AII and A17 amacrines in response to glutamate released by the RBC at the dyad synapse (Fig. 14A; Singer & Diamond 2003; Veruki et al., 2003; Chávez et al., 2006). It has also been previously demonstrated that these two cells express extrasynaptic NMDARs with complementary subunit composition (Fig. 14A; Veruki et al., 2019). However, there was no information about the sources of glutamate and endogenous co-agonist that activate these receptors. We also know that both AII and A17 amacrines could receive inhibitory synapses (Fig. 14B,D; Nelson & Kolb, 1985; Park et al., 2020). Very little is known about the identity and the influence of these inputs on both AII and A17 amacrines.

In this thesis, we have addressed several of the gaps in our knowledge of the cellular and biophysical basis of signal processing in these two cells. We have identified the sources of glutamate and the identity of co-agonist that activate extrasynaptic NMDARs on AII and A17 amacrine cells (Fig. 14A). We have also studied the biophysical properties and molecular identity of GABARs on both amacrine cells (Fig. 14B-D).

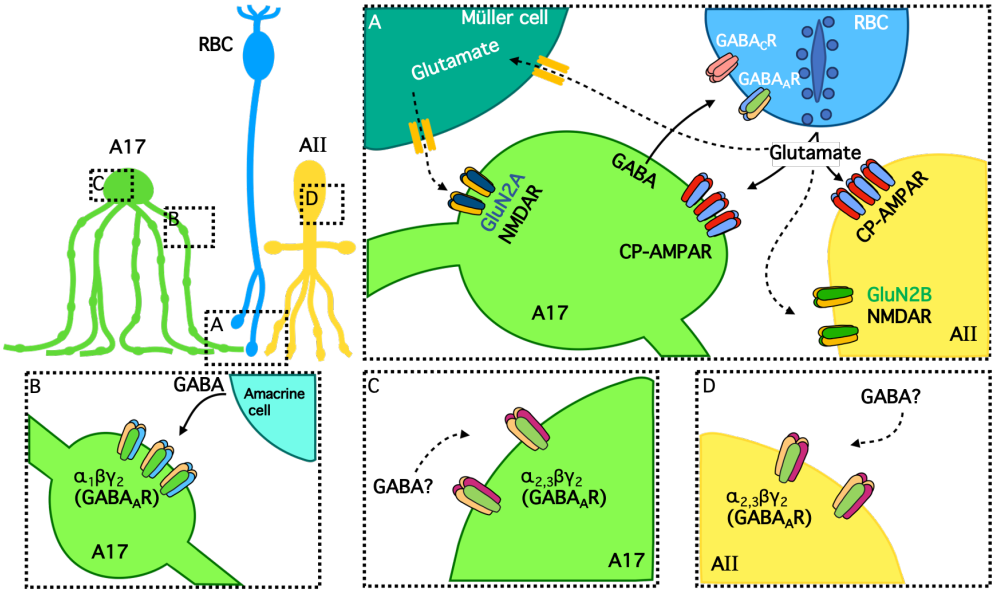

Figure 14. Summary of the findings of this thesis within the rod bipolar cell circuit. Diagram showing a simplified version of the rod pathway, including the rod bipolar cell (blue), the AII (yellow) and the A17 (green) amacrine cells. The color pattern is the same for all the panels in the figure. **A.** Diagram representing a dyad synapse between a rod bipolar cell, an AII and an A17 amacrine cell where findings from **Paper I** have been highlighted. Glutamate released by the RBC (black arrow) activate CP-AMPAR in both AII and A17 amacrines. Notice that the glutamate (dashed arrows) that activates extrasynaptic NMDARs on A17 comes from a Müller cell (dark green), whereas it comes from synaptic spillover in the case of AII amacrines. **B.** Diagram including findings from **Paper II** indicating the $GABA_AR$ subunit composition on a varicosity from a portion of a proximal dendrite of an A17 amacrine cells. **C.** Diagram including findings from **Paper II** indicating the extrasynaptic $GABA_AR$ subunit composition on A17 amacrine cells. **D.** Diagram including findings from **Paper III** indicating the extrasynaptic $GABA_AR$ subunit composition on AII amacrine cells.

5.1 Extrasynaptic NMDA receptors on AII and A17 amacrine cells

There is compelling evidence that demonstrates that AII and A17 amacrine cells express NMDARs (Hartveit & Veruki, 1997; Zhou et al., 2016) that are extrasynaptic and activated by ambient glutamate (Veruki et al., 2019). Activation of NMDARs by ambient glutamate has been reported in the hippocampus (Sah et al., 1989; Dalby &

Mody, 2003) and the retina (Gottesman & Miller, 2003). In Veruki et al. (2019) the authors demonstrated that extrasynaptic NMDARs on AII amacrine cells contain GluN2B subunits, whereas NMDARs on A17 amacrines contain GluN2A subunits. The differences between these two receptor populations are extended by our observations presented in **Paper I** that suggest that different sources of glutamate and different endogenous co-agonists activate the extrasynaptic NMDARs on AII and A17 amacrine cells.

5.1.1 Different sources of glutamate activate extrasynaptic NMDA receptors on AII and A17 amacrine cells

The source of neurotransmitters that activates synaptic receptors is generally obvious, although there are some exceptions where glia plays an active role (reviewed in Araque et al., 1999). This is not the case for extrasynaptic receptors, where the extracellular concentration of neurotransmitters might arise from different sources. Regarding a possible neuronal origin, one of the potential sources is spillover of synaptic glutamate from the RBC and cone bipolar cells (Veruki et al., 2006; Wersinger et al., 2006) that could contribute to the ambient pool of glutamate that activates extrasynaptic NMDARs on these two cells. However, Müller cells (glia) in the retina could also release glutamate to activate these receptors (reviewed in Bringmann et al., 2009).

In this project, we concluded that the glutamate that activates extrasynaptic NMDARs on AII amacrines is likely to be of neuronal origin (Fig. 14A). NMDA activation is significantly reduced after depleting synaptically released glutamate by treating retinal tissue with either Bafilomycin A1 or MSO. Under these conditions, AII amacrines did not show significant NMDA activation, suggesting a neuronal source of glutamate. Activation of NMDARs by synaptic spillover of glutamate has also been observed in ganglion cells in the retina (Chen & Diamond, 2002), in granule cells of the dentate gyrus (Dalby & Mody, 2003) or pyramidal cells in the cortex (Chalifoux & Carter, 2011). Under the same experimental conditions, A17 amacrines did show significant extrasynaptic NMDA activation, which clearly contrasts the result in AII amacrines and suggests a non-neuronal origin of glutamate, most likely glia (Fig. 14A;

Müller cells in the retina). Glial release of glutamate that contributes to NMDARs activation has been demonstrated before in hippocampal pyramidal cells (Le Meur et al., 2007; Savtchouk & Volterra, 2018). According to our observations, glial cells (Parpura et al., 1994; Malarkey & Parpura, 2008) such as the retinal Müller cell (Maguire et al., 1994; for review see Bringman et al., 2009) could participate in the activation of extrasynaptic NMDARs on A17 amacrine cells.

Both Bafilomycin A1 and MSO alter the glutamate cycle at different points, and it has been shown that after treatment with MSO, glutamate is redistributed and accumulated in Müller cells in the retina (Pow & Robinson, 1994; Winkler et al., 1999; Barnett et al., 2000). This is probably due to the expression pattern of the glutamine synthetase (the enzyme inhibited by MSO), located exclusively in Müller cells (Riepe & Norenburg, 1977). It has been proposed that following MSO treatment, the redistribution of glutamate from neurons to glial cells could lead to an increase in the extracellular concentration of glutamate that should be reflected in a higher NMDA activation (Le Meur et al., 2017). We did not observe such a change, most likely due to the ability of glutamate transporters to buffer increases in the extracellular concentration of glutamate (Le Meur et al., 2007). We investigated this possibility by blocking glutamate transporters with TBOA (Shimamoto et al., 1998) under the same experimental conditions as before. We found that blocking uptake of glutamate with TBOA led to an increase in membrane variance and evoked a large inward current. These results support the hypothesis that glutamate that activates extrasynaptic NMDARs on A17 amacrines is of glial origin.

5.1.2 D-serine acts as a co-agonist at extrasynaptic NMDA receptors on AII but not on A17 amacrine cells

As opposed to most neurotransmitter receptors, activation of NMDARs requires the binding of glutamate and a co-agonist that can be either glycine (Johnson & Ascher, 1987) or D-serine (Mothet et al., 2000), and a co-incident depolarization to expel the Mg^{+} that blocks the pore (Ascher et al., 1988; Ascher & Novak, 1988; reviewed in Traynelis et al., 2010). GluN2B-containing NMDARs display a higher affinity for D-serine and glycine than GluN2A-containing NMDARs, but each subtype has a similar

affinity for both co-agonists (reviewed in Mothet et al., 2015). Thus, the identity of the co-agonist that binds to NMDARs might depend on the spatial and temporal profile of co-agonist availability rather than the affinity of a specific subunit composition for a given co-agonist (Mothet et al., 2000; Chen et al., 2003; Tsai et al., 2004).

In this project, we also investigated the identity of the co-agonist that binds to extrasynaptic NMDARs on AII and A17 amacrine cells. As a first step, we tested if the co-agonist binding site was saturated in our preparations. Results showed that for both AII and A17 amacrine cells, addition of D-serine increases NMDA activation. This suggests that the co-agonist binding site of NMDARs is not saturated under physiological conditions on these cells. However, a complicating factor is that D-serine release is reduced following AMPA receptors blockade (Stevenson et al., 2003; Kalbaugh et al., 2009; Sullivan & Miller, 2010), which was needed to measure NMDA activity without contamination from other glutamate currents. Our results suggest that modulation of co-agonist levels could potentially provide a regulatory effect on the activation of these receptors on AII and A17 amacrines (Kalbaugh et al., 2009).

