ELECTROPHYSIOLOGICAL CHARACTERIZATION OF THE IONOTROPIC GLUTAMATE RECEPTORS IN THE MOUSE RETINAL AMACRINE CELLS

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ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CNS	central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dion
ConA	concanavalin A
CTZ	cyclothiazide
DNQX	6,7-dinitro-quinoxaline-2,3-dion
dNTP	deoxinucleoside triphosphate (nucleotides)
EC	extracellular solution
EGPF	enhanced green fluorescence protein
EPSC	excitatory postsynaptic current
Glu	glutamate
GluR	glutamate receptor
Gly	glycine
GlyT2	glycine transporter 2
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IC	intracellular solution
iGluR	ionotropic glutamate receptor
IPSC	inhibitory postsynaptic current
KA	kainic acid
LY	Lucifer yellow
mGluR	metabotropic glutamate receptor
NB	neurobiotin
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline
NMDA	N-methyl-D-aspartic acid
PCR	polymerase chain reaction
QA	quisqualate
Ø	diameter

CONTENTS

1. INTRODUCTION

1.1. ORGANIZATION OF THE RETINA

The retina is a transparent, 200 μm thick neural tissue which covers the interior of the eye. During development, it arises as an extension of the embryonic forebrain and is therefore part of the brain. An accessible part of the brain, containing more than 120 millions of neurons, interconnected in a precisely layered structure, which thus provides a basic model of the more complex circuits of higher brain areas. The function of the retina is to convert the light signals into electrical signals interpretable by the brain, as well as to attain the first steps of image processing.

At the input of the retina lie sensory neurons that respond to light, the photoreceptors, while at the output are the ganglion cells, whose axons form the optic nerve. The electrical message travels along the optic nerve towards the brain and it is relayed in the lateral geniculate nucleus before reaching the final destination in the occipital lobe, the visual cortex. The photoreceptors lie at the very back of the tissue, thus light must pass through the entire retina before reaching the visual pigments, the actual light sensors.

All vertebrate retinae are organized according to the same basic plan, with the cell bodies packed into three rows separated by two layers of neural processes and synaptic connections (*Fig. 1.1.*). The outer nuclear layer (ONL) contains the perikarya of the photoreceptors (rods and cones), the inner nuclear layer (INL) is built up by horizontal, bipolar and amacrine cell somata, while ganglion cell and displaced amacrine cell bodies form the ganglion cell layer (GCL). The photoreceptor axons contact bipolar and horizontal cells in the outer plexiform layer (OPL). In the inner plexiform layer (IPL), bipolar cell axons synapse either directly onto ganglion cell dendrites or onto amacrine cells, which in turn contact ganglion cells. Amacrine cells contact bipolar cells, other amacrine cells and ganglion cells. The main types of glia in the vertebrate retina are the Müller cells, which extend vertically through the tissue, and astrocytes, engaged with the optic nerve fibers.

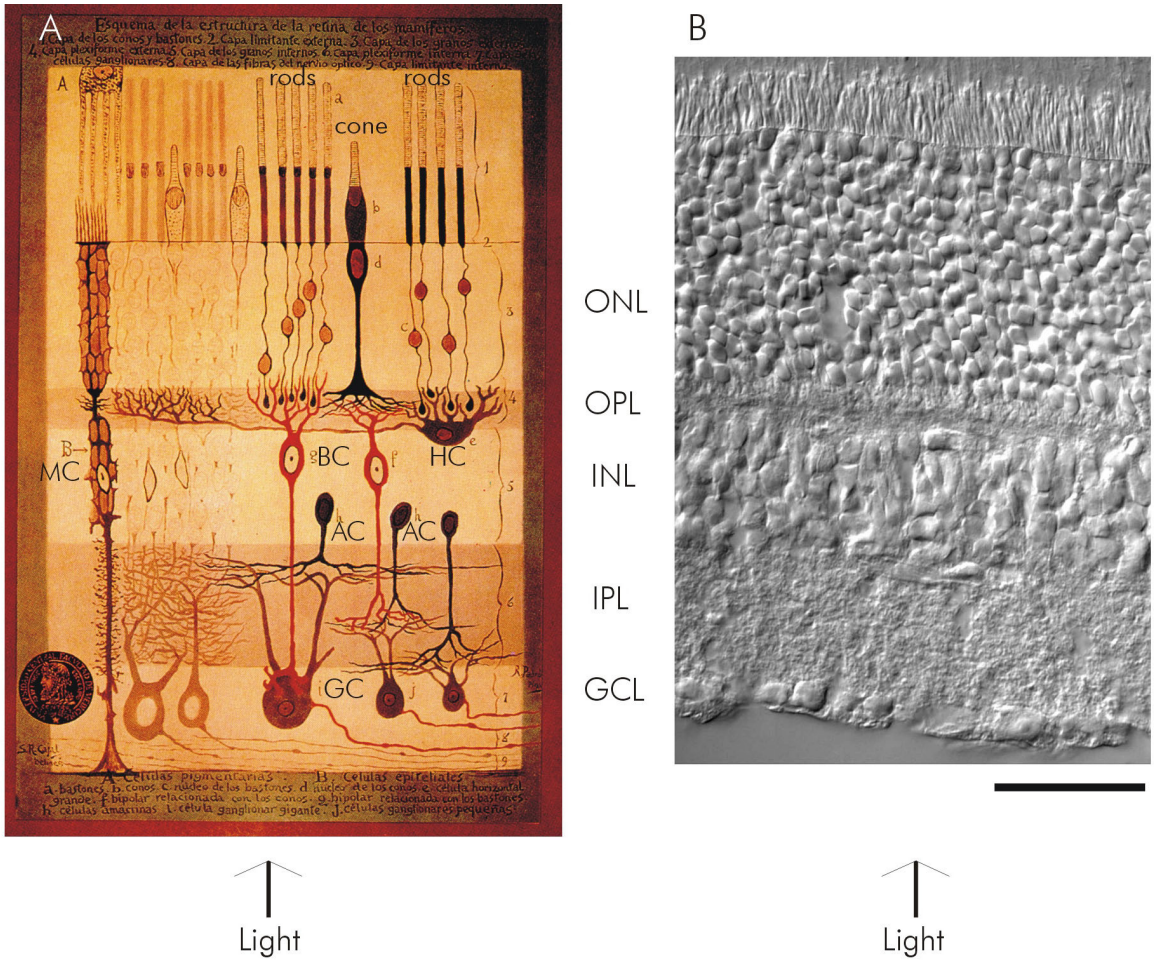

Figure 1.1. **Organization of the mammalian retina.**
(A) Schematic drawing of a vertical section through the retina, by Ramon y Cajal, based on Golgi stainings. The cell types are marked: rods and cones; HC horizontal cell; BC bipolar cell; AC amacrine cell; GC ganglion cell; MC Müller cell.
The following layers are indicated: ONL outer nuclear layer; OPL outer plexiform layer; INL inner nuclear layer; IPL inner plexiform layer; GCL ganglion cell layer.
(B) Nomarski image of a vertical section through the mouse retina. Scale bar 50 μm.

There are two basic types of photoreceptors, rods and cones (reviewed by *Rodieck 1998*). Rods are very sensitive to light and able to detect single photons, while cones are less sensitive and occur as three types for colour discrimination. Thus, rods are responsible for vision at low light levels, such as moonlight, whereas cones provide day and colour vision. In the dark, both rods and cones are depolarized and release glutamate as neurotransmitter. Upon illumination, they hyperpolarize and glutamate release is decreased.

Cone photoreceptors pass the signal to at least 10 different cone bipolar cells (*Ghosh et al. 2004*). There are OFF bipolar cells which, like the photoreceptors, hyperpolarize in response to light, but depolarize in the dark. Their axons stratify in the outer half of the

IPL, in the so-called OFF-sublamina. In contrast, ON cone bipolar cells depolarize upon illumination and they stratify in the inner half of the IPL, or ON-sublamina. The opposite responses of OFF versus ON bipolar cells are the result of different glutamate receptors present on their dendrites. OFF cells express ionotropic glutamate receptors, which open a cation channel upon activation, while ON cells possess metabotropic receptors, whose activation is linked to closure of cation channels (*Nakajima et al. 1993*, *Masu et al. 1995*). In the IPL, ON and OFF bipolar cell axons contact accordingly stratifying dendrites of ON and OFF ganglion cells, as well as different types of amacrine cells.

Rods contact only one type of bipolar cell, the rod bipolar, which is an ON cell. Rod bipolars do not contact ganglion cells directly, instead they send the signal to a specific type of amacrine, the AII cell. The outer (lobular) dendrites of the AII amacrine make inhibitory synapses onto cells of the OFF channel, while the inner (arboreal) dendrites excite cells of the ON channel via gap junctions (*Kolb 1979*, *Strettoi et al. 1992*). Thus, the rod pathway feeds into the cone pathway, allowing night vision to use the phylogenetically older circuits of day vision. It has been suggested that at higher light levels, the rod signals could take another route, skipping the rod bipolars by passing through gap junctions to cones and further to cone bipolar cells. Electrical connections between rod and cone photoreceptors have been in fact documented both anatomically (*Nelson 1977*) and physiologically (*Smith et al 1986*). Yet another alternative pathway has been proposed for the rodent retina: here, rod photoreceptors would be able to directly excite OFF cone bipolar cells (*Hack et al. 1999*, *Tsukamoto et al. 2001*).

Several vertical pathways therefore contribute to the visual processing in the retina. In addition, information is processed by lateral pathways in both plexiform layers.

In the OPL, both rods and cones receive lateral inhibitory input from horizontal cells, which leads to contrast enhancement and formation of receptive fields with excitatory center and inhibitory surround. In the IPL, light evoked responses are strongly modulated by amacrine cells, which provide lateral and feedback inhibitory input to bipolar cells and feedforward inhibition to ganglion cells. Amacrine cell circuits are essential for the formation of complex receptive field properties of ganglion cells, such as motion and directional selectivity. Around thirty types of amacrine cells have been described in the mammalian retina (reviewed by *Masland 1988*, *MacNeil and Masland 1998*, *Vaney 1991*) and they are thought to have distinct functions concerned with shaping and control of ganglion cell responses.

Ganglion cells are the output neurons of the retina. They can be viewed as neuronal filters extracting and transferring different informational aspects from the complex image projected onto the retina. For detailed review and further discussion of retinal cell types refer to Wässle and Boycott (*1991*) and Masland (*2001*).

1.2. GLUTAMATE RECEPTORS

Just like in other parts of the central nervous system (CNS), glutamate (Glu) is the main excitatory neurotransmitter in the retina. Glutamate mediates transmission in the vertical retinal pathway, while inhibition is provided by GABAergic and glycinergic cells in the horizontal circuits. Different types of retinal cells exhibit various light evoked responses, which are in part the result of their specific expression of neurotransmitter receptors. Therefore, knowing the composition in glutamate, GABA and glycine receptors of individual cell types is essential for understanding the functional diversity of the retina.

Glutamate effects on post-synaptic neurons are extremely diverse, thanks to an extensive family of glutamate receptors (GluRs), broadly divisible into ionotropic and metabotropic classes. Ionotropic glutamate receptors (iGluRs) form a non-selective cation channel and are further classified by their agonist selectivity into N-methyl-D-aspartate (NMDA) and non-NMDA (α-amino-3-hydroxy-5-methylisoxazolepropionic acid - AMPA, and kainic acid - KA) receptors. NMDA, AMPA and KA receptors differ in their pharmacology, ionic selectivity and kinetic properties. Metabotropic glutamate receptors (mGluRs) couple with G-proteins and activate multiple second messenger pathways. At least 14 iGluR subunits and 8 mGluR subtypes have been by now identified. In the following, a brief summary of iGluR functional diversity and localization in the brain and retina is given.

1.2.1. N-methyl-D-aspartate (NMDA) receptor properties

NMDA receptors are unique, as they are not only ligand-gated, but also exhibit voltage-dependence due to magnesium ions block (*Mayer et al. 1984*). At a physiological resting potential of -70 mV only a minimal fraction of the channels is open (*Jahr and Stevens 1990*) and membrane depolarization is required for Mg^{2+} unbinding and consequent receptor activation. NMDA receptors are also characterized by a high permeability to

calcium (*Mayer and Westbrook 1987*) and slow gating kinetics (*Lester et al. 1990*). These unique properties suggest that NMDA receptors form the molecular basis of synaptic plasticity. Ca^{2+} influx through NMDA receptors plays a key role in long-term potentiation (reviewed by *Kaczmarek et al. 1997*) and excitotoxicity.

Six NMDA subunits (NR1, NR2A, NR2B, NR2C, NR2D and NR3A) have been cloned to date (reviewed by *Hollmann and Heinemann 1994*). Some may assemble as homomers (*Monyer et al. 1992*), but functional NMDA receptors *in vivo* are thought to be heteromers. All functional NMDA receptors contain the NR1 subunit (*Monyer et al. 1992*). The exact subunit stoichiometry is not clear, different studies being split between a tetramer and a pentamer (reviewed by *Dingledine et al. 1999*).

NMDA receptors are structurally complex (reviewed by *Ozawa et al. 1997*, *Dingledine et al. 1999)*, with separate binding sites for glutamate, glycine, Mg^{2+}, zinc ions, a polyamine recognition site and an antagonist binding site for phencyclidine (PCP) and MK-801. The glutamate, glycine and Mg^{2+} binding sites are important for receptor activation and gating of the ion channel, while the others are regulatory sites, affecting channel efficacy. Both glutamate and glycine (*Johnson and Ascher 1987, Kleckner and Dingledine 1988*) have to bind to the receptor and the Mg^{2+} blockage has to be relieved in order for the channel to open. Zinc (Zn^{2+}), as well as cadmium ions (Cd^{2+}), inhibit NMDA receptors by both a weak voltage-dependent and a voltage-independent mechanism (*Westbrook and Mayer 1987*). The polyamine site (reviewed by *Williams 1997*) binds compounds such as spermine or spermidine, either potentiating (*Ransom and Stec 1988*, *Williams et al. 1994*) or inhibiting (*Williams et al. 1994*) the activity of the receptor, depending on the combination of subunits. NMDA receptors are also sensitive to extracellular protons and redox modulation. Native NMDA receptors are inhibited by physiologically relevant concentrations of extracellular protons, through a voltage-independent mechanism (reviewed by *Traynelis 1998*). The redox modulation is controlled by two cysteine residues, whose oxidation attenuates the receptor's response (reviewed by *McBain and Mayer 1994*).

Both NMDA and non-NMDA receptors are activated by the endogenous transmitter L-glutamate, while L-aspartate seems to activate NMDA receptors only (*Patneau and Mayer 1990*). Glycine is an essential coagonist at NMDA receptors (*Kleckner and Dingledine 1988*). Two glutamate and two glycine molecules appear to be required for maximum activation of an NMDA receptor (*Clements and Westbrook 1991*).