We also used a commercially available version of the enzyme D-amino acid oxidase (DAAO) that degrades D-serine (Dixon & Kleppe, 1965; Molla et al., 2006; Kalbaugh et al., 2009; Acton & Miles, 2017) to investigate the identity of the endogenous co-agonist that binds to extrasynaptic NMDARs on AII and A17 amacrine cells. We saw a significant decrease in NMDA activation following DAAO application on AII amacrines, but not on A17 amacrines cells. These results suggest that D-serine acts as a co-agonist at NMDARs on AII amacrines, whereas glycine is likely to be the main co-agonist at NMDARs on A17 amacrine cells. D-serine has been proposed to act primarily as a co-agonist at synaptic NMDRs, whereas glycine would act at extrasynaptic NMDARs (Papouin & Oliet, 2014). This comes from the rather oversimplified assumption that GluN2A receptors are predominantly synaptic and GluN2B receptors are located extrasynaptically in the adult CNS (Papouin et al., 2012; Shipton & Paulsen, 2014). Although this seems to be the case for certain brain areas and cell types, in other cases it has been shown that GluN2B subunit can be found at synapses (Kalbaugh et al., 2009) or that both GluN2A and GluN2B can be found

extrasynaptically (Veruki et al., 2019). There is also evidence for co-agonist overlap at NMDARs throughout the CNS (Li et al., 2013; Rosenberg et al., 2013; Mothet et al., 2015), and D-serine has been reported to bind to GluN2B subunits as well (Panatier et al., 2006). In the retina, both D-serine and glycine can act as endogenous co-agonists (Stevens et al., 2003; Gustafson et al., 2007). D-serine and its catalyzing enzyme have only been found in glial cells in the retina (Sullivan et al., 2003; Sullivan & Miller, 2010). Therefore, glial cells are likely to be the source of co-agonist in the case of NMDARs on AII amacrines. A possible functional explanation to different sources of glutamate and co-agonist could be that the levels of either one of them is always sufficiently high, but the other one permits and regulates NMDAR activation.

For A17 amacrine cells, we show that DAAO has little or no effect on NMDAR activation, which suggests that glycine and not D-serine could act as a co-agonist on A17 amacrine cells. In the salamander retina, the glycine transporter GlyT-1 has been demonstrated to contribute to the extracellular glycine that activates NMDARs on ganglion cells (Stevens et al., 2010), and given the number of amacrine cells that use glycine as their primary neurotransmitter (MacNeil & Masland, 1998; Menger et al., 1998), glycine is an obvious candidate as a co-agonist. Release of glycine to the extrasynaptic compartment could be due to glycine spillover from synapses as in the spinal cord (Ahmadi et al., 2003) or from glial release as demonstrated in hippocampal cell cultures (Rosenberg et al., 2013). However, only the transporter GlyT-1 has been found in the retina, and it has been shown to be exclusively localized in neurons, more precisely in amacrine cells (Menger et al., 1998; Pow & Hendrikson, 1998). It remains unclear whether glial cells could participate in NMDARs activation via glycine release through mechanisms other than reverse uptake through glycine transporters.

Altogether, our results suggest that the sources of both glutamate and co-agonist that activate extrasynaptic NMDARs on AII and A17 amacrine cells could be different, which could provide an additional level of regulation to the activation of these receptors.

5.1.3 Functional role of extrasynaptic NMDA receptors on AII and A17 amacrine cells

The role of extrasynaptic NMDA receptors on AII and A17 amacrine cells is not yet clearly understood. Amacrine cells, in general, have been shown to be especially vulnerable to NMDA excitotoxicity (Ullian et al., 2004), which could be interesting to study glutamate-associated retinal degenerative processes. There is evidence that NMDARs activation on AII amacrine cells is linked to the regulation of gap junction coupling between AII amacrines (Kothmann et al., 2012). That GluN2B subunit seems to be localized close to Cx36 labeling in AII amacrines (Veruki et al., 2019) supports this hypothesis. However, there is not much evidence for the role of NMDARs on A17 amacrine cells. It has been shown that the GABAergic feedback provided from the A17 amacrine to the RBC is increased when NMDA activation is enhanced (Veruki et al., 2019), although the mechanisms via which this happens are not elucidated and could include direct coupling of Ca^{2+} influx through NMDARs and vesicle release, activation of voltage-gated Ca^{2+} channels or both. What becomes clearer based on our findings is that AII and A17 amacrine cells differentially process the glutamate signals that activate extrasynaptic NMDARs: not only these two cells express different subunits (Veruki et al., 2019) with different single-channel properties (Erreger et al., 2005), but they seem to be activated by different sources of glutamate and different co-agonists.

Extrasynaptic NMDARs have also been traditionally linked to long-term synaptic plasticity processes. It has been proposed that extrasynaptic GluN2B-containing NMDARs are responsible for LTD (Liu et al., 2004; Massey et al., 2004), although this remains controversial (Weitlauf et al., 2005; Morishita et al., 2007). Furthermore, it has also been demonstrated that in some cases, extrasynaptic NMDARs contribute to LTP instead (Yang et al., 2017). The role of NMDARs co-agonist has also been linked to synaptic plasticity, and extrasynaptic receptors activated by D-serine could contribute to LTP, whereas those activated by glycine could contribute to LTD (Papouin et al., 2012). The disparity on the consequences of activating extrasynaptic NMDARs could be related to the wrong assumption of exclusive synaptic *vs.* extrasynaptic localization of specific subunits (Papouin et al., 2012; Shipton & Paulsen, 2014). Thus, it becomes

necessary to investigate the subunit composition, activation and distribution of these receptors in the CNS to understand their contribution to signal processing in neurons. It will be important to further investigate the functional role of these receptors on both AII and A17 amacrine cells to better understand their role on these neurons.

5.2 $GABA_A$ receptors on AII and A17 amacrine cells

In the studies outlined in **Paper II** and **Paper III** we studied the functional properties of synaptic $GABA_ARs$ on A17 amacrines and extrasynaptic $GABA_ARs$ on both AII and A17 amacrine cells. Previous research demonstrated the presence of $GABA_ARs$ on AII amacrine cells (Boos et al., 1993; Zhou & Dacheux, 2004; Zhou et al., 2016; Park et al. 2020). A17 amacrines have been shown to receive inputs from different amacrine cells at the proximal part of their dendrites (Nelson & Kolb, 1985) and to respond to GABA application (Menger & Wässle, 2000; Majumdar et al., 2009; Zhou et al., 2016). However, the biophysical properties and subunit composition of the GABARs that could mediate these inputs on AII and A17 amacrine cells are not well characterized, and we addressed this in **Papers II** and **III**.

5.2.1 Molecular identity of synaptic $GABA_A$ receptors on AII and A17 amacrine cells

We isolated and characterized spIPSCs mediated by $GABA_ARs$ on A17 amacrine cells. The kinetic analysis of spIPSCs shows events with very fast rise time and decay kinetics (Schubert et al., 2013; Dixon et al., 2014; Lagrange et al., 2018). This analysis also suggests that a large proportion of these events are generated close to the soma due to the fast rise times of spIPSCs and the close relationship between the E_{rev} and E_{Cl}. However, we did observe some degree of electrotonic filtering. It was previously described that A17 amacrine cells receive synaptic input from at least three different profiles of amacrine cells (Nelson & Kolb, 1985). Our data suggests some events display smaller amplitude and longer rise times (Gardner et al., 1999: Barberis et al., 2004). This variability could be explained if the events generated further from the soma are more heavily filtered, which would suggest that GABAergic synaptic currents may

be generated at different distances from the somas and potentially from different input sources. Non-stationary noise analysis of spIPSCs revealed a single-channel conductance of ~ 21 pS, which is in accordance with the value reported for the most common subunit combinations in other studies (Angelotti & Macdonald 1993; Fisher & Macdonald 1997; Brickley et al., 1999; Mortensen & Smart 2006).

The subunit composition of $GABA_A$Rs determines their kinetic properties (Lavoie et al., 1997; Haas & Macdonald, 2004). We investigated the composition of these receptors with different pharmacological agents. First, the high concentration of Zn^{2+} ($\geq$ 500 mM) required for the suppression of spIPSCs suggests that these receptors are composed of αβγ subunits. Furthermore, they are likely to be composed of α_1 subunits given that receptors containing this subunit show even less sensitivity to Zn^{2+} inhibition compared to other αβγ combinations (IC_{50} ~ 50-100 mM at αβγ and $IC_{50} \geq$ 250 mM at $\alpha_1\beta\gamma$; Draghun et al., 1990; Smart et al., 1991). The potentiation of spIPSCs by low concentrations of zolpidem (100 nM) strongly suggests the presence of $\alpha_1\gamma_2$ subunits (Pritchett & Seeburg, 1990; Wafford et al., 1993; Dämgen & Lüddens, 1999; reviewed in Möhler, 2006). Thus, these experiments suggest that the majority of synaptic $GABA_A$Rs of A17s amacrine cells are likely to be composed of $\alpha_1\beta\gamma_2$ subunits (Fig. 14B). This subunit combination is the most abundant in the adult CNS and represents 60% of all synaptic $GABA_A$Rs subunit combinations (Möhler, 2006). Given the fast kinetics of the GABAergic spIPSCs have, these are likely to mediate a quick but transient shunting of excitatory inputs (Jones & Westbrook, 1996; see Farrant & Nusser, 2005). However, it is unclear what the role of these inhibitory inputs at the proximal part of A17 amacrines dendrites could be on a neuron that has been proposed to operate as multiple and isolated circuits (Grimes et al., 2010).

Recent research demonstrated that AII amacrine cells receive GABAergic synaptic input from a wide-field amacrine cell (Park et al., 2020). However, in Gill et al. (2006) it was shown that adding the specific glycine receptor inhibitor strychnine blocked all spIPSCs in AII amacrines, which would presumably exclude the possibility of spontaneous GABAergic events on these cells. Consistent with previous research, we did not observe spontaneous GABAergic synaptic inputs (**Paper III**; Gill et al.,

2006). Nonetheless, it was somewhat surprising that we were unable to evoke GABAergic events in AII amacrines by application of high-K^+ or sucrose solutions. In Park et al. (2020) they described these inputs as TTX sensitive, but it should not be an obstacle to use it in our preparations as the depolarization required to evoke the release of vesicles is achieved with the application of high-K^+ or sucrose solutions (Bekkers & Stevens, 1995; Yu & Miller, 1995; Hartveit, 1996; Thoreson & Miller, 1996). Observations of synaptic GABAergic inputs to AII amacrines in Park et al. (2020) were made in a different animal model. That we were unable to evoke GABAergic synaptic inputs in our preparations as in Park et al. (2020) led us to think that $GABA_A$Rs receptors might be extrasynaptic in AII amacrine cells of the rat retina.

5.2.2 Molecular identity of extrasynaptic $GABA_A$ receptors on AII and A17 amacrine cells

We further examined the kinetic properties of these receptors by ultrafast perfusion of GABA (3 mM) to outside-out and nucleated outside-out patches excised from the somata of A17 and AII amacrine cells, respectively. Because there are not known synaptic inputs at the soma of the A17 amacrine cell, we argue that the receptors located here are extrasynaptic. We confirmed that responses evoked by GABA (3 mM) were solely mediated by $GABA_A$Rs by applying SR95531 (3 μM), which resulted in the reversible blockade of these responses for both AII and A17 amacrine cells.

We then analyzed the rise time, amplitude and decay kinetics in response to different pulse durations. We found that the kinetics of $GABA_A$Rs on A17 amacrine cells seemed to be slower compared to the kinetics of spIPSCs, which might suggest a different subunit composition (Barberis et al., 2007; Dixon et al., 2014; Lagrange et al., 2018). The decay kinetics of $GABA_A$ receptors from A17 outside-out patches we report (weighted τ_{decay} ~ 42 ms) are very similar to those reported for $\alpha_1\beta\gamma_2$ receptors (weighted τ_{decay} ~ 53 ms; Barberis et al., 2007) and $\alpha_2\beta\gamma_2$ receptors (weighted τ_{decay} ~ 45 ms at $\alpha_2\beta\gamma_2$ and ~ 10 ms at $\alpha_1\beta\gamma_2$; Dixon et al., 2014). We used the same methods and conditions in this thesis than those used in Barberis et al. (2007) and Dixon et al. (2014) to study $GABA_A$ receptors kinetics on outside-out patches, so it is surprising to see such discordant comparisons. Therefore, it is difficult to determine the subunit

composition of extrasynaptic $GABA_A$ receptors on A17 amacrine cells based on the kinetics of the responses.

We used nucleated patches to analyze the kinetic properties of $GABA_A$Rs on AII amacrine cells because of the low amplitude and quick rundown we observed when we tested outside-out patches. It is known that nucleated patches are more stable (Sather et al., 1992), and the kinetics of the receptors studied with these patches do not seem to be very different from those studied using conventional outside-out patches (discussed in sections **3.4.2** and **3.4.4**; Vandenberghe et al., 2000). Our data suggest that $GABA_A$Rs on AII amacrine cells seem to have slow kinetics (both rise-time and decay). Because α_1-containing $GABA_A$Rs exhibit faster kinetics than any other α subunits (Gingrich et al., 1995; Vicini et al., 2001; Ortinski et al., 2004; Barberis et al., 2007; Dixon et al., 2014), our results suggest that it is unlikely that α_1-containing $GABA_A$Rs are present to a significant extent in nucleated patches of AII amacrines. Instead, the decay kinetics we report for $GABA_A$ receptors on nucleated patches from AII amacrines (weighted $\tau_{decay} \sim 163$ ms) are fairly similar to those previously reported for $\alpha_3\beta\gamma_2$ receptors (weighted $\tau_{decay} \sim 185$ ms; Barberis et al., 2007) using similar methods.

Results from non-stationary noise analysis of these responses yielded a single-channel conductance of ~ 25 pS in A17 amacrines and ~ 23 pS in AII amacrine cells. This is in accordance with previous reports where single-channel conductance of most abundant subunit combinations was ~ 24 – 28 pS (Angelotti & Macdonald 1993; Fisher & Macdonald 1997; Brickley et al., 1999; Mortensen & Smart 2006). At the same time, this could indicate the absence of populations of receptors containing only αβ subunits that significantly contribute to these responses, since they have been reported to have a much smaller single-channel conductance of ~ 11 – 15 pS (Angelotti & Macdonald 1993; Mortensen & Smart 2006).

We next studied the subunit composition of extrasynaptic $GABA_A$Rs located at the soma of AII and A17 amacrine cells using pharmacological agents. Application of Zn^{2+} at 10 and 100 μM resulted in a significant blockade of the response for both AII

and A17 amacrine cells. These results suggest that $GABA_A$Rs are likely to be composed of αβγ subunits, but at the same exclude the presence of α_1-containing receptors since these are not as sensitive to Zn^{2+} inhibition (Draguhn et al., 1990; Smart et al., 1991). This is consistent with kinetics analysis that showed that these $GABA_A$ receptors display slower deactivation kinetics compared to spIPSCs on A17 amacrine cells and to previous research (Gingrich et al., 1995; Vicini et al., 2001; Ortinski et al., 2004; Barberis et al., 2007; Dixon et al., 2014). At the same time, populations of δ-containing receptors cannot be discarded (Draghun et al., 1990). The lack of potentiation effect at low concentrations of zolpidem (low nM range) in both cell types strongly suggests the absence of $\alpha_1\gamma_2$ combinations (Pritchett & Seeburg, 1990; Wafford et al., 1993; reviewed in Möhler, 2006). However, we found a significant effect of zolpidem 1 μM on both cells, which could suggest the presence of α subunits that are less sensitive to zolpidem potentiation in combination with γ_2, like $\alpha_{2,3}$ (Pritchett & Seeburg, 1990; Wafford et al., 1993). $\gamma_{1,3}$ subunits show reduced sensitivity to zolpidem (Puia et al., 1991; Dämgen & Lüddens, 1999), and therefore it is unlikely that they are found to a significant extent on extrasynaptic $GABA_A$ receptors from AII and A17 amacrine cells. Since $\alpha_{4,6}$ subunits also have less affinity for zolpidem (Criswell et al., 1997; Sur et al., 1999), and tend to co-localize with the δ subunit (Jones et al., 1997; Pirker et al., 2000; Sassoè-Pognetto et al., 2000; Zheleznova et al., 2009) which show similar Zn^{2+} sensitivity to the one we report (Draguhn et al., 1990), we studied the effect of THIP on these responses (Stórustovu & Ebert, 2003, 2006). We could not find activation of $GABA_A$Rs on either AII or A17 amacrine cells by THIP at low concentrations that unequivocally activate δ-containing receptors (Jia et al., 2005; Marowsky & Vogt, 2014), and therefore it is unlikely that δ-containing receptors contribute to GABA responses in these receptors. Because α_5 subunit is not present in the rat retina (Wässle et al., 1998), our results suggest that $GABA_A$Rs from somatic patches of AII and A17 amacrine cells are likely to be composed of $\alpha_{2,3}\beta\gamma_2$ subunits (Fig. 14C,D). At this point, and with the pharmacological agents available, we consider it is not possible to narrow down the subunit composition of these receptors.

5.2.3 Functional role of $GABA_A$ receptors on AII and A17 amacrine cells

The inhibition of inhibitory interneurons is a reiterated motif in cortical areas such as the visual cortex (Pfeffer et al., 2013; reviewed in Cardin, 2018) although relatively unexplored in the retina (e.g. Marc & Liu, 2000; Marc et al., 2014), where the ~ 60 types of inhibitory amacrine cells (interneurons; Yan et al., 2020) are known to interact with each other. This originates more complex spatiotemporal processing of signals and additional parallel processing pathways together with the main pathways of the retina (that includes bipolar to ganglion cell connections; Franke et al., 2017; reviewed in Diamond, 2017). However, it is not well understood what the role of this inhibition could be in AII and A17 amacrine cells. Recent research suggests that it could mediate the inhibitory surround properties of the receptive field of AII amacrine cells (Park et al., 2020). As of A17 amacrine cells, research published in the last 15 years suggests a compartmentalized functionality of the A17, where each varicosity could work as an isolated and independent processor (Chávez et al., 2006; Grimes et al., 2009: Grimes et al., 2010). This makes even more puzzling that these cells receive glutamatergic inputs from RBC at the distal parts of their dendrites and GABAergic inputs from amacrine cells at the proximal parts (Nelson & Kolb, 1985), which challenges the idea of isolated and independent microcircuits at each varicosity (Grimes et al., 2010). The segregation of excitatory and inhibitory inputs in A17 amacrines (Nelson & Kolb, 1985), together with our results suggests that signal integration in these cells might occur over larger regions of their dendrites than what has been previously described (Grimes et al., 2010).

Our results suggest that extrasynaptic $GABA_ARs$ on A17 amacrine cells show different kinetic properties compared to their synaptic counterparts. These differences could be due to a differential subunit composition as our results suggest (Gingrich et al., 1995; Vicini et al., 2001; Ortinski et al., 2004; Farrant & Nusser, 2005; Dixon et al., 2014). An alternative explanation is that the differences in kinetic properties and pharmacological profile could be due to differences in the modulatory state of these receptors. There are many known mechanisms to modulate $GABA_AR$ function, which

is likely to be relevant for both, synaptic receptors mediating phasic events and extrasynaptic receptors mediating tonic activation. Among these mechanisms we can count phosphorylation of intracellular loops of β and γ subunits (Hansra et al., 2004) or palmitoylation of γ_2 subunits (Keller et al., 2004). It has been demonstrated that these post-translational modifications can affect both biophysical properties (Jones & Westbrook, 1997; Hinkle & Macdonald, 2003) and localization (Rathenberg et al., 2004) of $GABA_A$Rs. We cannot overlook the possibility that somatic receptors on A17 amacrine cells might be identical to synaptic receptors but have undergone post-translational modifications.

Our results suggest that $GABA_A$Rs are likely to be composed of $\alpha_{2,3}\beta\gamma_2$ in AII amacrine cells and in fact, the kinetics we report correspond very well with the deactivation time course of $\alpha_3\beta\gamma_2$ kinetics described in previous research in a heterologous expression system (Barberis et al., 2007). The presence of $\alpha_3\beta\gamma_2$ on nucleated patches from AII amacrines is consistent with previous studies that localized the α_3 and α_1 subunit to synapses between dopaminergic amacrine cells and AII amacrine cells in the rat retina (Contini & Raviola, 2003). These dopaminergic neurons have been proposed to co-release dopamine and GABA and are thought to contact the AII amacrines near the soma, apical dendrite and lobular appendages (Contini & Raviola, 2003; Völgyi et al., 2014). These authors, however, could not definitively state that the α_3 and α_1 labeling belonged to AII amacrines. If extrasynaptic $GABA_A$ receptors on AII amacrine cells are representative for synaptic receptors, such slow kinetics could facilitate temporal summation of inhibitory signals with relatively low frequency. This contrasts with the fast kinetics of glycine receptors on AII amacrines (Gill et al., 2006), which are well-matched with the fast kinetics of the excitatory inputs (Veruki et al., 2003) and could mediate a transient shunt of excitatory inputs instead. In the mouse retina, AII amacrines have been shown to receive GABAergic inputs from NOS-1 amacrine cells (Park et al., 2020). That we were unable to evoke GABAergic synaptic inputs similar to that study could suggests that AII amacrines do not receive these synaptic inputs in the rat retina.

The deactivation kinetics are thought to play an important role in shaping the time course of the currents mediated by these receptors. The slower activation and deactivation kinetics exhibited by extrasynaptic $GABA_A$Rs on A17 but especially on AII amacrines suggest that these receptors might be involved in mediating signals with low temporal precision but sustained action (Barberis et al., 2007). There is a clear need of more research to unveil what the role of $GABA_A$Rs could be in both AII and A17 amacrine cells.