Antagonistic compounds can be grouped, on basis of their binding sites, into: competitive, binding at the agonist site, noncompetitive, binding at a different site, and uncompetitive, whose binding site is exposed only when the channel is open and can therefore act only on the activated receptors. The classical competitive antagonists of the glutamate site on NMDA receptors are phosphono derivatives of short-chain (5-7 carbons) amino acids, such as AP5 and AP7 (*Priestley et al. 1995*). Halogenated quinoxalinediones and kynurenic acid derivatives (*Priestley et al. 1995*) and, more recently, certain phthalazinedione and benzazepinedione derivatives (reviewed by *Dingledine et al. 1999*) are highly potent, selective glycine site antagonists. The most famous noncompetitive antagonists at NMDA receptor are ifenprodil and its analogues, while Mg^{2+}, cytoplasmic polyamines, PCP and MK-801 (*MacDonald et al. 1991*) are some of the most known uncompetitive blockers.

NMDA receptor gating is extremely slow in comparison to the fast gating of AMPA and KA receptors, but its affinity for glutamate is much higher. The EC_{50} values for peak response to fast glutamate application are around 1 μM for NMDA receptors (*McBain and Mayer, 1994*), while over 500 μM are necessary for non-NMDA receptors (summarized by *Dingledine et al. 1999*). In contrast to AMPA and KA receptors, NMDA receptors activate slowly, with a rise time constant of 10 to 50 ms and deactivate with a much slower time course (summarized by *Dingledine et al. 1999*). The decay time constants differ markedly depending on which NR2 subunit is assembled with NR1 (*Monyer et al. 1994*). Deactivation is much slower for receptors containing the NR2D subunit, which is expressed early in development (*Monyer et al. 1994*). The prolonged activation might be important for the formation, stabilization or elimination of synapses during development. In the continued presence of agonist, the NMDA receptors desensitize through a slow and complex process, which involves extracellular glycine, intracellular Ca^{2+} and some intracellular proteins (reviewed by *Mayer et al. 1995*).

The deactivation time of NMDA receptors is much longer than the time glutamate is present in most synaptic clefts (*Lester et al. 1990*) and will therefore determine the duration of the synaptic current. In addition to the slow unbinding of glutamate, desensitization at NMDA receptor also seems to contribute to the slow decay kinetics of the NMDA postsynaptic current (reviewed by *Ozawa et al. 1998*).

1.2.2. α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) receptor properties

AMPA receptors mediate fast excitatory neurotransmission at most synapses in the CNS. Functional AMPA receptors are composed of GluR1, GluR2, GluR3 or GluR4 subunits, assembled as either homo- or heteromers (reviewed by *Hollmann and Heinemann 1994*). All four subunits occur in two alternatively spliced variants, flip and flop (*Sommer et al. 1990*). The exact subunit stoichiometry is not clear, different studies being split between a tetramer, favoured at present, and a pentamer (reviewed by *Dingledine et al. 1999*).

AMPA receptors are more permeable to sodium (Na^+) and potassium ions (K^+) than Ca^{2+} (*Mayer and Westbrook 1987)*. By replacement of only one amino acid residue, glutamine (Q) by arginine (R), at the Q/R site (*Hume et al. 1991*, *Sommer et al. 1991*), GluR2 subunit is responsible for AMPA receptor Ca^{2+} permeability, rectification properties and sensitivity to polyamines (*Bochet et al. 1994, Geiger et al.* 1995, *Hollmann et al. 1991*). Homomeric GluR2 receptors display little permeability to Ca^{2+} and an outward rectification, while homomeric GluR1, GluR3 or GluR4 receptors are highly permeable to Ca^{2+} and display a strong inward rectification. GluR2 is dominant in determining the channel properties: coexpression of GluR2 with other AMPA subunits results in formation of receptors with little Ca^{2+} permeability and outward rectification (*Hollmann and Heinemann 1994*). The differently charged subunits confer the rectification properties of AMPA receptors, by determining whether intracellular polyamines, such as spermine and spermidine, will enter and block the channel pore (*Bowie and Mayer 1995*). Most brain neurons with Ca^{2+} permeable AMPA receptors are GABAergic and express the Ca^{2+} binding protein parvalbumin (*Bochet et al. 1994*, *Jonas and Burnashev 1995*).

AMPA receptors respond to glutamate, AMPA and quisqualate (QA) with desensitizing responses, but to KA with a sustained response. Other AMPA receptor agonists are found among analogs of AMPA itself and willardiine (reviewed by *Dingledine et al. 1999*). AMPA receptor activation apparently requires binding of two agonist molecules (*Clements et al. 1998*).

Quinoxalines and quinoxalinediones, such as NBQX, CNQX, DNQX, were the first generation of competitive blockers of non-NMDA receptors, but they show poor selectivity between AMPA and KA receptors. High concentrations of CNQX and DNQX, but not NBQX, also antagonize NMDA receptors. ATPO, a phosphono analog of AMPA, inhibits recombinant AMPA receptors, but has no effect on certain KA receptors (reviewed by

Dingledine et al. 1999). 2,3-benzodiazepines, such as GYKI 52466 and GYKI 53655, are selective antagonists at AMPA receptors, which they block in a noncompetitive manner (*Donevan and Rogawski 1993*, *Wilding and Huettner 1995*, *Bleakman et al. 1996*). The (-)-stereoisomer of GYKI 53655 is the most potent of these compounds, with an $IC_{50} < 1$ μM in rat cerebellar Purkinje neurons (*Bleakman et al. 1996*). As for uncompetitive antagonists, several polyamine amide toxins from arthropod venom, such as argiotoxin, philanthotoxin and Joro spider toxin (JSTX), have been shown to block non-NMDA receptors at nanomolar concentrations (reviewed by *Bowie et al. 1999*). JSTX selectively blocks Ca^{2+} permeable AMPA receptors (*Iino et al. 1996*).

Table 1.1. Overview of ionotropic glutamate receptor pharmacology.

	NMDA receptors	Non-NMDA receptors	
		AMPA Rs	KA Rs
Nonselective agonists	L-glutamate		
		AMPA, kainate	
Selective agonists	NMDA, L-aspartate	cyclothiazide	SYM 2081; concanavalin A
Nonselective antagonists		NBQX, CNQX, DNQX; Joro spider toxin	
Selective antagonists	AP5, AP7; ifenprodil; PCP, MK-801	GYKI 52466, GYKI 53655	NS 102

AMPA receptors are rapidly activated by high concentrations of glutamate, with a high probability of opening. They posses low-affinity binding sites for glutamate ($EC_{50} \sim 500$ μM) and are therefore thought to deactivate quickly because of the brief boundtime of the agonist. Rise times of AMPA receptors approach 0.2 - 0.4 ms (*Dingledine et al. 1999*), while their decay time constants range from 0.9 to 3.3 ms (*Geiger et al. 1995*). An important property of AMPA receptors is their rapid and profound desensitization, which tends to generate brief excitatory post-synaptic currents (EPSCs) and to prevent excitotoxicity. Desensitization time constants of AMPA receptor responses to 1-10 mM glutamate in outside-out patches from diverse central neurons were found to be between 1 and 16 ms (reviewed by *Ozawa et al. 1998*), while the time constants of the recovery from

desensitization ranged between 6 and 147 ms in recombinant AMPA receptors (summarized by *Dingledine et al. 1999*). The rate of desensitization and recovery depends on subunit composition and RNA alternative splicing at the flip/ flop region (*Mosbacher et al. 1994*). Several compounds have been identified that reduce desensitization at AMPA receptors – aniracetam, diaxozide, cyclothiazide (CTZ), PEPA (*Vyklicky et al. 1991*, *Bertolino et al. 1993*, *Palmer and Lodge 1993*), which act by different mechanisms (reviewed by *Ozawa et al. 1998*).

Due to the very brief time course of glutamate in the synaptic cleft (*Lester et al. 1990*), it is likely that deactivation rather than desensitization determines the decay rate of AMPA-EPSCs. However, desensitization will limit the duration of EPSCs when the synaptic glutamate concentration remains elevated for prolonged periods and this fact has been established for some synapses in the CNS (reviewed by *Ozawa et al. 1998*).

AMPA and NMDA receptors are in many cases colocalized at central synapses. The rapid activation and brief open time of AMPA receptors facilitates relief of NMDA receptors from Mg^{2+} block and thus participation of the more slowly activating NMDA receptors in synaptic currents. The NMDA receptor functions as a coincidence detector that can sense simultaneous presynaptic activity and postsynaptic depolarization, and mediates Ca^{2+} influx into the postsynaptic cell, which will initiate plastic changes in the strength of the synaptic connection.

1.2.3. Kainate (KA) receptor properties

As KA elicits a large nondesensitizing current at AMPA receptors, native KA receptor-mediated responses were until recently difficult to detect. Most of the studies concerning KA receptor properties were conducted on recombinant receptors. The investigation of KA receptors' function in synaptic transmission has only been possible since the discovery of AMPA receptor antagonists in 1995 (*Wilding and Huettner 1995*, *Bleakman et al. 1996*).

There are five KA receptor subunits (reviewed by *Hollmann and Heinemann 1994*), falling into two groups on basis of their sequence homology and agonist binding properties: GluR5, GluR6, GluR7 and KA1, KA2. GluR5, GluR6 and GluR7 are capable of forming functional homomeric channels, whereas a variety of heteromeric channels could be assembled (reviewed by *Huettner 2003*). The exact subunit stoichiometry is not

clear, different studies being split between a tetramer and a pentamer (reviewed by *Dingledine et al. 1999*).

Like the AMPA receptor subunit GluR2, KA receptor subunits GluR5 and GluR6 can undergo RNA editing at the Q/R site (*Sommer et al. 1991*), which influences permeation properties, single channel conductance and calcium permeability. Unedited receptors (Q variants) display inward rectification due to block by intracellular polyamines, a higher Ca^{2+} permeability and a higher unitary conductance (reviewed by *Ozawa et al. 1998* and *Huettner 2003*). Both edited and unedited homomeric GluR6 receptors show substantial Ca^{2+} permeability (*Ozawa et al. 1998*).

KA receptors are preferentially activated by glutamate, KA and domoate, while AMPA can also activate certain combinations of KA subunits (*Herb et al. 1992*). Unlike its effects at AMPA receptors, KA elicits a desensitizing response at KA receptors. Other KA receptor agonists include some willardiine analogues, the trifluoro-kainate analog DZKA, ATPA and (2S-4R)-4-methylglutamate (SYM 2081) (reviewed by *Dingledine et al. 1999*). SYM 2081 shows high selectivity for KA receptors (100-1000-fold in comparison to AMPA receptors) (*Gu et al. 1995*), where it induces fast and potent desensitization (*Zhou et al. 1997*, *Donevan et al. 1998*).

Quinoxalines and quinoxalinediones, such as NBQX, CNQX, DNQX, were the first generation of competitive blockers of non-NMDA receptors, but they show poor selectivity between AMPA and KA receptors. However, higher concentrations of these compounds are required for antagonizing KA receptors relative to AMPA receptors. Several competitive antagonists selective for GluR5 subunit have been reported (LY382884, LY293558, LY294486), but some of them also exhibit antagonism at AMPA receptors (reviewed by *Huettner 2003*). As for uncompetitive antagonists, several polyamine amide toxins from arthropod venom, such as argiotoxin, philanthotoxin and Joro spider toxin (JSTX), have been shown to block non-NMDA receptors at nanomolar concentrations (reviewed by *Bowie et al. 1999*). NS102 is an antagonist selective for KA receptors, but with limited solubility in physiological solutions (*Huettner 2003*). KA receptors can also be blocked with lanthanum (*Huettner et al. 1998*).

KA receptors possess basically the same kinetic properties as AMPA receptors. They are rapidly activated by high concentrations of glutamate, with a high probability of opening. They possess low-affinity binding sites for glutamate (EC_{50} ~ 500-600 μM), GluR7 unusually low (EC_{50} ~ 5900 μM) (summarized by *Dingledine et al. 1999*), and are

therefore thought to deactivate quickly because of the brief boundtime of the agonist. As AMPA receptors, KA receptors desensitize rapidly and profoundly in the continued presence of glutamate. KA receptors recover from desensitization with time constants approximately 10-fold slower than AMPA receptors, which is the main kinetic distinction between the two types of non-NMDA receptors (summarized by *Dingledine et al. 1999*). Concanavalin A (ConA) is a lectin that selectively relieves desensitization of KA receptors, without affecting AMPA receptor-mediated responses (*Partin et al. 1993*, *Wong and Mayer 1993*). ConA acts on GluR5 and GluR6 receptors, but not on GluR7 (*Schiffer et al. 1997*).

KA receptors are involved in bi-directional modulation of both excitatory and inhibitory transmission. Low to moderate KA receptor activation stimulates transmission, while strong activation inhibits transmission (reviewed by *Huettner 2003*). Synaptic currents mediated by KA receptors have lower peak amplitude and slower decay times than AMPA-EPSCs recorded in the same cell. KA receptors may also trigger a metabotropic cascade in some cases (reviewed by *Huettner 2003*).

1.2.4. Localization of iGluRs in the brain and in the retina

NMDA, AMPA and KA receptors are ubiquitously distributed throughout the CNS. Ligand binding studies have shown that NMDA receptors are predominantly localized in the forebrain (reviewed by *Ozawa et al. 1998*). [^{3}H-AMPA] binding studies have revealed high affinity binding sites in the hippocampus (the molecular layer of the dentate gyrus, CA1 and CA3 regions) and in the superficial layer of the cerebral cortex (reviewed by *Ozawa et al. 1998*). [^{3}H-KA] binding studies have showed presence of both low and high affinity KA binding sites, especially abundant in the hippocampal CA3 region and the granular layer of the cerebellum (reviewed by *Ozawa et al. 1998*). High affinity sites would correspond to KA1/ KA2 containing receptors, whereas assembles of GluR5, GluR6 and GluR7 subunits would make up receptors of low affinity (reviewed by *Huettner 2003*).

In the retina, all iGluR subunits are present, differently distributed in the two synaptic layers (see *Brandstätter et al. 1998*, *Thoreson and Witkovsky 1999* for review of vertebrate retina; *Haverkamp and Wässle 2000* for mouse; Qin *and Pourcho 1999 and 2000* for cat; *Grünert et al. 2002* for primate retina).

Photoreceptors: There is little physiological evidence for expression of iGluRs in the photoreceptors. Immunocytochemical and *in situ* hybridization studies have shown the presence of GluR1 (*Qin and Pourcho 1999*, cat) and NR1C2' (*Fletcher et al. 2000*, rat) in the cone pedicle and GluR6/7 in cone outer segments (*Peng et al. 1995*, goldfish).

Horizontal cells: Using the same methods, GluR3, GluR4 (*Qin and Pourcho 1999b*, cat), GluR6/7 (*Peng et al. 1995*, goldfish; *Brandstätter et al. 1997*, rat) and NR2A subunits (*Goebel et al. 1998*, cat) were identified on horizontal cells. Electrophysiological measurements in a variety of species also indicated presence of non-NMDA (*Zhou et al. 1993*, white perch; *Slaughter and Miller 1983*, mudpuppy; *Yang and Wu 1991*, salamander; *Eliasof and Jahr 1997*, catfish) and NMDA receptors (*O'Dell and Christensen 1989*, *Eliasof and Jahr 1997*, catfish) on horizontal cells.