6. Concluding remarks

A lack of detailed studies of the biophysical properties, activation and function of different neurotransmitter receptors results in a gap of knowledge about the specific contribution of specific receptors and ion channels to signal processing in neurons. The general scope of this thesis was to address this gap, and we focused our work on the study of synaptic and extrasynaptic receptors on two interneurons of the rod bipolar cell microcircuit. The work presented in this thesis is divided into two parts: First, we demonstrated that the sources of glutamate and the endogenous co-agonists that activate extrasynaptic NMDARs are different on AII and A17 amacrine cells. Second, we presented a detailed characterization of both synaptic and extrasynaptic $GABA_A$Rs on AII and A17 amacrine cells. The work has been carried out using a combination of electrophysiological and pharmacological approaches.

We have shown that extrasynaptic NMDARs on AII amacrine cells are activated by extracellular glutamate of neural origin, whereas it seems to be of glial origin in the case of A17 amacrine cells. Furthermore, D-serine could act as the endogenous co-agonist at extrasynaptic NMDARs on AII amacrine cells, but not on A17 amacrines. These results add valuable information towards understanding the role these receptors have in these two important inhibitory interneurons and provide evidence of a differential activation of extrasynaptic NMDRs within the same microcircuit.

The work outlined in this thesis also provides a comprehensive analysis of the biophysical properties and subunit composition of $GABA_A$Rs on AII and A17 amacrine cells. Synaptic $GABA_A$Rs on A17 amacrine cells have fast kinetics and are likely to be composed of $\alpha_1\beta\gamma_2$ subunits. We found no evidence for synaptic $GABA_A$Rs on AII amacrine cells. Extrasynaptic $GABA_A$ receptors on the somas of AII and A17 amacrine

cells displayed relatively slow deactivation kinetics and are likely to be composed of $\alpha_{2,3}\beta\gamma_2$ subunits.

The work in this thesis provides new and exciting evidence in the field of synaptic physiology, neurotransmitter receptors and their functional properties. This is important both for our basic understanding of synaptic transmission in the healthy central nervous system, and also for our understanding of disease processes where malfunction of specific receptors and ion channels can lead to serious consequences for human health for which we still struggle to find a cause and a cure.

7. References

Acton D, Miles GB (2017). Differential regulation of NMDA receptors by D-serine and glycine in mammalian spinal locomotor networks. *Journal of Neurophysiology*, *117*, 1877–1893.

Adesnik H (2018). Layer-specific excitation/inhibition balances during neuronal synchronization in the visual cortex. *Journal of Physiology*, *596,* 1639–1657.

Ahmadi S, Muth-Selbach U, Lauterbach A, Lipfert P, Neuhuber WL, Zeilhofer, HU (2003). Facilitation of spinal NMDA receptor currents by spillover of synaptically released glycine. *Science*, *300,* 2094–2097.

Angelotti TP, Macdonald RL (1993). Assembly of $GABA_A$ receptor subunits: Alpha 1 beta 1 and alpha 1 beta 1 gamma 2S subunits produce unique ion channels with dissimilar single- channel properties. *Journal of Neuroscience*, *13,* 1429–1440.

Araque A, Parpura V, Sanzgiri RP, Haydon P. (1999). Tripartite synapses: Glia, the unacknowledged partner. *Trends in Neurosciences*, *22,* 208–215.

Ascher P, Bregestovski P, Nowak L (1988). N-methyl-D-aspartate-activated channels of mouse central neurones in magnesium-free solutions. *Journal of Physiology*, *399,* 207–226.

Ascher P, Nowak L (1988). The role of divalent cations in the N-methyl-D-aspartate responses of mouse central neurones in culture. *Journal of Physiology*, *399*, 247–266.

Baden T, Berens P, Franke K, Román Rosón M, Bethge M, Euler T (2016). The functional diversity of retinal ganglion cells in the mouse. *Nature*, *529*, 345–350.

Barberis A, Mozrzymas JW, Ortinski PI, Vicini, S (2007). Desensitization and binding properties determine distinct α1β2γ2 and α3β2γ2 $GABA_A$ receptor-channel kinetic behavior. *European Journal of Neuroscience*, *25*, 2726–2740.

Barberis A, Petrini EM, Cherubini E (2004). Presynaptic source of quantal size variability at GABAergic synapses in rat hippocampal neurons in culture. *European Journal of Neuroscience*, *20*, 1803–1810.

Barbour B, Häusser M (1997). Intersynaptic diffusion of neurotransmitters. *Treds in Neuroscience*, *20*, 377–384.

Barnett NL, Pow DV, Robinson SR (2000). Inhibition of Müller cell glutamine synthetase rapidly impairs the retinal response to light. *Glia*, *30*, 64–73.

Baumann SW, Baur R, Sigel E (2003). Individual properties of the two functional agonist sites in $GABA_A$ receptors. *Journal of Neuroscience*, *23*, 11158–11166.

Baylor DA, Lamb TD, Yau KW (1979). Responses of retinal rods to single photons. *Journal of Physiology*, *288*(1), 613–634.

Bekkers JM, Stevens CF (1995). Quantal analysis of EPSCs recorded from small numbers of synapses in hippocampal cultures. *Journal of Neurophysiology*, *73*, 1145–1156.

Bennett MVL (2000). Electrical synapses, a personal perspective (or history). *Brain Research Reviews*, *32*, 16–28.

Bianchi MT, Macdonald RL.(2002). Slow phases of $GABA_A$ receptor desensitization: Structural determinants and possible relevance for synaptic function. *Journal of Physiology*, *544*, 3–18.

Bikoff JB, Gabitto MI, Rivard AF, Drobac E, Machado TA, Miri A, Brenner-Morton S, Famojure E, Diaz C, Alvarez FJ, Mentis GZ, Jessell TM (2016). Spinal inhibitory interneuron diversity delineates variant motor microcircuits. *Cell*, *165*, 207–219.

Boos R, Schneider H, Wassle H (1993). Voltage- and transmitter-gated currents of all-amacrine cells in a slice preparation of the rat retina. *Journal of Neuroscience*, *13*, 2874–2888.

Borden LA, Dhar TGM, Smith KE, Weinshank RL, Branchek TA, Gluchowski C (1994). Tiagabine, SK&F 89976-A, CI-966, and NNC-711 are selective for the cloned GABA transporter GAT-1. *European Journal of Pharmacology*, *269*, 219–224.

Bormann J, Feigenspan A (1995). GABAc receptors. *Trends in Neurosciences*, *18*, 515–519.

Brickley SG, Cull-Candy SG, Farrant M (1999). Single-channel properties of synaptic and extrasynaptic $GABA_A$ receptors suggest differential targeting of receptor subtypes. *Journal of Neuroscience*, *19*, 2960–2973.

Bringmann A, Pannicke T, Biedermann B, Francke M, Iandiev I, Grosche J, Wiedemann P, Albrecht J, Reichenbach A (2009). Role of retinal glial cells in neurotransmitter uptake and metabolism. *Neurochemistry International*, *54*, 143–160.

Cardin JA (2018). Inhibitory interneurons regulate temporal precision and correlations in cortical circuits. *Trends in Neurosciences*, *41*, 689–700.

Cavelier P, Hamann M, Rossi D, Mobbs P, Attwell D (2005). Tonic excitation and inhibition of neurons: Ambient transmitter sources and computational consequences. *Progress in Biophysics and Molecular Biology*, *87*, 3–16.

Chalifoux JR, Carter AG (2011). Glutamate spillover promotes the generation of NMDA spikes. *Journal of Neuroscience*, *31*, 16435–16446.

Chance FS, Abbott LF, Reyes, AD (2002). Gain modulation from background synaptic input. *Neuron*, *35*, 773–782.

Chávez AE, Singer JH, Diamond JS (2006). Fast neurotransmitter release triggered by Ca influx through AMPA-type glutamate receptors. *Nature*, *443*, 705–708.

Chen L, Muhlhauser M, Yang CR (2003). Glycine tranporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons in vitro and in vivo. *Journal of Neurophysiology*, *89*, 691–703.

Chen S, Diamond JS (2002). Synaptically released glutamate activates extrasynaptic NMDA receptors on cells in the ganglion cell layer of rat retina. *Journal of Neuroscience*, *22*, 2165–2173.

Clark BA, Farrant M, Cull-Candy SG (1997). A direct comparison of the single-channel properties of synaptic and extrasynaptic NMDA receptors. *Journal of Neuroscience*, *17*, 107–116.

Clements JD (1996). Transmitter timecourse in the synaptic cleft: Its role in central synaptic function. *Trends in Neurosciences*, *19*, 163–171.

Contini M, Raviola E (2003). GABAergic synapses made by a retinal dopaminergic neuron. *Proceedings of the National Academy of Sciences*, *100*, 1358–1363.

Cope DW, Wulff P, Oberto A, Aller MI, Capogna M, Ferraguti F, Halbsguth C, Hoeger H, Jolin HE, Jones A, Mckenzie ANJ, Ogris W, Poeltl A, Sinkkonen ST, Vekovischeva OY, Korpi ER, Sieghart W, Sigel E, Somogyi P, Wisden W (2004). Abolition of zolpidem sensitivity in mice with a point mutation in the $GABA_A$ receptor γ2 subunit. *Neuropharmacology*, *47*, 17–34.

Cox CL, Zhou Q, Sherman SM (1998). Glutamate locally activates dendritic outputs of thalamic interneurons. *Nature*, *394*, 478–482.

Criswell HE, McCown TJ, Moy SS, Mueller RA, Morrow AL, Breese GR (1997). Action of zolpidem on responses to GABA in relation to mRNAs for GABA(A) receptor alpha subunits within single cells: Evidence for multiple functional

GABA(A) isoreceptors on individual neurons. *Neuropharmacology*, *36*, 1641-1652

Cull-Candy S, Brickley S, Farrant M (2001). NMDA receptor subunits: Diversity, development and disease. *Current Opinion in Neurobiology*, *11*, 327–335.

Dalby NO, Mody I (2003). Activation of NMDA receptors in rat dentate gyrus granule cells by spontaneous and evoked transmitter release. *Journal of Neurophysiology*, *90*, 786–797.

Dämgen K, Lüddens H (1999). Zaleplon displays a selectivity to recombinant $GABA_A$ receptors different from zolipdem, zopiclone and benzodiazepines. *Neuroscience Research Communications*, *25*, 139–148.