Bipolar cells: Expression of non-NMDA glutamate receptors in OFF bipolar cells was attested by electrophysiological studies in many species (*Slaughter and Miller 1983*, mudpuppy; *Hensley et al. 1993*, salamander; *Sasaki and Kaneko 1996*, cat; *Euler et al. 1996*, rat). Immunocytochemistry and *in situ* hybridization revealed the presence of GluR1 (*Hack et al. 1999*, mouse and rat; *Qin and Pourcho 1999a*, cat), GluR2 (*Peng et al. 1995*, goldfish and rat; *Hack et al. 1999*, mouse and rat), GluR2/3 (*Peng et al. 1995*, rat), perhaps GluR4 (*Hughes 1997*, mouse), and KA2 (*Brandstätter et al. 1997*, rat) on bipolar cells. Immunostainings of the retina show that different iGluR subunits are differentially distributed in the synaptic layers, suggesting their involvement with different synaptic circuits and cell types. In the ground squirrel retina, DeVries (*2000*) showed that b2 OFF bipolar cells express AMPA receptors, while b3 and b7 cells exhibit KA receptors with distinct physiological properties. It is well established that ON bipolar cells use a metabotropic glutamate receptor (mGluR6) for reception of the visual signal from the photoreceptors (*Nakajima et al. 1993*, *Masu et al. 1995*). However, there is anatomical evidence suggesting iGluR expression in these cells (*Slaughter and Miller 1983*, *Peng et al. 1995*). In has been shown, for example, that the rod bipolar cells in the rat retina express GluR2 (*Hughes et al. 1992*), NR1 (*Hughes 1997*) and NR2D subunits (*Wenzel et al. 1997*). The physiological significance of these findings is not clear.

Amacrine cells: Amacrine cells were found, by immunocytochemistry and *in situ* hybridization, to express all types of iGluRs. GluR1, GluR2/3 (*Peng et al. 1995*), GluR6 and NR2A-C (*Brandstätter et al. 1994*) were identified on amacrine cells in the rat, and GluR2/3 and GluR4 also in the cat retina (*Qin and Pourcho 1999*). GluR2/3 and GluR4

antibody staining was shown to be associated with AII amacrine cells (*Qin and Pourcho 1999b*, cat; *Ghosh et al. 2001*, monkey and rabbit; *Li et al. 2002*, rabbit), while ∂1/2 subunits were found on AI amacrines in monkey (*Ghosh et al. 2001*) and S1 cells in rabbit (*Li et al. 2002*). AI amacrine cells may also express GluR6/7 and KA2 kainate receptor subunits (*Brandstätter et al. 1997*, cat). NMDA receptor subunits were found on the glycinergic amacrine cells in the primate retina (*Grünert et al. 2002*).

Non-NMDA as well as NMDA receptor activity was recorded in amacrine cells from a large variety of species (*Slaughter and Miller 1983*, mudpuppy; *Massey and Miller 1988*, *Zhou and Dacheux 2004,* rabbit; *Dixon and Copenhagen 1992*, *Tran et al. 1999*, salamander; *Boos et al. 1993*, *Hartveit and Veruki 1997*, rat). Light-evoked responses of amacrine cells are blocked by non-NMDA receptor antagonists, whereas NMDA receptor blockers only modify the light responses (*Slaughter and Miller 1983*, *Massey and Miller 1988*, *Dixon and Copenhagen 1992*). AMPA receptor mediated activity is well established in AII (*Mørkve et al. 2002*, *Veruki et al. 2003, Singer and Diamond 2003*) and A17 amacrine cells in the rat retina (*Singer and Diamond 2003*), and in starburst amacrine cells of the rabbit retina (*Firth et al. 2003*). Starburst amacrines also express NMDA receptors (*Zhou and Fain 1995*), while AII cells show little or no response to NMDA in the physiological voltage range (*Boos et al. 1993*, *Hartveit and Veruki 1997*, rat).

Ganglion cells: GluR1-7, KA2, NR1 and NR2A-C subunits have been identified in the ganglion cell layer by immunocytochemistry and *in situ* hybridization in some mammalian retinae (*Hughes et al. 1992*, *Hamassaki-Britto et al. 1993*, *Brandstätter et al. 1994*, *Peng et al. 1995*, rat; *Hamassaki-Britto et al. 1993*, *Qin and Pourcho 1999*, cat). In the primate retina, the NR2A subunit was found on the dendrites of midget and parasol ganglion cells, where it partially colocalizes with AMPA subunits, probably GluR2/3-GluR4 heteromers (*Grünert et al. 2002*). Many electrophysiological studies testify expression of both non-NMDA and NMDA receptors on ganglion cells, but while blockage of non-NMDA activity strongly suppresses the light-evoked responses of these cells, blockage of NMDA receptors generally only modifies them (*Slaughter and Miller 1983*, *Coleman and Miller 1988*, mudpuppy; *Aizenman et al. 1988*, rat; *Diamond and Copenhagen 1993*, salamander; *Cohen and Miller 1994*, primate; *Massey and Miller 1988*, *1990* rabbit).

1.3. AMACRINE CELLS

Approximately 40% of all neurons in the INL of the mammalian retina are amacrine cells (*Strettoi and Masland 1995*), inhibitory interneurons essential for shaping the ganglion cell responses before transmission of visual signals to the brain. During more than one century of retinal studies, the diversity of amacrine cells has never ceased to be a surprise. As many as thirty types of amacrine cells have been described in different mammalian retinae (*Boycott and Dowling 1969*, *Kolb and Nelson 1981*, *Wässle and Boycott 1991*, *MacNeil et al. 1999*). They differ not only in morphology and biochemistry, but also in their functional roles (reviewed by *Masland 1988*, *MacNeil and Masland 1998*). The different roles of various amacrine cells can be inferred from their different stratification patterns, retinal coverage, secreted neurotransmitters and synaptic partners.

Most amacrine cells contain the inhibitory neurotransmitters γ-aminobutyric acid (GABA) or glycine. Glycinergic amacrines have rather small dendritic trees (*Menger et al. 1998*), while GABAergic cells are usually wide-field. Grouping amacrine cells into general narrow- (under 100 μm) and wide-field (more than 100 μm) classes is useful, as size and density suggest a general category of functions that these cells could carry out. Narrow-field cells could transmit or modify information passing down the retinal vertical pathway from bipolar to ganglion cells, while wide-field cells might be expected to mediate more complex operations, including horizontal interactions across larger areas. Differently sized amacrine cells also seem to differ in their stratification patterns: while narrow-field cells frequently extend their processes between sublayers (diffused cells), wide-field cells generally extend their dendrites in only one of the IPL sublaminae (*MacNeil and Masland 1998*).

The most completely understood amacrine cell to date is the AII, the cell linking the rod with the cone pathway. It is a narrow-field cell with a pear-shaped body and generally one main thick process giving off lobular appendages in sublamina *a* of the IPL and arboreal dendrites in sublamina *b*. The AII cell receives input from rod bipolar cells contacting the lower dendrites. Its main synaptic output is upon OFF cone bipolar and OFF ganglion cells through the lobular appendages, while the lower dendrites also contact ON cone bipolar cells via gap junctions (*Kolb 1979*, cat; *Strettoi et al. 1992*, rabbit).

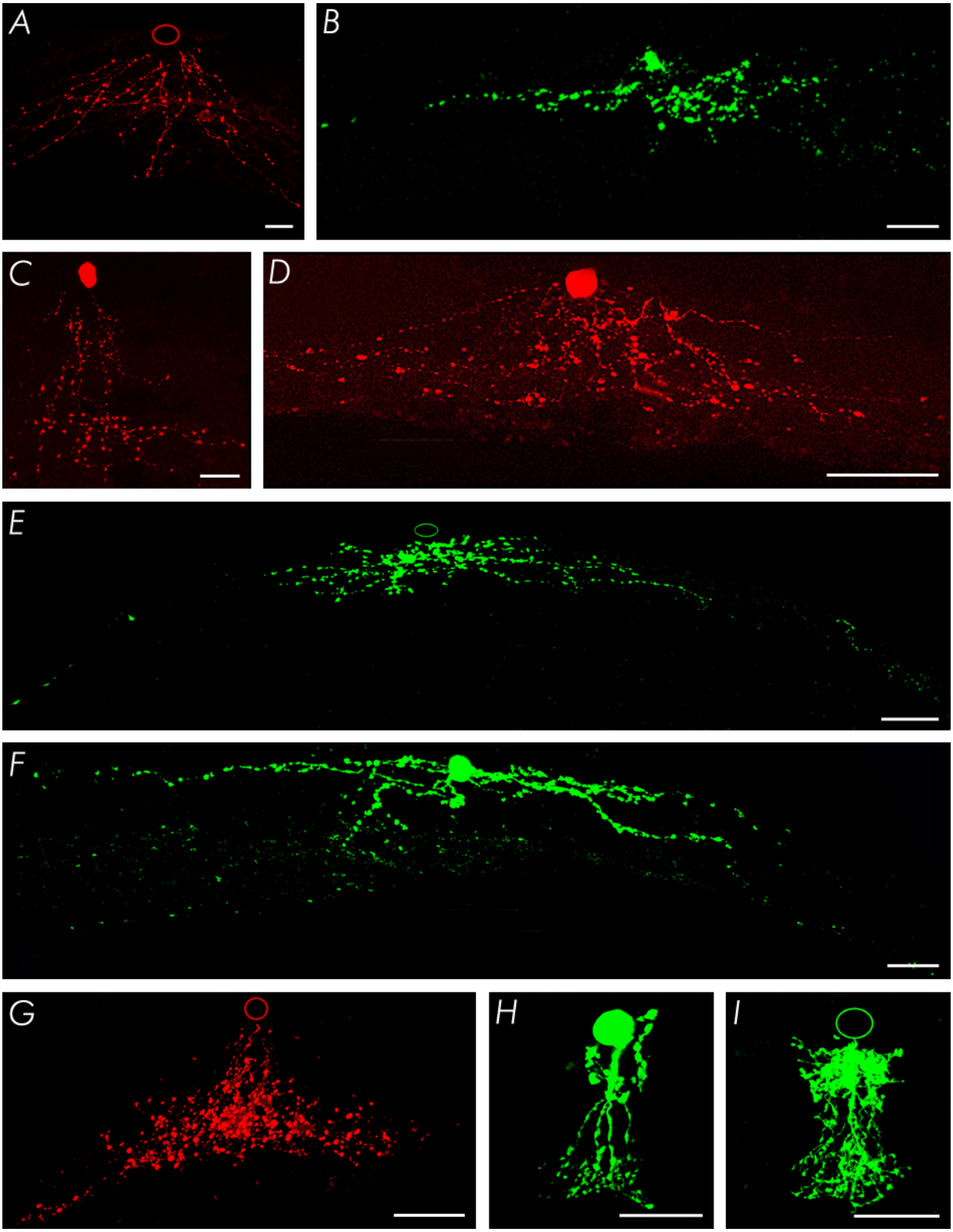

Figure 1.2. **Amacrine cell types in the mouse retina.** Cells were filled with neurobiotin during patch-clamp recordings in slices and then stained with fluorescently marked streptavidin. All scale bars 20 μm.

(A) - (G) Wide-field amacrine cells.

(H) - (I) Narrow-field amacrine cells. The cell in (H) is a typical AII amacrine.

The other cell postsynaptic at the dyads of the rod bipolar cell is the AI, also named A17 in cat and rat retinae (*Menger and Wässle 2000*), or serotonin-/indoleamine-accumulating amacrine S1 and 2 in rabbit retina. The AI is a diffuse wide-field amacrine, whose dendrites can span up to a millimetre through the IPL. Its fine dendrites bear varicosities and they mainly expand in sublamina b of the IPL. AI cells do not appear to contact any other amacrines or ganglion cells, but they merely interconnect rod bipolars by reciprocal inhibitory synapses.

Another type of amacrine that has received much interest is the starburst cell. Starburst amacrines are wide-field cells with regularly spaced, evenly radiating dendrites and use acetylcholine as a neurotransmitter. Because of the asymmetry of their synaptic connections to directional selective ganglion cells (*Famiglietti 1991*), starburst amacrines have been thought to be the source of direction selectivity in these cells. They have been later shown to only potentiate the responses of ganglion cells to moving stimuli (*He and Masland 1997*).

The A18 is a wide-field dopaminergic amacrine cell, stratifying almost exclusively in the first sublamina of the IPL. In the fish retina, dopaminergic amacrines are interplexiform cells, with synaptic connections in both OPL and IPL (*Teranishi and Negishi 1986, Mangel and Dowling 1987*). Dopamine uncouples cells connected via gap junctions, such as the AII amacrines in the mammalian retina (*Hampson et al. 1992*).

Every amacrine cell whose function is known has a clear task. It is therefore expected that each of the numerous amacrine types described has a role of its own in the transmission of information through the inner retina. This is the only apparent explanation to account for the huge variability of these interneurons and it is in agreement with the distinct branching pattern of various amacrines, i. e. their distinct connectivity.

2. MATERIALS AND METHODS

2.1. ANIMALS

Two mouse lines were used in the present study: wild-type C57 Black6 and transgenic GlyT2-EGFP animals (*Zeilhofer et al. 2003*). The latter express EGFP under the control of the neuronal glycine transporter GlyT2 promoter and were thus useful for visualizing glycinergic neurons in the living slice preparations.

2.1.1. GlyT2-EGFP mice genotyping

GlyT2-EGFP mice were backcrossed with wild-type animals, to prevent retinal degeneration, a phenotype occurring among homozygotic transgenic mice. It was thus necessary to genotype the offspring, in order to identify the animals expressing the GlyT2-EGFP construct.

Small amounts of tissue (tail cuts) from each animal were collected in Eppendorf tubes, which were then filled with lysis buffer and proteinase K (*Table 2.1.*) and left overnight at 55 °C. The lysis reaction was ended by heating up the probes to 95 °C for 15 minutes. The probes were centrifuged 5 minutes at high speed (13000 rpm) and 100 μl of the supernatant was transferred in new tubes. The supernatant was not further processed for extracting the DNA, but was directly used in the PCR (polymerase chain reaction).

21 μl master-mix (*Table 2.1.*) were added to 4 μl probe in PCR tubes kept on ice. A Qiagen PCR kit (Taq DNA-polymerase) and Roche dNTPs were used. The two primers (5' - AAT GTG TGC ATC TGT GTA TGC AGA C - 3' and 5' - CTT GAA GAA GAT GGT GCG CTC CTG - 3') were ordered from MWG Biotech. *Table 2.1.* lists the PCR cycle parameters.

Table 2.1. Solutions and protocols for GlyT2-EGFP mice genotyping.