Demb JB, Singer JH (2012). Intrinsic properties and functional circuitry of the AII amacrine cell. *Visual Neuroscience*, *29*, 51–60.

Diamond JS (2017). Inhibitory interneurons in the retina: Types, circuitry, and function. *Annual Review of Vision Science*, *3*, 1–24.

Dixon C, Sah P, Lynch JW, Keramidas A (2014). $GABA_A$ receptor α and γ subunits shape synaptic currents via different mechanisms. *Journal of Biological Chemistry*, *289*, 5399–5411.

Dixon M, Kleppe K (1965). D-amino acid oxidase I. Dissociation and recombination of the holoenzyme. *Biochimica et Biophysica Acta*, *96*, 357–367.

Draguhn A, Verdorn TA, Ewert M, Seeburg PH, Sakmann B (1990). Functional and molecular distinction between recombinant rat $GABA_A$ receptor subtypes by Zn^{2+}. *Neuron*, *5*, 781–788.

Drew CA, Johnston GAR, &Weatherby RP (1984). Bicuculline-insensitive GABA receptors: Studies on the binding of (−)-baclofen to rat cerebellar membranes. *Neuroscience Letters*, *52*, 317–321.

Dröse S, Altendorf K (1997). Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. *Journal of Experimental Biology*, *200*, 1–8.

Enz R, Brandstätter JH, Wässle H, Bormann J (1996). Immunocytochemical localization of the GABAC receptor ρ subunits in the mammalian retina. *Journal of Neuroscience*, *16*, 4479–4490.

Erreger K, Dravid SM, Banke TG, Wyllie DJA, Traynelis SF (2005). Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signaling profiles. *Journal of Physiology*, *563*, 345–358.

Euler T, Detwiler PB, Denk W (2002). Directionally selective calcium signals in dendrites of starburst amacrine cells. *Nature*, *418*, 845–852.

Famiglietti EV, Kolb H (1975). A bistratified amacrine cell and synaptic circuitry in the inner plexiform layer of the retina. *Brain Research*, *84*, 293–300.

Famiglietti EV, Edward V (1992). Polyaxonal amacrine cells of rabbit retina: Morphology and stratification of PA1 cells. *Journal of Comparative Neurology*, *316*, 391–405.

Farrant M, Nusser Z. (2005). Variations on an inhibitory theme: Phasic and tonic activation of $GABA_A$ receptors. *Nature Reviews Neuroscience*, *6*, 215–229.

Feigenspan A, Weiler R (2004). Electrophysiological properties of mouse horizontal cell $GABA_A$ receptors. *Journal of Neurophysiology*, *92*, 2789–2801

Feigenspan A, Wässle H, Bormann J (1993). Pharmacology of GABA receptor CI − channels in rat retinal bipolar cells. *Nature*, *361*, 159–162.

Fisher JL, Macdonal, RL (1997). Single channel properties of recombinant $GABA_A$ receptors containing gamma 2 or delta subtypes expressed with alpha 1 and beta 3 subtypes in mouse L929 cells. *The Journal of Physiology*, *505*, 283–297.

Franke K, Baden T (2017). General features of inhibition in the inner retina. *The Journal of Physiology*, *595*, 5507–5515.

Franke K, Berens P, Schubert T, Bethge M, Euler T, Baden T (2017). Inhibition decorrelates visual feature representations in the inner retina. *Nature*, *542*, 439–444.

Gardner SM, Trussell LO, Oertel D (1999). Time course and permeation of synaptic AMPA receptors in cochlear nuclear neurons correlate with input. *Journal of Neuroscience*, *19*, 8721–8729.

Gill SB, Veruki ML, Hartveit E (2006). Functional properties of spontaneous IPSCs and glycine receptors in rod amacrine (AII) cells in the rat retina. *Journal of Physiology*, *575*, 739–759.

Gingrich KJ, Roberts WA, Kass RS (1995). Dependence of the $GABA_A$ receptor gating kinetics on the alpha-subunit isoform: Implications for structure-function relations and synaptic transmission. *Journal of Physiology*, *489*, 529–543.

Gollisch T, Meister M (2010). Eye smarter than scientists believed: Neural computations in circuits of the retina. *Neuron*, *65*, 150–164.

Gómez-Gonzalo M, Zehnder T, Requie LM, Bezzi P, Carmignoto G (2018). Insights into the release mechanism of astrocytic glutamate evoking in neurons NMDA receptor-mediated slow depolarizing inward currents. *Glia*, *66*, 2188–2199.

Gottesman J, Miller RF (2003). N-methyl-D-aspartate receptors contribute to the baseline noise of retinal ganglion cells. *Visual Neuroscience*, *20*, 329–333.

Greferath U, Grünert U, Fristchy JM, Stephenson A, Möhler H, Wässle H (1995). $GABA_A$ receptor subunits have diffrential distributions in the rat retina: In situ hybridization and immunohistochemistry. *Journal of Comparative Neurology*, *353*, 553–571.

Grimes WN, Li W, Chávez AE, Diamond JS (2009). BK channels modulate pre- and postsynaptic signaling at reciprocal synapses in retina. *Nature Neuroscience*, *12*, 585–592.

Grimes WN, Zhang J, Graydon CW, Kachar B. Diamond JS (2010). Retinal parallel processors: More than 100 independent microcircuits operate within a single interneuron. *Neuron*, *65*, 873–885.

Groc L, Heine M, Cousins SL, Stephenson FA, Lounis B, Cognet L, Choquet D (2006). NMDA receptor surface mobility depends on NR2A-2B subunits. *Proceedings of the National Academy of Sciences*, *103*, 18769–18774.

Gustafson EC, Stevens ER, Wolosker H, Miller RF (2007). Endogenous D-serine contributes to NMDA-receptor–mediated light-evoked responses in the vertebrate retina. *Journal of Neurophysiology*, *98*, 122–130.

Gustincich S, Feigenspan A, Sieghart W, Raviola E (1999). Composition of the $GABA_A$ receptors of retinal dopaminergic neurons. *Journal of Neuroscience*, *19*, 7812–7822

Gutiérrez A, Khan ZU, De Blas AL (1996). Immunocytochemical localization of the α_6 subunit of the γ-aminobutyric $acid_A$ receptor in the rat nervous system. *Journal of Comparative Neurology*, *365*, 504–510.

Guzulaitis R, Hounsgaard J (2017). Synaptic excitation in spinal motoneurons alternates with synaptic inhibition and is balanced by outward rectification during rhythmic motor Network activity. *Journal of Neuroscience*, *37*, 9239–9248.

Haas KF, Macdonald RL (1999). $GABA_A$ receptor subunit γ2 and δ subtypes confer unique kinetic properties on recombinant $GABA_A$ receptor currents in mouse fibroblasts. *Journal of Physiology*, *514*, 27–45.

Hamberger AC, Chiang GH, Nylén ES, Scheff SW, Cotman CW (1979). Glutamate as a CNS transmitter. I. Evaluation of glucose and glutamine as precursors for the synthesis of preferentially released glutamate. *Brain Research*, *168*, 513–530.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv*, *391*, 85–100.

Hansra N, Arya S, Quick, MW (2004). Intracellular domains of a rat brain GABA transporter that govern transport. *Journal of Neuroscience*, *24*, 4082–4087.

Hardingham GE, Bading H (2003). The Yin and Yang of NMDA receptor signaling. *Trends in Neurosciences*, *26*, 81–89.

Hardingham GE, Fukunaga Y, Bading H (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nature Neuroscience*, *5*, 405–414.

Harris AZ, Pettit DL (2007). Extrasynaptic and synaptic NMDA receptors form stable and uniform pools in rat hippocampal slices. *Journal of Physiology*, *584*, 509–519.

Hartveit E (1996). Membrane currents evoked by ionotropic glutamate receptor agonists in rod bipolar cells in the rat retinal slice preparation. *Journal of Neurophysiology*, *76*, 401–422.

Hartveit E, Veruki ML (1997). AII amacrine cells express functional NMDA receptors. *NeuroReport*, *8*, 1219–1223.

Hartveit E, Veruki ML (2007). Studying properties of neurotransmitter receptors by non-stationary noise analysis of spontaneous postsynaptic currents and agonist-evoked responses in outside-out patches. *Nature Protocols*, *2*, 434–448.

Hartveit E, Veruki ML (2019). Combining multiphoton excitation microscopy with fast microiontophoresis to investigate neuronal signaling. In: *Multiphoton Microscopy*, Hartveit E (ed.) 131–159. Humana Press. New York, USA.

Heinemann SH, Conti F (1992). Nonstationary noise analysis and application to patch clamp recordings. *Methods in Enzymology 207,*131–148.

Helmstaedter M, Briggman KL, Turaga SC, Jain V, Seung HS, Denk W (2013). Connectomic reconstruction of the inner plexiform layer in the mouse retina. *Nature*, *500*, 168–174.

Henn FA, Goldstein MN, Hamberger A (1974). Uptake of the neurotransmitter candidate glutamate by glia. *Nature*, *249*, 663–664.

Hinkle, D. J., & Macdonald, R. L. (2003). β subunit phosphorylation selectively increases fast desensitization and prolongs deactivation of $\alpha1\beta1\gamma2L$ and $\alpha1\beta3\gamma2L$ $GABA_A$ receptor currents. *Journal of Neuoscience, 23*, 11698–11710

Hodgkin AL, Huxley AF (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *Journal of Physiology*, *117*, 500–544.

Hoon M, Okawa H, Della Santina L, Wong ROL (2014). Functional architecture of the retina: Development and disease. *Progress in Retinal and Eye Research*, *42*, 44–84.

Horowitz P, Hill W (1989). *The art of electronics*. Cambridge University Press. Cambridge, England.

Jia F, Pignataro L, Schofield CM, Yue M, Harrison NL, Goldstein PA (2005). An extrasynaptic $GABA_A$ receptor mediates tonic inhibition in thalamic VB neurons. *Journal of Neurophysiology*, *94*, 4491–4501.

Johnson JW, Ascher P (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, *325*, 529–531.

Jonas P (1995). Fast application of agonists to isolated membrane patches. In: *Single-Channel Recording*. Sakmann B., Neher E. (eds.), 231–243. Plenum Press. New York, USA.

Jones A, Korpi ER, McKernan RM, Pelz R, Nusser Z, Mäkelä R, Mellor JR, Pollard S, Bahn S, Stephenson FA, Randall AD, Sieghart W, Somogyi P, Smith AJH, Wisden W (1997). Ligand-gated ion channel subunit partnerships: $GABA_A$ receptor α6 subunit gene inactivation inhibits δ subunit expression. *Journal of Neuroscience*, *17*, 1350–1362.