Solutions	
Lysis buffer (ml)	
NaCl 5M	0,5
TrisHCl 1M, pH 8	0,25
H_2O	24,25
Proteinase K	10 mg/ ml in PBS
Lysis mixture (μl)	
Lysis buffer	500
Proteinase K	20

PCR		
Master-mix (μl)		
$MgCl_2$ *(Qiagen)*	1,75	
10x PCR buffer *(Qiagen)*	2,5	
Q solution *(Qiagen)*	5	
dNTPs *(Roche)*	0,5	
Primer 1 *(MWG)*	0,25	
Primer 2 *(MWG)*	0,25	
Taq polymerase *(Qiagen)*	0,25	
H_2O	10,5	
PCR cycle parameters		
95 °C	5 min	
95 °C	45 sec	40 cycles
58 °C	45 sec	
72 °C	1 min	
72 °C	2 min	
4 °C	forever	

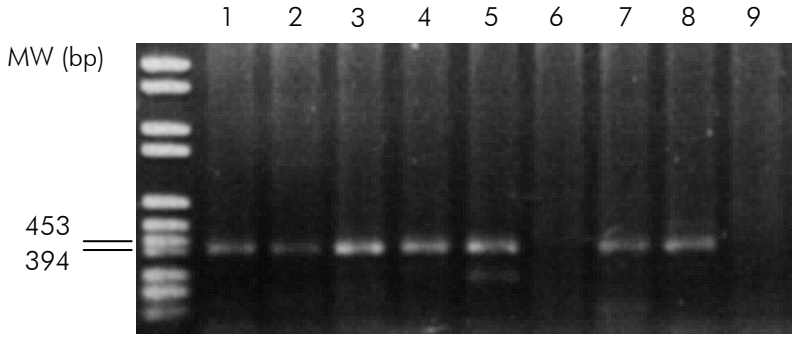

Figure 2.1. **Identification of GlyT2-EGFP positive mice.** Detection by PCR of a 421-bp fragment of the GlyT2 gene from the total DNA extract of tail-cuts collected from 9 mice. Animals 1-5, 7 and 8 were found positive.

2.2. EYE DISSECTION & PREPARATION OF RETINA SLICES

Adult mice of both wild-type and transgenic background were deeply anesthetized with 4% halothane and decapitated (procedure approved by the local animal care committee). After decapitation, the eyes were immediately enucleated and cut around the midline (*ora serrata*) for removing the cornea and lens. The retina was then carefully detached from the eyecup, the vitreous removed, and a rectangular piece of tissue was cut out for imbedding into agar. The dissection of the eye, as well as the later incubation of the retina slices, was done at room temperature in AMES medium, buffered with sodium hydrogen carbonate and continuously bubbled with carbogen (95% O_2, 5% CO_2).

The retina was afterwards embedded in 3% low gelling temperature agarose. 300 mg agarose, 9 ml AMES and 1 ml distillate water were cooked until boiling in a microwave oven and then kept during the dissection at 36 °C in a water bath. Immediately before tissue embedding, the agarose was cooled down to 32 °C. When solid, the agarose around the tissue was cut out and the small block containing the retina was fixed with a drop of Uhu superglue onto the tray of the vibratome (D.S.K. Microslicer, Japan). 200 μm thick vertical slices were cut, which were then collected and incubated in AMES and could be used for recordings up to six hours after the preparation.

2.3. ELECTROPHYSIOLOGY

2.3.1. Set-up

The slices were viewed under an up-right microscope (Axioplan, Zeiss, Germany) with a x40 water immersion objective, 16x oculars and DIC (differential interference contrast) optic. To visualize the cells labeled with EGFP or filled with fluorescent dyes, epifluorescence was employed, using a mercury lamp (Osram) and LY filter set (Zeiss, Nr5).

One slice was fixed with a custom-made platinum grid in the circular recording chamber mounted onto the microscope stage. The tissue was constantly perfused from one site of the chamber with fresh medium, at a rate of one - two milliliter per minute. On the opposite side of the chamber, the old medium was removed by suction, using a peristaltic

pump. Drugs were locally applied onto the cells using a voltage controlled 12-line superfusion system (DAD-12, ALA, USA).

Two Ag/AgCl electrodes were employed for electrical measurements - the reference electrode in the bath and the recording electrode placed in a glass pipette. Pipettes were obtained by pulling borosilicate filament capillaries (outer ∅ 1,5 mm, inner ∅ 0,86 mm; Harvard, UK) on a horizontal DMZ-Universal puller (Zeitz, Germany).

The electrodes were connected to the headstage of a patch-clamp amplifier (EPC 9/ 2, HEKA, Germany). The headstage was mounted onto a micromanipulator (Luigs & Neumann, Germany). Pulse v8.53 software was used for stimulus generation and simultaneous data acquisition, at a sampling rate of 1 kHz.

2.3.2. Identification of amacrine cells in retina slices

To record from amacrine cells, somata in the proximal (lower) part of the INL were chosen for patching. Due to their characteristic morphology with an elongated soma and one thick main dendrite, AII cells could be easily recognized. Other amacrine cell types could be classified only after filling with LY. After recording, the slices were processed for recovering the morphology of the cells (*section 2.4.*).

2.3.3. Whole-cell experiments

The electrophysiological recordings were performed in the whole-cell configuration of the patch-clamp technique (*Hamill et al. 1981*). The whole-cell configuration ensures electrical continuity between the cytoplasm and the intracellular (IC) solution and allows monitoring the activity of all channels throughout the membrane.

A glass pipette was half filled with IC solution (*section 2.3.4.*) and fixed onto the headstage holding the recording electrode. The pipette was placed in the bath, over the tissue, and then directed to the surface of a cell using the micromanipulator. Pipettes with a resistance of 7-10 MΩ were used, which corresponds to a tip size in 1 μm range. The pipette capacitance was automatically compensated by the amplifier and positive pressure was applied to the pipette, in order to prevent bath solution entering the tip. Upon reaching the cell, the pipette was slightly pressed against the membrane and the positive pressure released, which lead to the formation of a gigaseal (a contact of GΩ order

resistance between membrane and pipette wall). The whole-cell configuration was then established by rupturing the membrane patch in the pipette tip through gentle suction. Access resistance and membrane capacitance were manually compensated.

2.3.4. Solutions & protocols

Two basic solutions were used: an external solution in the recording chamber and an internal solution in the recording pipette (*Table 2.2.*).

Table 2.2. Solutions used intra- and extracellularly during electrophysiological recordings.

IC		EC		DAD	
Ingredient	Concentration (mM)	Ingredient	Concentration (mM)	Ingredient	Concentration (mM)
Cs gluconate	125	NaCl	120	NaCl	195
HEPES	10	KCl	3	KCl	2,5
EGTA	10	$CaCl_2$	1,5	$CaCl_2$	2
TBA	5	$MgSO_4$	1,2	$MgCl_2$	1
$MgSO_4$	4,6	$NaHCO_3$	22,5	HEPES	1
Na-ATP	4	KH_2PO_4	0,5		
Na-GTP	0,4	D-glucose	6		
$CaCl_2$	1	Aminoacids, vitamins			
Neurobiotin	15				
Lucifer Yellow	1%				
pH 7,42 adjusted with CsOH		pH 7,4		pH 7,4 adjusted with NaOH	

For best mimicking *in-vivo* conditions, the retina was kept in an extracellular solution (EC) based on AMES medium buffered with 23 mM sodium hydrogen carbonate and permanently bubbled with carbogen. The EC solution was freshly made up before each experiment.

The IC solution was prepared including all ingredients and small aliquots were frozen at -20 °C. Before each experiment, one aliquot was defrosted and Lucifer yellow (LY) and neurobiotin (NB) were added to the solution.

During the recordings, the cells were clamped at -75 mV, close to their physiological resting potential. Glutamate receptor-potent drugs were locally applied to the dendrites, in order to discriminate the contribution of the different receptor subtypes to the agonist-induced current. The diverse combinations of the drugs used can be found in *Table 2.3*.

Table 2.3. NMDA and non-NMDA receptor potent drugs applied during the electrophysiological recordings.

NMDA Receptors	Non-NMDA Receptors	
	AMPA Rs	KA Rs
100/ 500 μM NMDA 2 μM Gly 1 μM strychnine in Mg-free solution	100/ 200 μM AMPA	100/ 200 μM KA
	100/ 200 μM AMPA 100 μM CTZ	100/ 200 μM KA 20 μM SYM 2081
	100/ 200 μM AMPA 20 μM GYKI 52466/ GYKI 53655	50 μM SYM 2081
	100/ 200 μM AMPA 10 μM NBQX	100/ 200 μM KA 10 μM NBQX

Refer to Table 1.1. in the Introduction for the pharmacological effects of the different drugs..

Expression of NMDA receptors was tested by application of 100 μM NMDA in presence of small amounts of the co-agonist glycine and strychnine, for preventing activation of glycine receptors. Magnesium-free solution was used in this case, in order to hinder blocking of the NMDA receptors by Mg ions. As NMDA responses were often difficult to obtain, the agonist concentration was increased to 500 μM and the holding potential was changed to positive values (5, 25 and 45 mV) in later experiments.

Non-NMDA glutamate receptors were activated by application of the basic agonists AMPA and kainate (KA). The EC_{50} values in AII cells of the rat retina were found by other investigators to be 118 μM for AMPA and 169 μM for KA (*Mørkve et al. 2002*). In the present study, 100 and 200 μM of agonist were used. AMPA and KA induced currents were blocked by the non-selective non-NMDA antagonist NBQX (*Sheardown et al. 1990, Lodge et al. 1991*).

Discrimination of AMPA from KA receptor-mediated responses was made using the selective agonists cyclothiazide (CTZ) and SYM 2081, as well as the selective AMPA receptor antagonists GYKI 52466 and GYKI 53655. CTZ is known to remove the rapid desensitization at AMPA receptors and thus increase the AMPA response, while also

prolonging its decay time (*Vyklicky et al. 1991*, *Bertolino et al. 1993*, *Palmer and Lodge 1993*). SYM 2081 is a KA receptor-selective agonist, with antagonist-like effect. SYM 2081 blocks transmission at KA receptors by producing a brief activation followed by a maintained desensitization (*Zhou et al. 1997, Donevan et al. 1998*). Finally, GYKI 52466 and GYKI 53655 selectively inhibit the activity of AMPA receptors (*Donevan and Rogawski 1993*, *Wilding and Huettner 1995*, *Bleakman et al. 1996*) and are therefore useful in establishing the contribution of each non-NMDA receptor type to the total current.

Drugs, in the combinations given in *Table 2.3.*, were applied from a twelve-line superfusion system, through an application pipette with a diameter of 100 μm. The applications followed a standard sequence: basic agonist – selective agonist/ antagonist – basic agonist (selective agonist/ antagonist wash-out). A thirteenth flush line, active between applications, insured the removal of any previous drug left in the pipette tip before a new application. The time between applications ranged from 1,5 to 2 minutes, for allowing receptor recovery from desensitization. Each sequence of drugs was applied several times to make sure that the effects were reproducible.

2.3.5. Data analysis

Electrophysiological data were recorded and saved using the Pulse software (HEKA, Germany) installed on the computer connected with the set-up. Data analysis was consequently performed with the IgorPro 4 program (WaveMetrics Inc., USA) and the statistics and graphs were completed in Excel (Microsoft) and Sigma-Plot (Aspire Software International, USA).

The liquid junction potential (LJP) was calculated with the JPCalc program (*Barry 1994*) and the found value of 15 mV was later subtracted from the raw data for correction. Much of the data presented in this study has a qualitative nature, therefore series resistance and space clamp errors are not important.

Expression of NMDA, AMPA and KA receptors was determined by analysing the effects of the receptor-selective drugs on the current induced by the basic agonist. The effects were quantitatively evaluated on basis of the current integrals. Integrals, representing the area between current trace and baseline, are proportional to the amount of charge moving across the membrane and are therefore a good measure of the currents.

2.4. MORPHOLOGICAL RECOVERY OF RECORDED CELLS

Somata lying proximally in the INL, belonging to a large variety of amacrine cell types, were patched. The recorded cells could be visualized at the end of the experiment due to LY diffusion throughout the dendritic tree. The morphology of the cells was recovered by staining for NB, also included in the IC solution.

2.4.1. Staining of recorded cells

The staining was carried out according to the standard protocols described in *Table 2.4*. All incubation steps were done at room temperature, in darkness. In some cases, the fluorescent staining was intensified by using anti-LY antibodies, followed by secondary antibodies carrying a green fluorophore (*Table 2.5.*).

Table 2.4. Protocols for recovering the morphology of recorded cells.

DAB reaction	Flourescent staining
1h fixation in 4% paraformaldehyde	30 min fixation in 4% paraformaldehyde
3 x 10 min wash in 0,1 M PB	
30 min incubation in 1% triton X in 0,1 M PB	
3 x 10 min wash in 0,1 M PB	
45 min incubation in extravidine-peroxidase 1:500 in 0,1 M PB	overnight incubation in streptavidine-Texas red/ streptavidine-fluorescein 1:100 in 0,1 M PB
3 x 15 min wash in 0,1 M PB	
1-4 min in 950 μl 0,1 M PB + 50 μl 1% DAB + 2 μl 3% H_2O_2	
3 x 5 min wash in 0,1 M PB	
mounting in Aqua polymount	

2.4.2. Confocal microscopy

All fluorescent specimens were viewed using a Zeiss confocal upright microscope. Stacks of confocal images could be obtained by changing the confocal plane. Using Pascal software, images of such stacks were collapsed onto a single image with extended depth of focus and all images further shown resulted from such projections. Images were processed using Adobe Photoshop 6.0 software.

Table 2.5. List of all chemicals used, with their sources.

Chemical	Source
Agarose	Sigma Chemical Co., St. Louis, MS, USA
AlexaFluor 488 hydrazide	MoBiTec, Göttingen, Germany
AMES medium	Sigma Chemical Co., St. Louis, MS, USA
(S)-AMPA ((S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)	Biotrend (Tocris), Cologne, Germany
Anti-Lucifer yellow, rabbit antibodies IgG, biotin-xx conjugate	Molecular Probes Europe, Leiden, The Netherlands
Aqua PolyMount	Polysciences Europe, Eppelheim, Germany
Na-ATP (adenosine triphosphate, sodium salt)	Sigma Chemical Co., St. Louis, MS, USA
Caesium gluconate	Christopher Habermann, Frankfurt/ Main, Germany
Caesium hydroxide (CsOH)	Sigma-Aldrich Chemie Gmbh, Münich, Germany
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	Sigma Chemical Co., St. Louis, MS, USA
Carbogen	Nenner, Messer Griesheim, Germany
Chemiblocker	Chemicon Ldt., Hofheim, Germany
Cyclothiazide	Biotrend (Tocris), Cologne, Germany
DAB (diaminobenzidine)	Sigma Chemical Co., St. Louis, MS, USA
DMSO (dimethylsulfoxide)	Merck, Darmstadt, Germany

EGTA (ethylene glycol-bis(ß-aminoethyl ether)N,N,N′,N′-tetraacetic acid)	Sigma Chemical Co., St. Louis, MS, USA
Extravidine-peroxidase	Sigma Chemical Co., St. Louis, MS, USA
Glycine	Biotrend (Tocris), Cologne, Germany
GYKI 52466 ((R)-(-)-α-methylhistamine hydrochloride)	Sigma Chemical Co., St. Louis, MS, USA
GYKI 53655 (1-(4-aminophenyl)-3-methylcarbamyl-4- methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine)	Dr. Carl Romano, Washington University, St. Louis, USA
Na-GTP (guanosine triphosphate, sodium salt)	Sigma Chemical Co., St. Louis, MS, USA
Halothane	Eurim Pharma, Piding, Germany
HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid])	Sigma Chemical Co., St. Louis, MS, USA
Kainic acid	Biotrend (Tocris), Cologne, Germany
Lucifer yellow	Sigma Chemical Co., St. Louis, MS, USA
Magnesium sulfate ($MgSO_4$)	Sigma Chemical Co., St. Louis, MS, USA
Methanol	J. T. Baker, Germany
Natriumazid	Merck, Darmstadt, Germany
NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo(f)quinoxaline-7-sulfonamide disodium salt)	Biotrend (Tocris), Cologne, Germany
Neurobiotin	Linaris (Vector)
NMDA (N-methyl-D-aspartic acid)	Biotrend (Tocris), Cologne, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
PCR kit	Qiagen, Heiden, Germany
PCR primers	MWG Biotech AG, Ebersberg, Germany
Proteinase K	Sigma Chemical Co., St. Louis, MS, USA
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Darmstadt, Germany
Streptavidin fluorescein	Amersham Biosciences Europe, Freiburg, Germany

Streptavidin Texas red	Amersham Biosciences Europe, Freiburg, Germany
Strychnine	Sigma Chemical Co., St. Louis, MS, USA
SYM 2081 ((2S,4R)-4-methylglutamate)	Calbiochem
TBA (tetrabutylammonium chloride)	Sigma Chemical Co., St. Louis, MS, USA
Triton	Sigma Chemical Co., St. Louis, MS, USA
H_2O_2 (hydrogen peroxide)	Merck, Darmstadt, Germany

3. RESULTS

Amacrine cells make up the most diverse population of all retinal neurons, both morphologically and physiologically. These interneurons receive information from bipolar cells through glutamatergic input, which they filter, feed back to bipolar cells and further transmit to ganglion cells, the output neurons of the retina. The present study aimed to determine whether amacrine functional diversity is based on a differential expression of glutamate receptors among cell populations and types. The patch-clamp technique was employed for this purpose. Electrophysiological recordings were made in a slice preparation of the mouse retina and expression of AMPA, KA and NMDA receptors was pharmacologically investigated. Selective agonists and antagonists of the different receptor types were used, such as cyclothiazide (CTZ), GYKI 52466, GYKI 53655, SYM 2081 and NBQX, and their effects were evaluated in comparison with the responses to the basic agonists AMPA, KA and NMDA.

3.1. AMACRINE CELL CLASSIFICATION

All cells were filled with both a fluorescent dye (Lucifer yellow or Alexa 488) and neurobiotin. It was thus possible to visualize the cells after recording and to photograph them later, after fixation and coupling of neurobiotin with a fluorescent marker.

At least 30 morphologically different types of amacrines have been estimated to exist. They have been anatomically described and classified only in few retinae, such as in rabbit (*MacNeil and Masland 1998*) and partially in rat (*Perry and Walker 1980, Menger et al. 1998*). The correspondence of types across species is not completely clear (e.g. *MacNeil et al. 1999*).

In this study, the recorded cells were grouped according to their dendritic field size (see *Section 1.3.*) into narrow-field and wide-field cells. Cells with dendrites extending less than 100 μm in the inner plexiform layer (IPL) were included in the former class, while cells with dendritic trees larger than 100 μm in the later. *Figure 3.1. B* and *C* shows one example of a narrow-field and one example of a wide-field cell, but cells with different dendritic morphology and stratification patterns are lumped together within each of the groups (*Figure 1.2., Introduction*). Due to their characteristic morphology with an elongated soma and one thick main dendrite, AII amacrine cells could be easily recognized and characterized as a type of their own (*Figure 3.1. A*). They were not included in the general analysis of narrow-field cells.

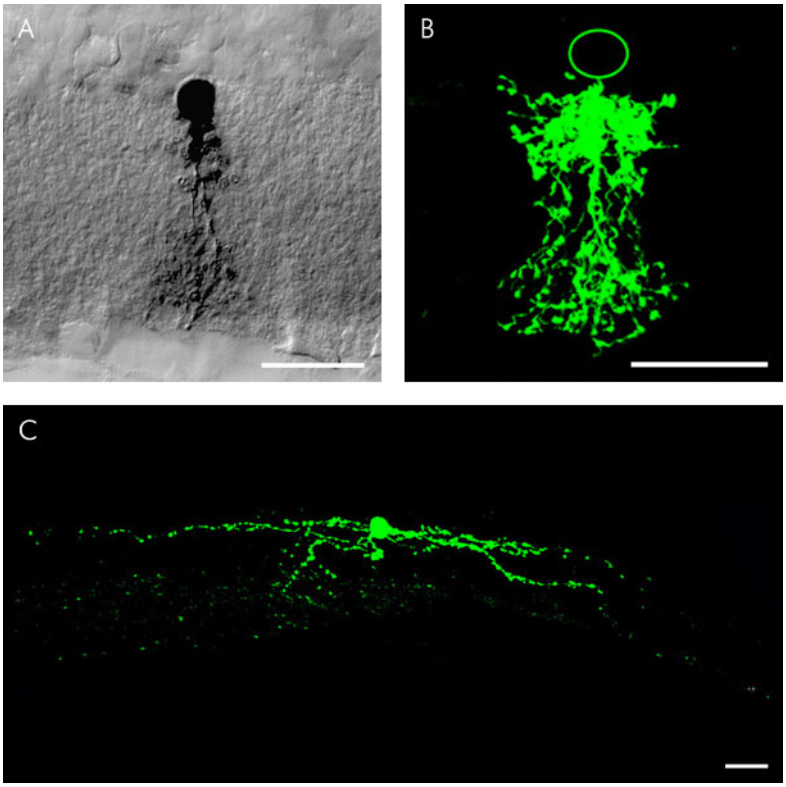

Figure 3.1. **Narrow-field *vs.* wide-field amacrine cells.** Depending on the size of their dendritic field, amacrine cells have been classified as narrow-field (< 100 μm) and wide-field cells (> 100 μm).

(A) Example of an AII amacrine cell, with lobular appendages in the outer part and arboreal dendrites in the inner part of the IPL.

(B) Example of a narrow-field amacrine cell, with a dendritic diameter smaller than 20 μm, stratifying all throughout the IPL.

(C) Example of a wide-field amacrine cell, with a dendritic diameter of ca. 200 μm, stratifying mostly in the outer sublaminae of the IPL.

Scale bars 20 μm.

In addition, a transgenic mouse line was used. The glycinergic neurons of the GlyT2-EGFP mice express EGFP and therefore allowed classification of amacrine cells on basis of their neurotransmitter. *Figure 3.2. A* shows a vertical section through the inner retina of a GlyT2-EGFP mouse, stained for the neurotransmitter GABA (in red). Most of the EGFP labelled cells are glycinergic, but some GABAergic amacrines were also found to express the transgene. In our experiments, we considered EGFP-labelled cells glycinergic and all other not labelled amacrine cells GABAergic.

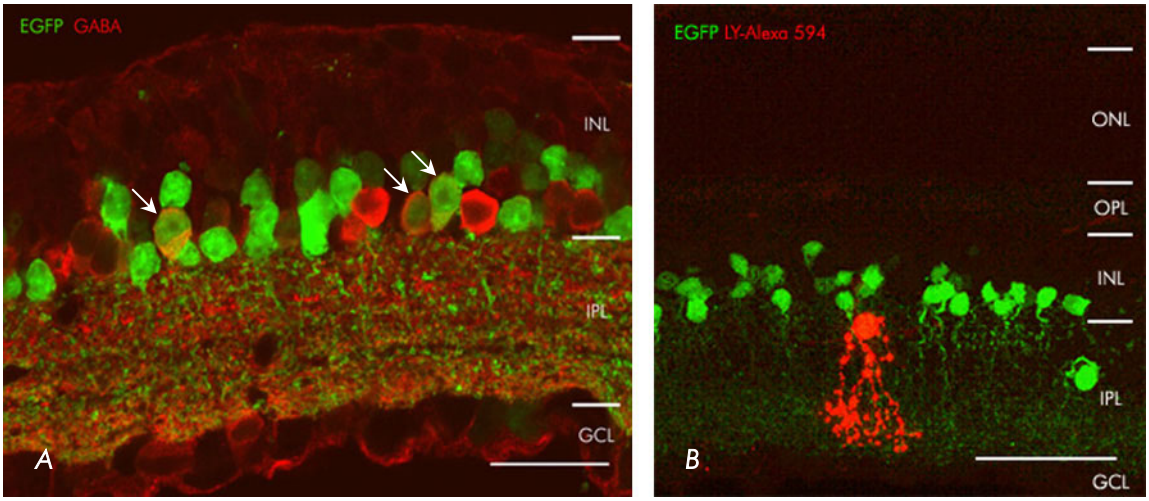

Figure 3.2. **Vertical sections through the retina of GlyT2-EGFP mice.**
(A) GlyT2-EGFP retina stained for GABA. Glycinergic cells express EGFP and appear green; GABAergic cells were stained for GABA, in red. As indicated by arrows, some GABAergic amacrines also express the transgene. Scale bar 20 μm (*courtesy of Silke Haverkamp*)
(B) AII cell patched in a GlyT2-EGFP mouse. Following filling with LY, the morphology of the cell could be recovered by immunostaining with anti-LY antibodies marked with Alexa 594. Scale bar 50 μm.
The following layers are indicated: ONL outer nuclear layer; OPL outer plexiform layer; INL inner nuclear layer; IPL inner plexiform layer; GCL ganglion cell layer.

3.2. ELECTROPHYSIOLOGY

Electrophysiological experiments have attested presence of both non-NMDA and NMDA receptors on amacrine cells in different species, including mudpuppy, salamander, rat and rabbit (*Slaughter and Miller 1983*, *Massey and Miller 1988*, *Dixon and Copenhagen 1992*, *Boos et al. 1993*, *Hartveit and Veruki 1997, Tran et al. 1999, Zhou and Dacheux 2004*). Virtually all ionotropic glutamate receptor subunits have been identified by immunocytochemistry also in the IPL of the mouse retina (e.g. *Haverkamp and Wässle 2000*). In order to test expression of different GluR types among amacrine cells in the mouse retina, pharmacological experiments using AMPA, KA and NMDA receptor agonists and antagonists were performed in this study.

More than 300 amacrine cells were patched and recordings from 130 cells were included in the analysis. The cells were voltage clamped to -75 mV and the recording time varied between 10 and 25 min.

Some cells exhibited spontaneous synaptic activity, including both excitatory (EPSCs) and inhibitory post-synaptic currents (IPSCs). As the reversal potential for both cations and chloride ions was around 0 mV, all spontaneous events recorded at -75 mV were inward currents. The excitatory or inhibitory nature of the synaptic currents was inferred from their kinetics: EPSCs display small amplitude and fast decay, while IPSCs have significantly larger amplitudes and decay slowly. *Figure 3.3.* shows a continuous recording from a cell with large IPSCs, ranging from 50 to 350 pA in amplitude. PSCs were no further investigated with pharmacological tools.

Some cells were actively firing (*Figure 3.4.*). Almost all cells which fired action potentials were identified as AII amacrine cells. It has been previously shown that AII cells typically exhibit Na^+ action currents, which can be blocked by TTX (*Boos et al. 1993*) and can be considered the "electrophysiological signature" of these cells (*Veruki et al. 2003*).

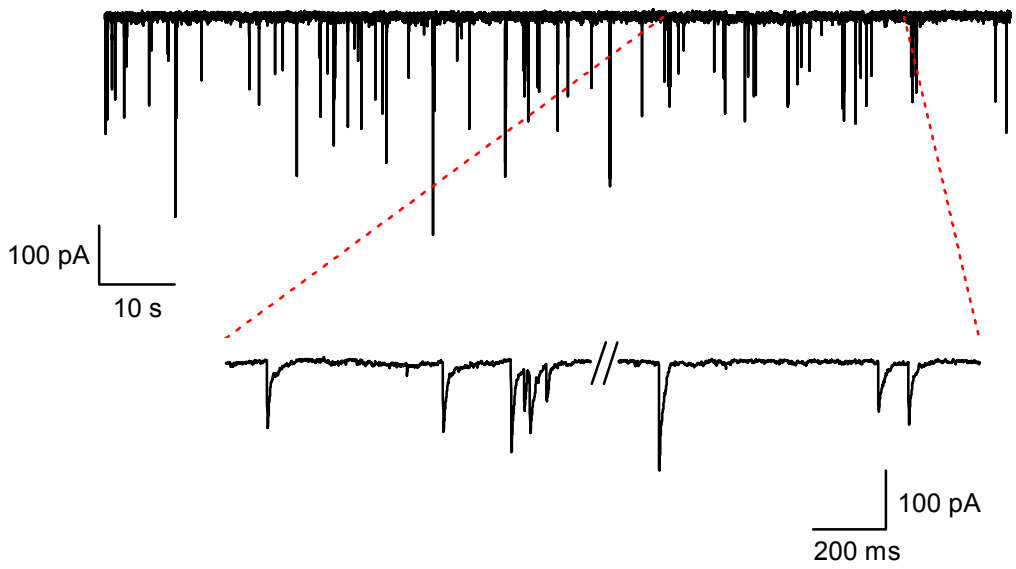

Figure 3.3. **Spontaneous synaptic activity recorded in an amacrine cell.**
Up: 2 minutes continuous recording, with spontaneous events ranging from 50 to 350 pA.
Down: 50x time scale zoom-out of a part of the recording above. The spontaneous events represent inhibitory post-synaptic currents (IPSCs), because of their relatively large amplitudes and slow deactivation profiles. Occasional overlapping of several IPSCs could be seen.

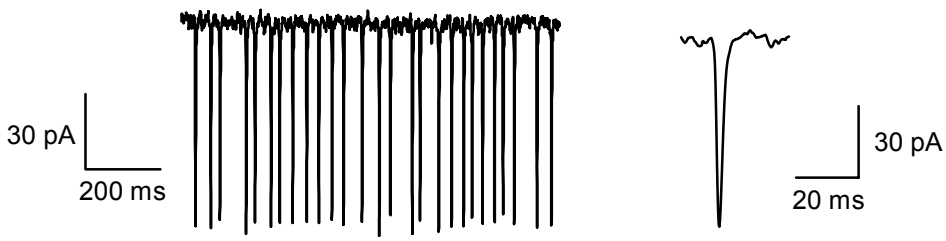

Figure 3.4. **Spontaneous firing recorded in an AII amacrine cell.**
Left: Half a minute continuous recording of spontaneous action currents with mean amplitude of 60 pA, fired at a frequency of 175 Hz.
Right: Isolated action current magnified 10x.

3.2.1. Amacrine cell responses to glutamate agonists

All cells recorded (n = 300) responded to application of the basic non-NMDA receptor agonists AMPA and KA. The EC_{50} values in AII cells of the rat retina were found by other investigators to be 118 μM for AMPA and 169 μM for KA (*Mørkve et al. 2002*). We used 100 or 200 μM of each agonist.