Jones MV, Westbrook GL (1995). Desensitized states prolong $GABA_A$ channel responses to brief agonist pulses. *Neuron*, *15*, 181–191.

Jones MV, Westbrook GL (1996). The impact of receptor desensitization on fast synaptic transmission. *Trends in Neurosciences*, *19*, 96–101.

Jones MV, Westbrook GL (1997). Shaping of IPSCs by endogenous calcineurin activity. *Journal of Neuroscience*, *17*, 7626–7633.

Kalbaugh TL, Zhang J, Diamond JS (2009). Coagonist release modulates NMDA receptor subtype contributions at synaptic inputs to retinal ganglion cells. *Journal of Neuroscience*, *29*, 1469–1479.

Katz B, Miledi R (1967). The timing of calcium action during neuromuscular transmission. *Journal of Physiology*, *189*, 535–544.

Khan ZU, Gutiérrez A, Mehta AK, Miralles CP, De Blas AL (1996). The α_4 subunit of the $GABA_A$ receptors from rat brain and retina. *Neuropharmacology*, *35*, 1315–1322.

Keller CA, Yuan X, Panzanelli P, Martin ML, Alldred M, Sassoè-Pognetto M, Lüscher B (2004). The γ2 subunit of $GABA_A$ receptors is a substrate for palmitoylation by GODZ. *Journal of Neuroscience*, *24*, 5881–5891.

Kleckner NW, Dingledine R (1988). Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. *Science*, *241*, 835–837.

Kolb H (1979). The inner plexiform layer in the retina of the cat: Electron microscopic observations. *Journal of Neurocytology*, *8*, 295–329.

Kolb H, Famigilietti EV (1974). Rod and cone oathways in the inner plexiform layer of cat retina. *Science*, *186*, 47–49.

Kothmann WW, Trexler EB, Whitaker CM, Li W, Massey SC, O'Brien J (2012). Nonsynaptic NMDA receptors mediate activity-dependent plasticity of gap junctional coupling in the AII amacrine cell network. *Journal of Neuroscience*, *32*, 6747–6759.

Lagrange AH, Hu N, Macdonald RL (2018). GABA beyond the synapse: Defining the subtype-specific pharmacodynamics of non-synaptic $GABA_A$ receptors. *Journal of Physiology*, *596*, 4475–4495.

Lavoie AM, Tingey JJ, Harrison NL, Pritchett DB, Twyman RE (1997). Activation and deactivation rates of recombinant GABA(A) receptor channels are dependent on alpha-subunit isoform. *Biophysical Journal*, *73*, 2518–2526.

Lerma J, Herranz AS, Herreras O, Abraira V, del Rio RM (1986). In vivo determination of extracellular concentration of amino acids in the rat hippocampus. A method based on brain dialysis and computerized analysis. *Brain Research*, *384*, 145–155.

Lerma J, Zukin RS, Bennett MV (1990). Glycine decreases desensitization of N-methyl-D-aspartate (NMDA) receptors expressed in Xenopus oocytes and is required for NMDA responses. *Proceedings of the National Academy of Sciences*, *87*, 2354–2358.

Le Meur K, Galante M, Angulo MC, Audinat E (2007). Tonic activation of NMDA receptors by ambient glutamate of non-synaptic origin in the rat hippocampus. *Journal of Physiology*, *580*, 373–383.

Li Y, Sacchi S, Pollegioni L, Basu AC, Coyle JT, Bolshakov VY (2013). Identity of endogenous NMDAR glycine site agonist in amygdala is determined by synaptic activity level. *Nature Communications*, *4*, 1760.

Liu D, Yang Q, Li S (2013). Activation of extrasynaptic NMDA receptors induces LTD in rat hippocampal CA1 neurons. *Brain Research Bulletin*, *93*, 10–16.

Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science*, *304*, 1021–1024.

Loiola EC, Ventura ALM (2011). Release of ATP from avian Müller glia cells in culture. *Neurochemistry International*, *58*, 414–422.

López-Muñoz F, Boya J, Alamo C (2006). Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramón y Cajal. *Brain Research Bulletin*, *70*, 391–405.

Lüscher B, Keller CA (2004). Regulation of $GABA_A$ receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. *Pharmacology & Therapeutics*, *102*, 195–221.

MacNeil MA, Masland RH (1998). Extreme diversity among amacrine cells: Implications for function. *Neuron*, *20*, 971–982.

Maguire G, Simko H, Weinreb RN, Ayoub G (1998). Transport-mediated release of endogenous glutamate in the vertebrate retina. *European Journal of Physiology*, *436*, 481–484.

Major G (1993). Solutions for transients in arbitrarily branching cables: III. Voltage clamp problems. *Biophysical Journal, 65*, 469-491.

Majumdar S, Weiss J, Wässle H (2009). Glycinergic input of widefield, displaced amacrine cells of the mouse retina. *Journal of Physiology*, *587*, 3831–3849.

Malarkey EB, Parpura V (2008). Mechanisms of glutamate release from astrocytes. *Neurochemistry International*, *52*, 142–154.

Manookin MB, Beaudoin DL, Ernst ZR, Flagel LJ, Demb JB (2008). Disinhibition combines with excitation to extend the operating range of the OFF visual pathway in daylight. *Journal of Neuroscience*, *28*, 4136–4150.

Marc RE, Anderson JR, Jones BW, Sigulinsky CL, Lauritzen JS (2014). The AII amacrine cell connectome: a dense network hub. *Frontiers in Neural Circuits*, *8*, 104.

Marc RE, Liu WLS (2000). Fundamental GABAergic amacrine cell circuitries in the retina: Nested feedback, concatenated inhibition, and axosomatic synapses. *Journal of Comparative Neurology*, *425*, 560–582

Marowsky A, Vogt KE (2014). Delta-subunit-containing $GABA_A$-receptors mediate tonic inhibition in paracapsular cells of the mouse amygdala. *Frontiers in Neural Circuits*, *8*, 1–27

Martel MA, Wyllie DJA, Hardingham GE (2009). In developing hippocampal neurons, NR2B-containing N-methyl-d-aspartate receptors (NMDARs) can mediate signaling to neuronal survival and synaptic potentiation, as well as neuronal death. *Neuroscience*, *158*, 334–343.

Marty A, Neher E (1983). Tight-Seal Whole-Cell Recording. In: *Single-Channel Recording*, Bert Sakmann & E. Neher (eds.). Springer. Boston, USA.

Masland RH (1988). Amacrine cells. *Trends in Neurosciences*, *11*, 405–410.

Masland RH (2012). The neuronal organization of the retina. *Neuron*, *76*, 266–280.

Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI (2004). Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. *Journal of Neuroscience*, *24*, 7821–7828.

McKernan RM, Whiting PJ (1996). Which $GABA_A$-receptor subtypes really occur in the brain? *Trends in Neurosciences*, *19*, 139–143.

Menger N, Pow DV, Wässle H (1998). Glycinergic amacrine cells of the rat retina. *Journal of Comparative Neurology*, *401*, 34–46.

Menger N, Wässle H (2000). Morphological and physiological properties of the A17 amacrine cell of the rat retina. *Visual Neuroscience*, *17*, 769–780.

Miles R, Tóth K, Gulyás AI, Hájos N, Freund TF (1996). Differences between somatic and dendritic inhibition in the hippocampus. *Neuron*, *16*, 815–823.

Mody I, De Koninck Y, Otis TS, Soltesz I (1994). Bridging the cleft at GABA synapses in the brain. *Trends in Neurosciences*, *17*, 517–525.

Möhler H (2006). $GABA_A$ receptor diversity and pharmacology. *Cell and Tissue Research*, *326*, 505–516.

Molla G, Sacchi S, Bernasconi M, Pilone MS, Fukui K, Pollegioni L (2006). Characterization of human d-amino acid oxidase. *FEBS Letters*, *580*, 2358–2364.

Molleman A (2003). *Patch clamping: An introductory guide to patch clamp electrophysiology*. John Wiley & Sons. Chichester, UK.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron*, *12*, 529–540.

Moore AK, Weible AP, Balmer TS, Trussell LO, Wehr M (2018). Rapid rebalancing of excitation and inhibition by cortical circuitry. *Neuron*, *97*, 1341-1355.e6.

Moran O (1995). Patch-clamp technique. In: *Experimental techniques in bioelectrochemistry. Bioelectrochemistry: Principles and practice*, vol 3, Brabec V, Walz D, Milazzo G (eds), Birkhäuser, Switzerland.

Morishita W, Lu W, Smith GB, Nicoll RA, Bear MF, Malenka RC (2007). Activation of NR2B-containing NMDA receptors is not required for NMDA receptor-dependent long-term depression. *Neuropharmacology*, *52*, 71–76.

Mortensen M, Smart TG (2006). Extrasynaptic αβ subunit $GABA_A$ receptors on rat hippocampal pyramidal neurons. *Journal of Physiology*, *577*, 841–856.

Mothet JP, Bail ML, Billard JM (2015). Time and space profiling of NMDA receptor co-agonist functions. *Journal of Neurochemistry*, *135*, 210–225.

Mothet JP, Parent AT, Wolosker H, Brady RO, Linden DJ, Ferris CD, Rogawski MA, Snyder SH (2000). D-Serine is an endogenous ligand for the glycine site of the N-methyl-d-aspartate receptor. *Proceedings of the National Academy of Sciences*, *97*, 4926–4931.

Mozrzymas JW, Żarnowska ED, Mercik K (2003). Modulation of $GABA_A$ receptors by hydrogen ions reveals synaptic GABA transient and a crucial role of the desensitization process. *Journal of Neuroscience*, *23*, 7981–7992.

Murphy GJ, Rieke F (2006). Network variability limits stimulus-evoked spike timing precision in retinal ganglion cells. *Neuron*, *52*, 511–524.

Nelson R, Kolb H (1985). A17: A broad-field amacrine cell in the rod system of the cat retina. *Journal of Neurophysiology*, *54*, 592–614.

Newman EA (2015). Glial cell regulation of neuronal activity and blood flow in the retina by release of gliotransmitters. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*, 20140195.

Neyton J, Paoletti P (2006). Relating NMDA receptor function to receptor subunit composition: Limitations of the pharmacological approach. *Journal of Neuroscience*, *26*, 1331–1333.

Nusser Z, Naylor D, Mody I (2001). Synapse-specific contribution of the variation of transmitter concentration to the decay of inhibitory postsynaptic currents. *Biophysical Journal*, *80*, 1251–1261.