Generally, wide-field cells showed smaller currents than narrow-field cells, which can be partially accounted for by the lower drug concentrations that reach the distant dendrites by diffusion. The current amplitudes may also be reduced in the wide-field cells because their dendritic fields may be truncated in the slice preparation.

AMPA and KA-induced currents showed rather profound rundown, despite ATP and GTP being included in the intracellular solution. The responses decreased typically by 20 to 40% after 10 minutes of recording, as the example in *Figure 3.5.* shows.

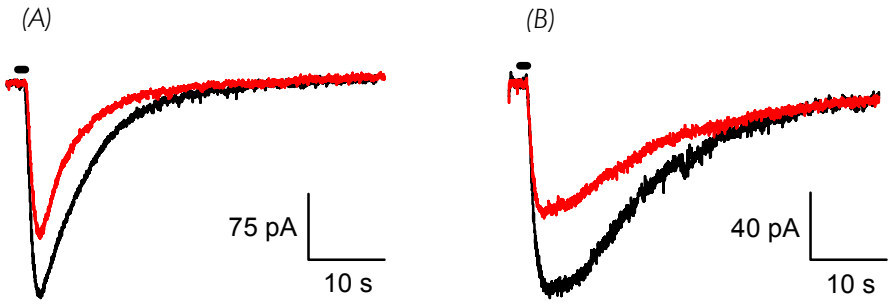

Figure 3.5. **Rundown of non-NMDA-mediated currents normally observed in the recorded cells.**
(A) KA-induced response at the beginning of the experiment (black trace) and 10 minutes later (red trace). A reduction of 35% of the current was measured.
(B) AMPA-induced responses in the same cell, recorded at a 10 minute interval. The later response (red trace) is 37% smaller than the initial current (black trace).
The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

In the same cell, KA application usually evoked a significantly faster deactivating current than AMPA application. *Figure 3.6.* shows the currents induced by application of 100 μM AMPA and 100 μM KA in a narrow-field and a wide-field cell. The decay time constant (τ) of the AMPA-induced current in the narrow-field cell was 6,1 s compared with only 1,8 s for the KA-induced response (*panel B*). In the wide-field cell, the AMPA-evoked current decayed with a time constant of 7,0 s, while a τ value of 3,2 s was found for the KA-

evoked response (*panel A*). Similar results were obtained in the majority of the recorded cells. It is known that AMPA and KA receptor kinetics and affinities are different for the two agonists. AMPA induces a desensitizing response at AMPA receptors, while KA triggers a sustained response at AMPA receptors and a desensitizing response at KA receptors (reviewed by *Dingledine et al. 1999*). However, desensitization cannot account for the different effects obtained in our experiments, as the agonists were applied for only a short time (1 s), in which the rising phase of the current just developed. Even for longer applications (5 s) it has been shown for the AII cells of the rat retina that AMPA- and KA-induced currents do not desensitize (*Mørkve et al. 2002*). It is rather a matter of affinity – it takes a higher concentration of KA than AMPA to open the same amount of AMPA receptors ($EC_{50\ KA} > EC_{50\ AMPA}$ at AMPA receptors) and *vice versa* ($EC_{50\ KA} < EC_{50\ AMPA}$ at KA receptors). We used both agonists in the same concentration, so that a faster deactivating KA-induced current suggests presence of AMPA receptors in the cell (while not excluding the possibility of KA receptors being as well expressed).

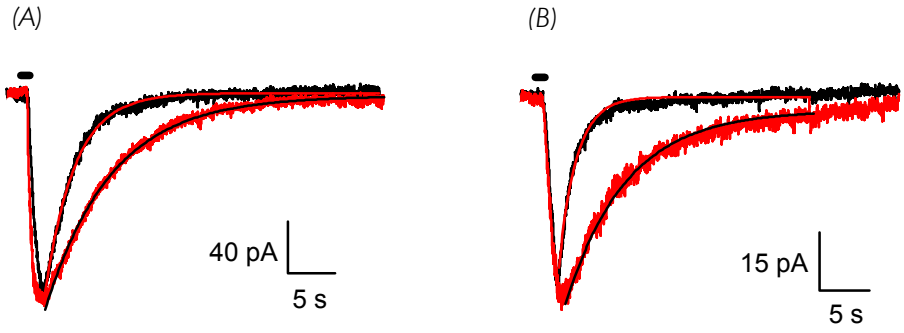

Figure 3.6. **AMPA- (I_{AMPA}) and KA-induced (I_{KA}) currents in amacrine cells.** Deactivation slopes of the currents were fitted with single-exponential functions (superimposed traces). The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

(A) AMPA- (red trace) and KA-induced (black trace) responses recorded in a wide-field amacrine cell. A decay time constant (τ) of 7,0 s was found for I_{AMPA} and 3,2 s for I_{KA}.

(B) AMPA- (red trace) and KA-induced (black trace) responses recorded in a narrow-field amacrine cell. I_{AMPA} showed a τ of 6,1 s, much slower than I_{KA}, for which a τ of 1,8 s was found.

The reversal potential calculated for AMPA- and KA-evoked currents was 0,7 ± 5 mV (n = 7) (*Figure 3.7.*), which confirms mediation by non-NMDA channels.

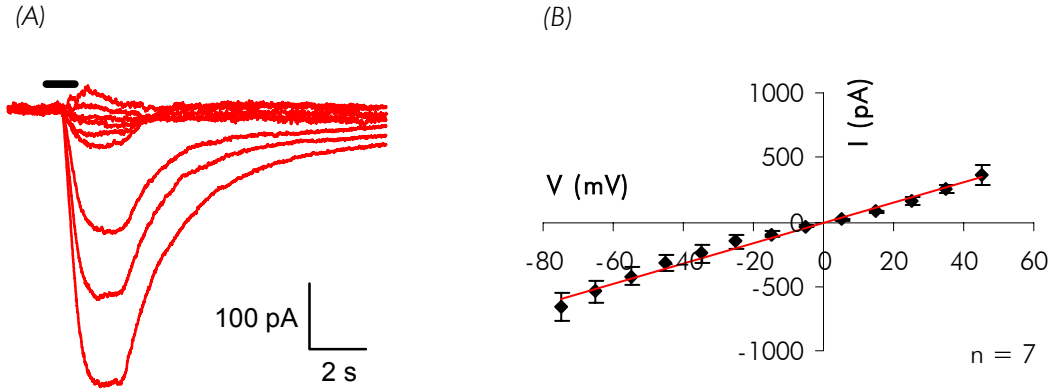

Figure 3.7. **Reverse potential of AMPA- and KA-evoked currents in amacrine cells.**
(A) Series of KA-induced responses recorded in an amacrine cell between -65 and 15 mV, in 10 mV voltage steps. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).
(B) Current-voltage (I-V) relationship of KA-evoked currents, based on recordings from 7 amacrine cells. The average reversal potential was found to be 0,7 mV (± 5 mV).

AMPA and KA-induced currents could be blocked with NBQX, a non-selective non-NMDA receptor blocker. In all cells tested (n = 20), application of 10 μM NBQX led to a strong (90-100%) inhibition of the currents evoked by the basic agonists (*Figure 3.8.*).

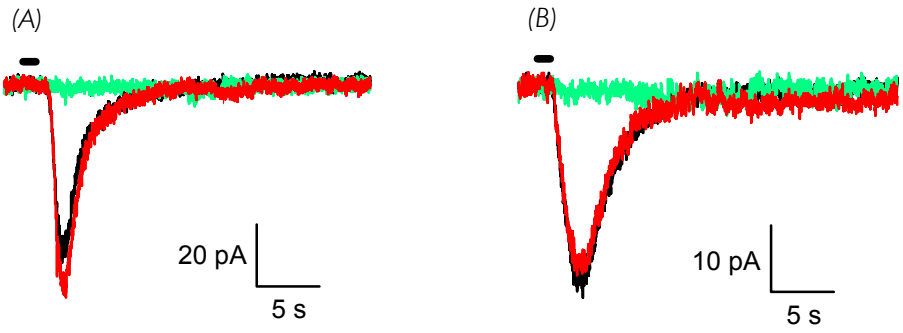

Figure 3.8. **Blockage of non-NMDA-mediated currents by application of NBQX.**
Application of 10 μM NBQX completely blocked AMPA- *(A)* and KA-induced *(B)* currents. The recordings were made in two different cells. Responses to basic agonists (AMPA and KA) are shown in black, drug applications in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

In contrast to the non-NMDA agonists, NMDA application elicited responses in some cells only (*Figure 3.9.*). The cells were clamped to -75 mV and 100 μM NMDA were applied in Mg-free solution and in the presence of small amounts of the co-agonist glycine (2 μM) and strychnine (1 μM), to prevent activation of glycine receptors. Under these circumstances, small responses could be observed in most of the wide-field (6 of 8) and half of the narrow-field amacrine cells tested (4 of 8). No AII amacrine cell (n = 10) showed NMDA-mediated currents under the same conditions (*Figure 3.9., panel A*). When the agonist concentration was increased to 500 μM and the holding potential switched to depolarizing values (-35 to 45 mV), NMDA responses could be elicit in 4 out of 5 wide-field (*panels E-F*) and 3 out of 7 narrow-field cells (*panel D*). In addition, NMDA responses could be also seen in 3 out of 12 AII amacrine cells tested (*panel B*).

In the EGFP mouse, only two glycinergic cells were tested with NMDA and none of them showed any response (*panel G*). In 2 out of 3 GABAergic cells tried, NMDA elicited currents at different holding potentials and concentrations.

In all cells tested, the NMDA-evoked currents were significantly smaller than the AMPA and KA-evoked responses. Their amplitudes ranged from a few to 60 pA in the best cases. No cell has been encountered, that expressed exclusively NMDA receptors.

The results of the NMDA experiments are summarised in the diagram of *Figure 3.10.*. The coloured bars indicate the percentage of the cells tested that were found to express NMDA receptors, while the grey domains stand for the cells that have no NMDA receptors. More wide-field and GABAergic than narrow-field and glycinergic cells tend to express NMDA receptors. Only a small proportion of the AII amacrine cells showed NMDA activity, otherwise not in the physiological voltage range.

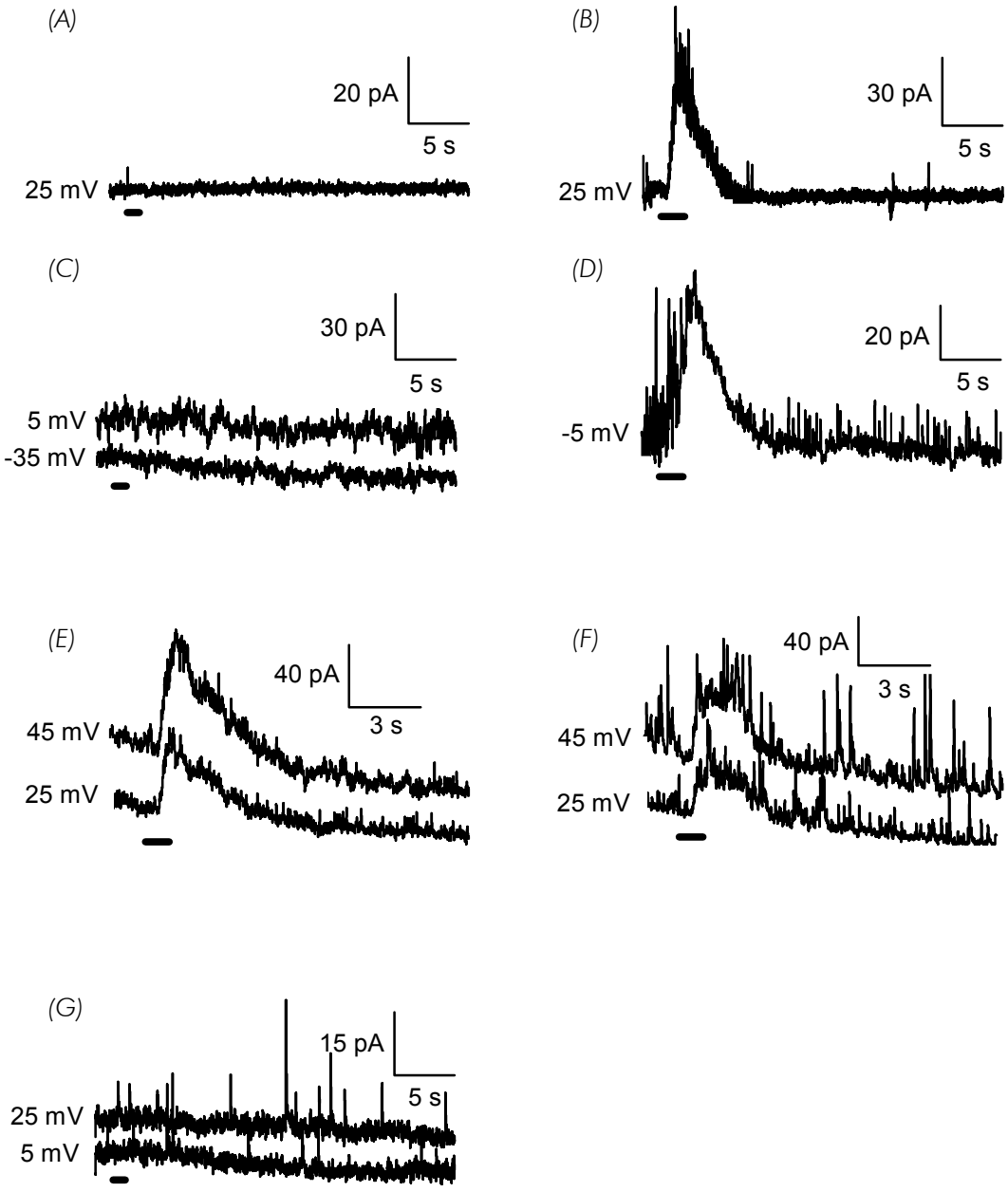

Figure 3.9. NMDA-evoked responses in different amacrine cells.

(A) Application of 100 μM NMDA evoked no response in an AII cell clamped to 25 mV.

(B) Application of 500 μM NMDA evoked a current of 40 pA peak amplitude in another AII cell, clamped to 25 mV.

(C) Application of 500 μM NMDA could elicit no current in a narrow-field cell hold at -35 and 5 mV.

(D) A current of 50 pA peak amplitude was evoked by the application of 500 μM NMDA in another narrow-field cell, clamped to -5 mV.

(E) Application of 500 μM NMDA evoked currents of up to 60 pA in a wide-field cell hold at 25 and 45 mV.

(F) Similar responses in another wide-field cell, under the same conditions.

(G) Application of 500 μM NMDA had no effect in a glycinergic cell clamped to depolarizing potentials (5 and 25 mV).

The duration of drug application is indicated by the horizontal bars under of the traces (1 or 2 s).

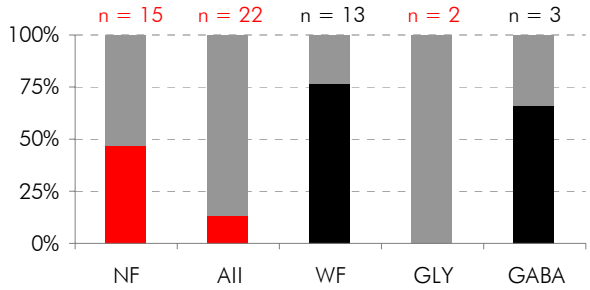

Figure 3.10. **Statistics of NMDA receptor expression in different cell classes.** Coloured bars indicate the proportion of cells that express NMDA receptors; grey domains stand for the percentage of cells that do not possess NMDA receptors. Almost half of the narrow-field cells tested (n = 15), but no glycinergic cell (n = 2) were found to have NMDA receptors. The great majority of AII cells (86%, n = 22) showed no NMDA receptor activity. More than 75% of the wide-field (n = 13) and 2 out of 3 GABAergic cells were found to express NMDA receptors.

AMPA and KA activate both non-NMDA receptor types. Simple application of AMPA or KA is therefore not enough to decide whether a cell expresses AMPA receptors, KA receptors or both. Receptor selective drugs have to be used for this purpose. Cyclothiazide and GYKI compounds are known to specifically act on AMPA receptors, while having no effect on KA receptors. SYM 2081 has been shown to act on KA receptors, without affecting AMPA activity. In the following, an account of the experiments performed with these drugs is given.

3.2.2. Amacrine cell responses to AMPA receptor selective drugs

Expression of AMPA receptors was tested by application of the selective AMPA receptor agonist cyclothiazide (CTZ) and the selective antagonists GYKI 52466 and GYKI 53655. CTZ is known to remove the rapid desensitization at AMPA receptors and thus increase the AMPA response, while also prolonging its decay time (*Vyklicky et al. 1991*, *Bertolino et al. 1993*, *Palmer and Lodge 1993*). GYKI 52466 and GYKI 53655 are compounds which selectively block AMPA receptors (*Donevan and Rogawski 1993*, *Wilding and Huettner 1995*, *Bleakman et al. 1996*). These drugs were applied in combination with the basic agonists and their effects were evaluated in comparison with the control current. The applications followed a standard sequence: basic agonist – basic agonist plus selective agonist/ antagonist – basic agonist (selective agonist/ antagonist wash-out). For example, AMPA – AMPA plus CTZ – AMPA; KA – KA plus GYKI – KA. The time between applications ranged from 1,5 to 2 minutes, for allowing receptor recovery from possible desensitization.

3.2.2.1. Cyclothiazide (CTZ) effects

100 μM CTZ were applied in combination with 100 or 200 μM AMPA. Application of CTZ had different effects in different cells, ranging from strong stimulation to no effect at all. The whole range of effects could be observed among cells of each population.

Figure 3.11. shows three examples of narrow-field amacrine cells displaying different patterns of CTZ-activated currents. In half of the narrow-field cells tested (14 of 31) the AMPA-evoked current showed two different components, as if the agonist would activate two types of receptors with different kinetic properties – a fast activating - fast deactivating and a slower activating - slower deactivating component. The latter normally disappears with application of CTZ and remains reduced during the recovery (*Figure 3.11. A*). AMPA receptors have a slightly faster kinetics than KA receptors and a higher affinity for AMPA. One could therefore attribute the two components of the response to activation of AMPA and KA receptors, respectively. This could also explain why the second component cannot be seen during the application of CTZ; the strong activation of AMPA receptors by CTZ

would mask the smaller current mediated by KA receptors. Only 1 out of 9 AII amacrine cells showed a similar pattern, while 4 out of 13 wide-field cells displayed slightly faster deactivating currents at application of CTZ, which persisted during the recovery (*Figure 3.12. A*).

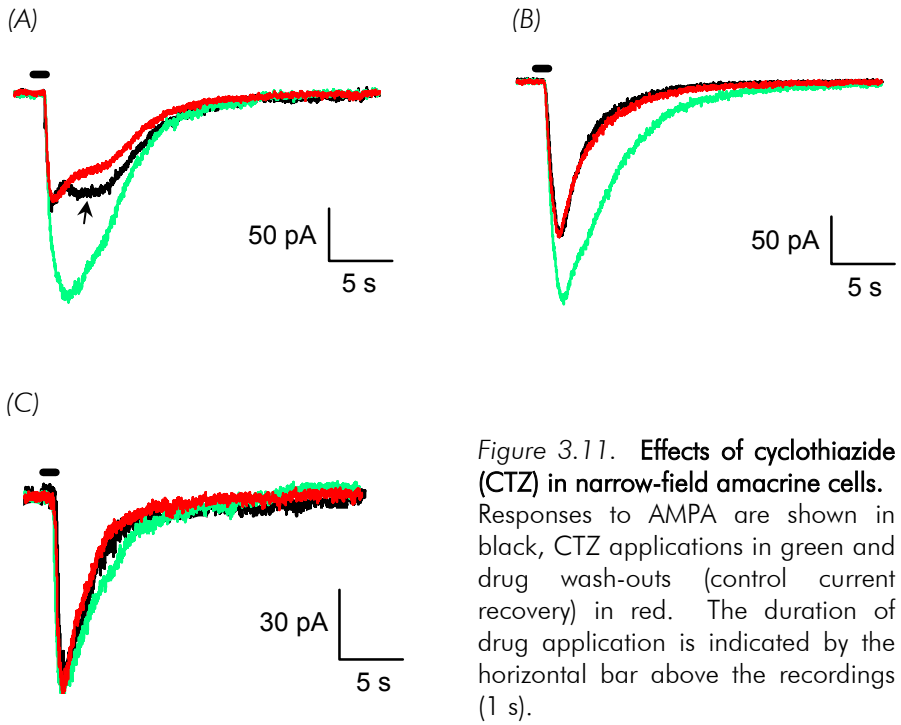

Figure 3.11. **Effects of cyclothiazide (CTZ) in narrow-field amacrine cells.** Responses to AMPA are shown in black, CTZ applications in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

(A) Strong effect of CTZ. Peak amplitude was increased by 86% and the overall current by 78% in this cell. The second component of the AMPA response (*arrow*) disappeared following application of CTZ.

(B) CTZ produced an increase of 43% in the peak amplitude of the AMPA-induced response, while the overall current was two times larger than the control. The decay time of the drug response was also much prolonged (τ_{AMPA} = 2,5 s, τ_{CTZ} = 4,5 s).

(C) With the exception of a slight reduction of the decay time at drug application, CTZ had no noticeable effect in this cell.

In other narrow-field, as well as wide-field cells, CTZ produced an increase in both the peak amplitude and the decay time of the current (*B panels*). One third of the narrow-field cells and a half of the wide-field cells tested did not respond to application of CTZ (*C panels*).

A quantification of the CTZ effects in different cell populations and types is given in the charts in *Figure 3.13.*, while a statistics of AMPA receptor expression is provided in the next section (*Figure 3.21.*).

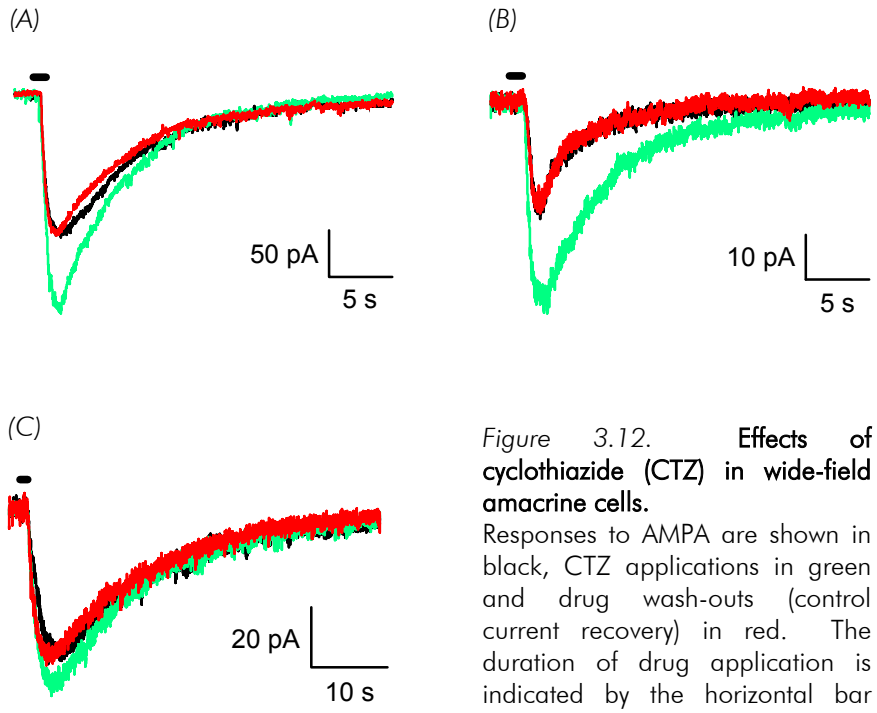

Figure 3.12. **Effects of cyclothiazide (CTZ) in wide-field amacrine cells.**
Responses to AMPA are shown in black, CTZ applications in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

(A) Moderate effect of CTZ. Peak amplitude was increased by 53% and the overall current by 22% in this cell. A slight reduction in the decay time at application of CTZ is to be noticed (τ_{AMPA} = 7,0 s, τ_{CTZ} = 5,9 s).
(B) CTZ produced an increase of 77% in the peak amplitude of the AMPA-induced response, while the overall current was 2,5 times larger than the control. The decay time of the drug response was prolonged in this case (τ_{AMPA} = 3,4 s, τ_{CTZ} = 4,9 s).
(C) No noticeable effect of CTZ.

Both peak amplitudes and integrals (transferred charge over duration of the response) were considered for the analysis of the effects of CTZ. The parameters of the CTZ-activated current were measured and divided by those of the control current. The relative effect of the drug compared to the control is given in percents. Thus, a value of 100% reflects no change in the peak amplitude and integral of the current at drug application in comparison with the current evoked by the basic agonist. Values over 100% show an increase, while values under 100% reflect a reduction of the control current by the drug. Increments of at least 25% in both the amplitude and the integral of the CTZ-triggered current in comparison with the control current were considered evidence for AMPA receptor activity. For each cell, if possible, the results of the CTZ experiments were further confronted with the outcome of the GYKI experiments, before including them in the statistics of AMPA receptor expression.

Figure 3.13. (*upper panel*) shows the average of the drug response integral and peak amplitude reported to the integral and peak amplitude of the control current in AII, narrow-field and wide-field amacrine cells. In the AII cells, CTZ increased the peak amplitude of the AMPA-evoked current by 50 ± 14% and the overall current (integral) was 140 ± 34% larger, on average (n = 8). Narrow-field cell responses to CTZ were 60 ± 7% larger than the control (n = 31), whereas the wide-field cells showed increments of 40 ± 7% on average (n = 13). However, while CTZ effects in the AII amacrines are rather homogeneous, a large variability is to be noticed among the narrow-field and wide-field cells (*Figure 3.13., lower panels*).

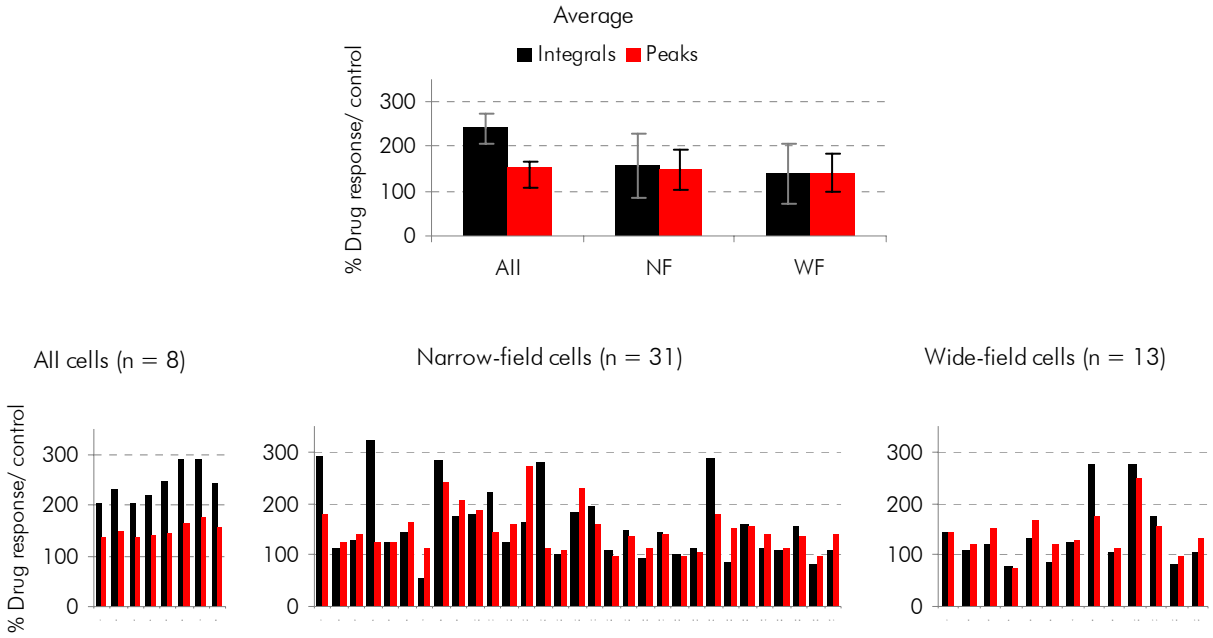

Figure 3.13. **Quantitative analysis of cyclothiazide (CTZ) effects in narrow- (NF) vs. wide-field (WF) amacrine cells.** Integrals (black) and peak amplitudes (red) have been measured for the drug response and the control current. The values obtained for the drug were divided by those obtained for the control and the ratio is shown in percents.

Up: Mean values calculated for the different cell classes. In the AII cells (n = 8), CTZ produced average increase of 51 ± 14% in the peak amplitude and 141 ± 34% in the overall current. In the narrow-field cells (n = 31), the average increase in the overall current was 62 ± 7% and the peak amplitude rose by 51 ± 4%. Wide-field cells (n = 13) responded on average with a 40% rise in both peak amplitude (± 4%) and overall current (± 7%).

Down: Individual values measured for each cell in the three classes. A considerable variability is to be noticed among the narrow- and wide-field cells, which responded very differently to application of CTZ. The values measured for the AII group were rather similar among different cells.

The same analysis in case of the cells recorded in GlyT2-EGFP mice shows that, on average, CTZ increased the AMPA-triggered current by 22 ± 24% in the glycinergic cells (n =11) (*Figure 3.14., upper panel*). For both glycinergic and GABAergic groups, some variability can be noticed among the individual cells (*Figure 3.14., lower panels*), although not as striking as in the case of narrow- *vs.* wide-field amacrines. In three of the glycinergic cells and one of the two GABAergic cells tested the drug had no effect.