Nusser Z, Sieghart W, Somogyi P (1998). Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *Journal of Neuroscience*, *18*, 1693–1703.

Ortinski PI, Lu C, Takagaki K, Fu Z, Vicini S (2004). Expression of distinct α subunits of $GABA_A$ receptor regulates inhibitory synaptic strength. *Journal of Neurophysiology*, *92*, 1718–1727.

Pál B (2018). Involvement of extrasynaptic glutamate in physiological and pathophysiological changes of neuronal excitability. *Cellular and Molecular Life Sciences*, *75*, 2917–2949.

Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA, Oliet SHR (2006). Glia-Derived D-Serine Controls NMDA Receptor activity and synaptic memory. *Cell*, *125*, 775–784.

Paoletti P, Bellone C, Zhou Q (2013). NMDA receptor subunit diversity: Impact on receptor properties, synaptic plasticity and disease. *Nature Reviews Neuroscience*, *14*, 383–400.

Paoletti P, Neyton J (2007). NMDA receptor subunits: Function and pharmacology. *Current Opinion in Pharmacology*, *7*, 39–47.

Papouin T, Ladépêche L, Ruel J, Sacchi S, Labasque M, Hanini M, Groc L, Pollegioni L, Mothet JP, Oliet SHR (2012). Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists. *Cell*, *150*, 633–646.

Papouin T, Oliet SHR (2014). Organization, control and function of extrasynaptic NMDA receptors. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*, 20130601.

Park SJ, Lieberman EE, Ke JB, Rho N, Ghorbani P, Rahmani P, Jun NY, Lee HL, Kim IJ, Briggman KL, Demb JB, Singer JH (2020). Connectomic analysis reveals an interneuron with an integral role in the retinal circuit for night vision. *ELife*, *9*, e56077.

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994). Glutamate-mediated astrocyte–neuron signaling. *Nature*, *369*, 744–747.

Parsons MP, Raymond LA (2014). Extrasynaptic NMDA receptor involvement in central nervous system disorders. *Neuron*, *82*, 279–293.

Perrais D, Ropert N (1999). Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic $GABA_A$ receptors in central synapses. *Journal of Neuroscience*, *19*, 578–588.

Petralia RS, Wang YX, Hua F, Yi Z, Zhou A, Ge L, Stephenson FA, Wenthold RJ (2010). Organization of NMDA receptors at extrasynaptic locations. *Neuroscience*, *167*, 68–87.

Pfeffer CK, Xue M, He M, Huang ZJ, Scanziani M (2013). Inhibition of inhibition in visual cortex: The logic of connections between molecularly distinct interneurons. *Nature Neuroscience*, *16*, 1068–1076.

Pinault D, Smith Y, Deschênes M (1997). Dendrodendritic and axoaxonic synapses in the thalamic reticular nucleus of the adult rat. *Journal of Neuroscience*, *17*, 3215–3233.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000). $GABA_A$ receptors: Immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience*, *101*, 815–850.

Potapenko ES, Biancardi VC, Florschutz RM, Ryu PD, Stern JE (2011). Inhibitory-excitatory synaptic balance is shifted toward increased excitation in magnocellular neurosecretory cells of heart failure rats. *Journal of Neurophysiology*, *106*, 1545–1557.

Pouille F, Marin-Burgin A, Adesnik H, Atallah BV, Scanziani M (2009). Input normalization by global feedforward inhibition expands cortical dynamic range. *Nature Neuroscience*, *12*, 1577–1585.

Pow DV, Hendrikson AE (1998). Distribution of the glycine transpoter GlyT-1 in mammalian and nonmammalian retinae. *Visual Neuroscience*, *16*, 231–239.

Pow DV, Robinson SR (1994). Glutamate in some retinal neurons is derived solely from glia. *Neuroscience*, *60*, 355–366.

Price JL, Powell TPS (1970). The synaptology of the granule cells of the olfactory bulb. *Journal of Cell Science*, *7*, 125–155.

Pritchett DB, Seeburg PH (1990). γ-Aminobutyric $acid_A$ receptor α_5-subunit creates novel type II benzodiazepine receptor pharmacology. *Journal of Neurochemestry*, *54*, 1802–1804

Pritchett DB, Sontheimer H, Shiver BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PH (1989). Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature*, *338*, 582–585.

Puia G, Vicini S, Seeburg PH, Costa E (1991). Influence of recombinant γ-aminobutyric $acid_A$ receptor subunit composition on the action of allosteric modulators of γ-aminobutyric acid-gated Cl^- currents. *Molecular Pharmacology*, *39*, 691–696.

Purves D (ed.) (2019). *Neuroscience*, 6th ed. Oxford University Press. New York, USA.

Ramón y Cajal S (1989). *Recollections of my life*. MIT Press. Cambridge, USA.

Rathenberg J, Kittler JT, Moss SJ (2004). Palmitoylation regulates the clustering and cell surface stability of $GABA_A$ receptors. *Molecular and Cellular Neuroscience*, *26*, 251–257.

Reingruber J, Holcman D, Fain GL (2015). How rods respond to single photons: Key adaptations of a G-protein cascade that enable vision at the physical limit of perception. *BioEssays*, *37*, 1243–1252.

Riepe RE, Norenburg MD (1977). Müller cell localisation of glutamine synthetase in rat retina. *Nature*, *268*, 654–655.

Ronzio RA, Rowe WB, Meister A (1969). Mechanism of inhibition of glutamine synthetase by methionine sulfoximine. *Biochemistry*, *8*, 1066–1075.

Rosenberg D, Artoul S, Segal AC, Kolodney G, Radzishevsky I, Dikopoltsev E, Foltyn VN, Inoue R, Mori H, Billard JM, Wolosker H (2013). Neuronal D-serine and glycine release via the Asc-1 transporter regulates NMDA receptor-dependent synaptic activity. *Journal of Neuroscience*, *33*, 3533–3544.

Royer S, Zemelman BV, Losonczy A, Kim J, Chance F, Magee JC, Buzsáki G (2012). Control of timing, rate and bursts of hippocampal place cells by dendritic and somatic inhibition. *Nature Neuroscience*, *15*, 769–775.

Rubin R, Abbott LF, Sompolinsky H (2017). Balanced excitation and inhibition are required for high-capacity, noise-robust neuronal selectivity. *Proceedings of the National Academy of Sciences*, *114*, E9366–E9375.

Rudolph U, Möhler H (2014). $GABA_A$ Receptor Subtypes: Therapeutic potential in down syndrome, affective disorders, schizophrenia and autism. *Annual Review of Pharmacology and Toxicology*, *54*, 483–507.

Rusakov DA, Dityatev AE (2014). Brain circuitry outside the synaptic cleft. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*, 20130591.

Sah P, Hestrin S, Nicoll RA (1989). Tonic activation of NMDA receptors by ambient glutamate enhances excitability of neurons. *Science*, *246*, 815–818.

Sakmann B, Neher E (1984). Patch clamp yechniques for studying ionic channels in excitable membranes. *Annual Review of Physiology*, *46*, 455–472.

Sandell JH, Masland RH (1986). A system of indoleamine-accumulating neurons in the rabbit retina. *Journal of Neuroscience*, *6*, 3331–3347.

Sandell JH, Masland RH, Raviola E, Dacheux RF (1989). Connections of indoleamine-accumulating cells in the rabbit retina. *Journal of Comparative Neurology*, *283*, 303–313.

Sassoè-Pognetto M, Panzanelli P, Sieghart W, Fritschy JM (2000). Colocalization of multiple $GABA_A$ receptor subtypes with gephyrin at postsynaptic sites. *Journal of Comparative Neurology*, *420*, 481–498.

Sather W, Dieudonné S, MacDonald JF, Ascher P (1992). Activation and desensitization of N-methyl-D-aspartate receptors in nucleated outside-out patches from mouse neurones. *Journal of Physiology*, *450*, 643–672.

Savtchouk I, Volterra A (2018). Gliotransmission: Beyond black-and-white. *Journal of Neuroscience*, *38*, 14–25.

Saxena NC, Macdonald RL (1996). Properties of putative cerebellar gamma-aminobutyric acid A receptor isoforms. *Molecular Pharmacology*, *49*, 567–579.

Schell MJ, Molliver ME, Snyder SH (1995). D-serine, an endogenous synaptic modulator: Localization to astrocytes and glutamate-stimulated release. *Proceedings of the National Academy of Sciences*, *92*, 3948–3952.

Schorge S, Colquhoun D (2003). Studies of NMDA receptor function and stoichiometry with truncated and tandem subunits. *Journal of Neuroscience*, *23*, 1151–1158.

Schubert T, Hoon M, Euler T, Lukasiewicz PD, Wong ROL (2013). Developmental regulation and activity-dependent maintenance of GABAergic presynaptic inhibition onto rod bipolar cell axonal terminals. *Neuron*, *78*, 124–137.

Schwiening CJ (2012). A brief historical perspective: Hodgkin and Huxley. *Journal of Physiology*, *590*, 2571–2575.

Scimemi A, Beato M (2009). Determining the neurotransmitter concentration profile at active synapses. *Molecular Neurobiology*, *4*, 289–306.

Scimemi A, Fine A, Kullmann DM, Rusakov DA (2004). NR2B-containing receptors mediate cross talk among hippocampal synapses. *Journal of Neuroscience*, *24*, 4767–4777.

Sherrington CS (1906). *The integrative action of the nervous system*. C Scribner and Sons. New York, USA.

Shimamoto K, Lebrun B, Yasuda-Kamatani Y, Sakaitani M, Shigeri Y, Yumoto N, Nakajima T (1998). Dl-threo-β-Benzyloxyaspartate, a potent blocker of excitatory amino acid transporters. *Molecular Pharmacology*, *53*, 195–201.

Shipton OA, Paulsen O (2014). GluN2A and GluN2B subunit-containing NMDA receptors in hippocampal plasticity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*, 20130163.

Sieghart W, Sperk G (2002). Subunit composition, distribution and function of GABA-A receptor subtypes. *Current Topics in Medicinal Chemistry*, *2*, 795–816.

Sieghart W (2015). Allosteric modulation of $GABA_A$ receptors via multiple drug-binding sites. *Advances in Pharmacology*, *72*, 53–96.

Sigel E, Buhr A (1997). The benzodiazepine binding site of $GABA_A$ receptors. *Trends in Pharmacological Sciences*, *18*, 425–429.

Silver RA, Colquhoun D, Cull-Candy SG, Edmonds B (1996). Deactivation and desensitization of non-NMDA receptors in patches and the time course of EPSCs in rat cerebellar granule cells. *Journal of Physiology*, *493*, 167–173.

Singer JH, Diamond JS (2003). Sustained Ca^{2+} entry elicits transient postsynaptic currents at a retinal ribbon synapse. *Journal of Neuroscience*, *23*, 10923–10933.

Smart TG, Moss SJ, Xie X, Huganir RL (1991). $GABA_A$ receptors are differentially sensitive to zinc: Dependence on subunit composition. *British Journal of Pharmacology*, *103*, 1837–1839.

Stevens ER, Esguerra M, Kim PM, Newman EA, Snyder SH, Zahs KR, Miller RF (2003). D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. *Proceedings of the National Academy of Sciences*, *100*, 6789–6794.

Stevens ER, Gustafson EC, Miller RF (2010). Glycine transport accounts for the differential role of glycine vs. D-serine at NMDA receptor coagonist sites in the salamander retina. *European Journal of Neuroscience*, *31*, 808–816.

Stórustovu Sí, Ebert B (2003). Gaboxadol: In vitro interaction studies with benzodiazepines and ethanol suggest functional selectivity. *European Journal of Pharmacology*, *467*, 49–56.

Stórustovu Sí, Ebert B (2006). Pharmacological characterization of agonists at δ-containing $GABA_A$ receptors: functional selectivity for extrasynaptic receptors is dependent on the absence of γ2. *Journal of Pharmacology and Experimental Therapeutics*, *316*, 1351–1359.

Strettoi E, Dacheux RF, Raviola E (1990). Synaptic connections of rod bipolar cells in the inner plexiform layer of the rabbit retina. *Journal of Comparative Neurology*, *295*, 449–466.

Strettoi E, Dacheux RF, Raviola E (1994). Cone bipolar cells as interneurons in the rod, pathway of the rabbit retina. *Journal of Comparative Neurology*, *347*(1), 139–149.

Strettoi E, Raviola E, Dacheux RF (1992). Synaptic connections of the narrow-field, bistratified rod amacrine cell (AII) in the rabbit retina. *Journal of Comparative Neurology*, *325*, 152–168.

Sullivan SJ, Miller RF (2010). AMPA receptor mediated D-serine release from retinal glial cells. *Journal of Neurochemistry*, *115*, 1681–1689.

Sur C, Farrar SJ, Kerby J, Whiting PJ, Atack JR, McKernan RM (1999). Preferential coassembly of α_4 and δ subunits of the γ-aminobutyric $acid_A$ receptor in rat thalamus. *Molecular Pharmacology*, *56*, 110–115.

Swanson LW (2017). *The beautiful brain*. Newman EA, Araque A, Dubinski JM (eds.). Abrams. New York, USA.

Thoreson WB, Miller RF (1996). Removal of extracellular chloride suppresses transmitter release from photoreceptor terminals in the mudpuppy retina. *Journal of General Physiology*, *107*, 631–642.

Tovar KR, Westbrook GL (1999). The Incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *Journal of Neuroscience*, *19*, 4180–4188.

Traynelis SF, Angus Silver R, Cull-Candy SG (1993). Estimated conductance of glutamate receptor channels activated during EPSCs at the cerebellar mossy fiber-granule cell synapse. *Neuron*, *11*, 279–289.

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R (2010). Glutamate receptor ion channels: Structure, regulation, and function. *Pharmacological Reviews*, *62*, 405–496.

Tsai G, Ralph-Williams RJ, Martina M, Bergeron R, Berger-Sweeney J, Dunham KS, Jiang Z, Caine SB, Coyle JT (2004). Gene knockout of glycine transporter 1: Characterization of the behavioral phenotype. *Proceedings of the National Academy of Sciences*, *101*, 8485–8490.

Ulbrich MH, Isacoff EY (2008). Rules of engagement for NMDA receptor subunits. *Proceedings of the National Academy of Sciences*, *105*, 14163–14168.

Ullian EM, Barkis WB, CHen S, Diamond JS, Barres BA (2004). Invulnerability of retinal ganglion cells to NMDA excitotoxicity. *Molecular and Cellular Neuroscience*, *26*, 544–557.

Vandenberghe W, Ihle EC, Patneau DK, Robberecht W, Brorson JR (2000). AMPA receptor current density, not desensitization, predicts selective motoneuron vulnerability. *Journal of Neuroscience*, *20*, 7158–7166.

Vaney DI (1986). Morphological identification of serotonin-accumulating neurons in the living retina. *Science*, *233*, 444–446.

Vaney DI (2004). Type 1 nitrergic (ND1) cells of the rabbit retina: Comparison with other axon-bearing amacrine cells. *Journal of Comparative Neurology*, *474*, 149–171.

Veruki ML, Zhou Y, Castilho Á, Morgans CW, Hartveit E (2019). Extrasynaptic NMDA receptors on rod pathway amacrine cells: Molecular composition, activation, and signaling. *Journal of Neuroscience*, *39*, 627–650.

Veruki ML, Hartveit E (2002). AII (rod) amacrine cells form a network of electrically coupled interneurons in the mammalian retina. *Neuron*, *33*, 935–946.

Veruki ML, Mørkve SH, Hartveit E (2003). Functional properties of spontaneous EPSCs and non-NMDA receptors in rod amacrine (AII) cells in the rat retina. *Journal of Physiology*, *549*, 759–774.

Veruki ML, Mørkve SH, Hartveit E (2006). Activation of a presynaptic glutamate transporter regulates synaptic transmission through electrical signaling. *Nature Neuroscience*, *9*, 1388–1396.

Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, Homanics GE (2001). $GABA_A$ receptor α1 subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. *Journal of Neuroscience*, *21*, 3009–3016.

Vijay P (1983). Release of [3H]gamma-aminobutyric acid from glial (Müller) cells of the rat retina: Effects of K^+, veratridine, and ethylenediamine. *Journal of Neuroscience*, *3*, 10.

Völgyi B, Debertin G, Balogh M, Popovich E, Kovács-Öller T (2014). Compartment-specific tyrosine hydroxylase-positive innervation to AII amacrine cells in the rabbit retina. *Neuroscience*, *270*, 88–97.

Wafford KA, Whiting PJ, Kemp JA (1993). Diferences in affinity and efficacy of benzodiazepine receptor ligands at recombinant γ-aminobutyric acid A receptor subtypes. *Molecular Pharmacolgy*, *43*, 240–244.

Weitlauf C, Honse Y, Auberson YP, Mishina M, Lovinger DM, Winder DG (2005). Activation of NR2A-containing NMDA receptors is not obligatory for NMDA receptor-dependent long-term potentiation. *Journal of Neuroscience*, *25*, 8386–8390.

Wersinger E, Schwab Y, Sahel JA, Rendon A, Pow DV, Picaud S, Roux MJ (2006). The glutamate transporter EAAT5 works as a presynaptic receptor in mouse rod bipolar cells. *Journal of Physiology*, *577*, 221–234.

Wilson NR, Runyan CA, Wang FL, Sur M (2012). Division and subtraction by distinct cortical inhibitory networks in vivo. *Nature*, *488*, 343–348.

Winkler BS, Kapousta-Bruneau N, Arnold MJ, Green DG (1999). Effects of inhibiting glutamine synthetase and blocking glutamate uptake on b-wave generation in the isolated rat retina. *Visual Neuroscience*, *16*, 345–353.

Wright LL, Vaney DI (2004). The type 1 polyaxonal amacrine cells of the rabbit retina: A tracer-coupling study. *Visual Neuroscience*, *21*, 145–155.

Wu YW, Grebenyuk S, McHugh TJ, Rusakov DA, Semyanov A (2012). Backpropagating action potentials enable detection of extrasynaptic glutamate by NMDA receptors. *Cell Reports*, *1*, 495–505.

Wässle H (2004). Parallel processing in the mammalian retina. *Nature Reviews Neuroscience*, *5*, 747–757.

Wässle H, Chun MH, Müller F (1987). Amacrine cells in the ganglion cell layer of the cat retina. *Journal of Comparative Neurology*, *265*, 391–408.

Wässle H, Koulen P, Brandstätter JH, Fletcher EL, Becker CM (1998). Glycine and GABA receptors in the mammalian retina. *Vision Research*, *38*, 1411–1430.

Yamashita T, Kanda T, Eguchi K, Takahashi T (2009). Vesicular glutamate filling and AMPA receptor occupancy at the calyx of Held synapse of immature rats. *Journal of Physiology*, *587*, 2327–2339.

Yan W, Laboulaye MA, Tran NM, Whitney IE, Benhar I, Sanes JR (2020). Mouse retinal cell atlas: Molecular identification of over sixty amacrine cell types. *Journal of Neuroscience*, *40*, 5177–5195.

Yang Q, Zhu G, Liu D, Ju JG, Liao ZH, Xiao YX, Zhang Y, Chao N, Wang J, Li W, Luo JH, Li ST (2017). Extrasynaptic NMDA receptor dependent long-term potentiation of hippocampal CA1 pyramidal neurons. *Scientific Reports*, *7*, 3045.

Yu W, Miller RF (1995). Hyperosmotic activation of transmitter release from presynaptic terminals onto retinal ganglion cells. *Journal of Neuroscience Methods*, *62*, 159–168.

Zhang M, Biancardi VC, Stern JE (2017). An increased extrasynaptic NMDA tone inhibits A-type K+ current and increases excitability of hypothalamic neurosecretory neurons in hypertensive rats. *Journal of Physiology*, *595*, 4647–4661.

Zhang R, Du W, Prober DA, Du J (2019). Müller glial cells participate in retinal waves via glutamate transporters and AMPA receptors. *Cell Reports*, *27*, 2871-2880.e2.

Zheleznova NN, Sedelnikova A, Weiss DS (2009). Function and modulation of δ-containing $GABA_A$ receptors. *Psychoneuroendocrinology*, *34*, 67–73.

Zhou C, Dacheux RF (2004). AII amacrine cells in the rabbit retina possess AMPA-, NMDA-, GABA-, and glycine-activated currents. *Visual Neuroscience*, *21*, 181–188.

Zhou Y, Tencerová B, Hartveit E, Veruki ML (2016). Functional NMDA receptors are expressed by both AII and A17 amacrine cells in the rod pathway of the mammalian retina. *Journal of Neurophysiology*, *115*, 389–403.

Zhu Y, Xu J, Hauswirth WW, DeVries SH (2014). Genetically Targeted Binary Labeling of Retinal Neurons. *Journal of Neuroscience*, *34*, 7845–7861.

Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

uib.no

ISBN: 9788230868355 (print)
9788230864296 (PDF)