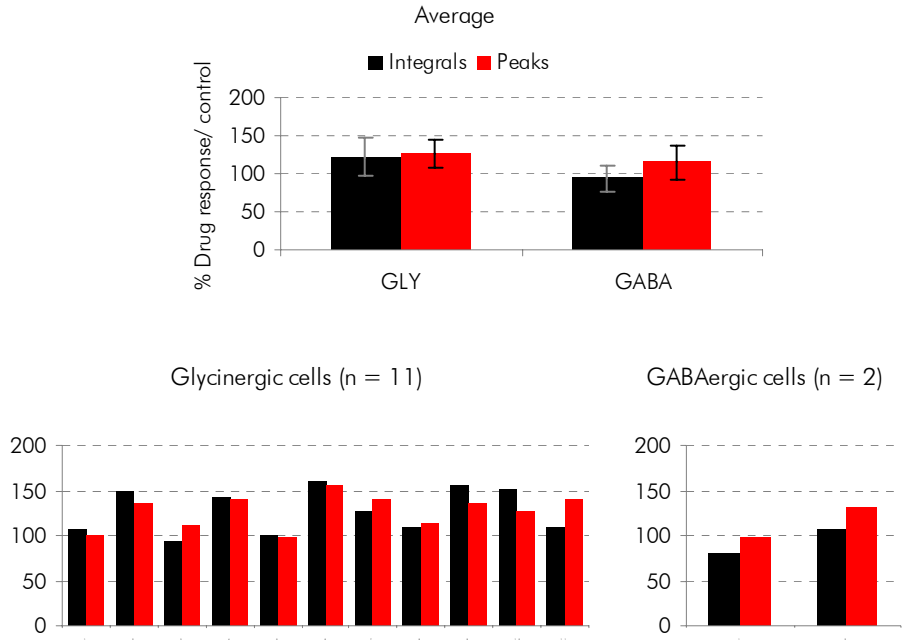

Figure 3.14. **Quantitative analysis of cyclothiazide (CTZ) effects in glycinergic (GLY) *vs.* GABAergic (GABA) amacrine cells.** Integrals (black) and peak amplitudes (red) have been measured for the drug response and the control current. The values obtained for the drug were divided by those obtained for the control and the ratio is shown in percents.

Up: Mean values calculated for the two cell classes. In the glycinergic cells (n = 11), CTZ produced an average increase of 27 ± 18% in the peak amplitude and 22 ± 24% in the overall current. In the GABAergic cells (n = 2), no gain in the overall current could be seen, while the peak amplitude weakly increased by 14 ± 23%.

Down: Individual values measured for each cell in the two classes. Some variability in the CTZ effects can be noticed among the individual glycinergic and GABAergic cells.

3.2.2.2. GYKI 52466 and GYKI 53655 effects

Expression of AMPA receptors was also verified by application of a selective antagonist. Two compounds were used for this purpose, GYKI 52466 and GYKI 53655. GYKI 53655 is a more effective inhibitor of AMPA receptors and could be obtained from a private source. However, most experiments were performed using GYKI 52466, which is commercially available. A direct comparison between the two compounds is not shown here, but their effects can be compared by confronting the diagrams in *Figures 3.18.* and *3.19.* (*upper panels*).

20 μM GYKI 52466 or 53655 were applied together with either AMPA or KA (100 or 200 μM). In all cells tested with both agonists (n = 5), the KA-activated current was more sensitive to inhibition by GYKI than the AMPA-activated current (*Figure 3.15.*). This is a consequence of the different effects of the two agonists at AMPA receptors, with KA inducing a more sustained current than AMPA. Thus, KA provides more current to be blocked by GYKI. However, the current left after application of GYKI is in both cases similar or even slightly larger with KA (*Figure 3.15.*).

Most of the experiments were done using KA as an agonist. The experiments in which AMPA was used have been documented separately.

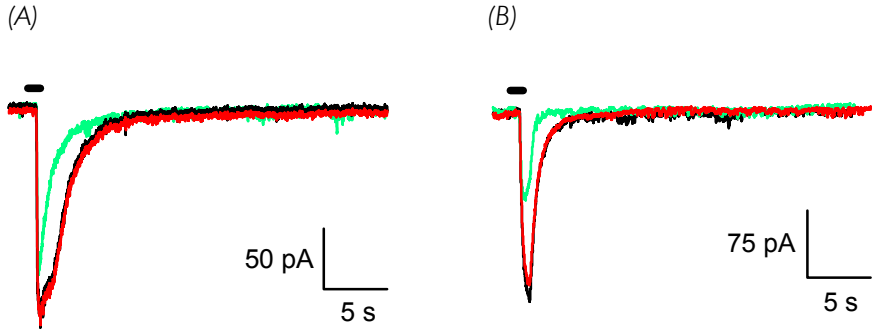

Figure 3.15. **Effects of GYKI on AMPA- and KA-induced currents in the same cell.** Responses to AMPA or KA (200 μM) are shown in black, GYKI 53655 applications (20 μM) in green and drug wash-outs in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

(A) AMPA-induced current inhibited by GYKI 53655. An overall current reduction of 68% was measured, while the peak amplitude decreased by 33% at the application of the drug.

(B) KA-induced current inhibited by GYKI 53655, in the same cell as in *(A)*. An overall current reduction of 75% was found in this case, while the peak amplitude got more than two times smaller.

Just like in the case of CTZ, GYKI had different effects in different cells. Both narrow-field and wide-field cells showed the whole spectrum of effects, from strong to partial to no inhibition at all. The same holds true for the glycinergic and GABAergic cells recorded in the GlyT2-EGFP mice.

In *Figures 3.16.* and *3.17.*, three examples of experiments performed in narrow-field and wide-field cells, respectively, are shown. GYKI 52466 often produced only a partial inhibition of the agonist-driven current (*B panels*). Half of the wide-field cells (n = 11) and about a fifth of the narrow-field cells tested (n =15) showed strong inhibition (*A panels*), while a minority of cells did not react to the application of the drug (*C panels*). GYKI 53655 produced more homogeneous effects among the cells of each population (not illustrated). In AII cells, GYKI 52466 reduced the non-NMDA current by circa 50 ± 4% (n = 5), while GYKI 53655 (n = 3) had a slightly stronger effect.

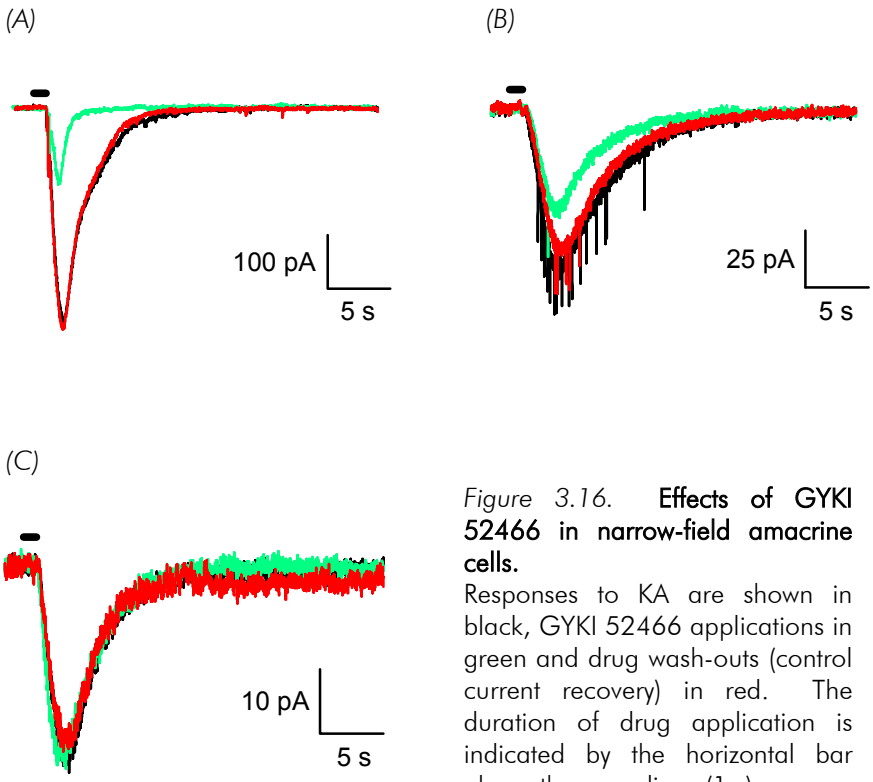

Figure 3.16. **Effects of GYKI 52466 in narrow-field amacrine cells.**
Responses to KA are shown in black, GYKI 52466 applications in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

(A) Strong effect of GYKI 52466. The peak amplitude dropped to 36% and the overall current to 20% of the initial value.
(B) In this cell, GYKI 52466 determined a reduction of 26% in the peak amplitude of the KA-induced response, while the overall current dropped to 57% of the control value.
(C) No noticeable effect of GYKI 52466.

In the majority of cells responsive to the GYKI compounds, the drugs induced not only a reduction of the total current, but also a decrease in the decay time of the currents. Thus, in most of the AII (6 of 8) and narrow-field cells (10 of 15), as well as in half of the wide-field amacrines tested (8 of 15), GYKI responses deactivated up to 2 times faster than the control current.

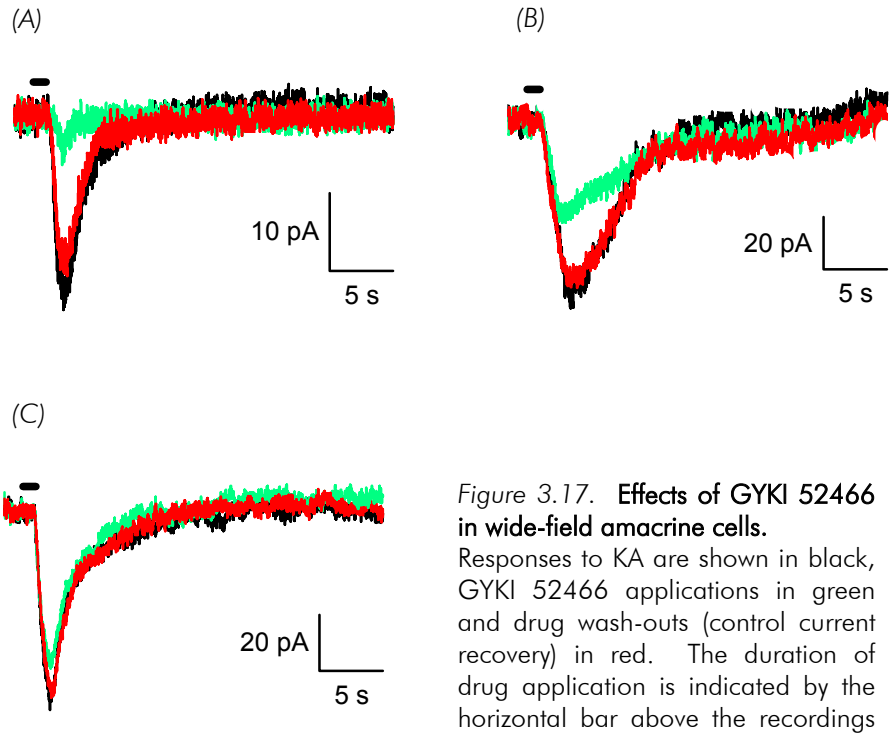

Figure 3.17. **Effects of GYKI 52466 in wide-field amacrine cells.**
Responses to KA are shown in black, GYKI 52466 applications in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

(A) Strong effect of GYKI 52466. The peak amplitude dropped to 25% and the overall current to 11% of the initial value.
(B) In the presence of GYKI 52466, the overall current dropped to 63% and the peak amplitude was half smaller than the control.
(C) GYKI 52466 had no noticeable effect in this cell.

For the quantification of GYKI effects in different cells both peak amplitude and integral of the responses have been taken into account. The data was plotted in the same manner as for the CTZ experiments described in the previous section. The charts in *Figures 3.18.* and *3.19.* show the percentage ratio of the drug response to control current parameters. A value of 100% reflects no change in the agonist-activated current at the application of the drug, while values under 100% show reduction of the control current by the drug. Cells in which GYKI 52466 induced a reduction of at least 25% and GYKI 53655 of at

least 15% in both the peak amplitude and the integral of the control current were considered to express AMPA receptors. For each cell, where possible, the results were compared with the outcome of the CTZ experiments, before including them in the statistics of AMPA receptor expression.

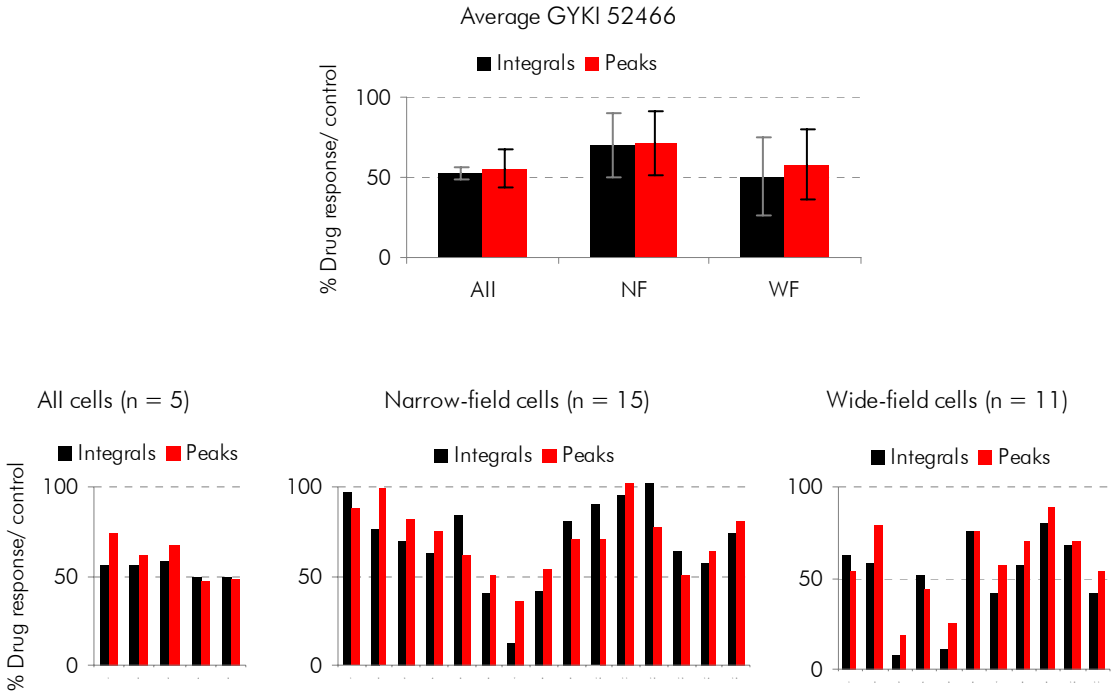

Figure 3.18. **Quantitative analysis of GYKI 52466 effects in narrow- (NF) vs. wide-field (WF) amacrine cells.** Integrals (black) and peak amplitudes (red) have been measured for the drug response and the control current. The values obtained for the drug were divided by those obtained for the control and the ratio is shown in percents.

Up: Mean values calculated for the different cell classes. In the AII cells (n = 5), the peak amplitude and the overall current dropped by almost 50% (± 12%, ± 14%) on average when GYKI 52466 was applied. Narrow-field cells (n = 15) responded on average with a 30% decrease in both peak amplitude (± 20%) and overall current (± 26%). For the wide-field cells (n = 11), the mean reduction in the overall current was 51 ± 24% and the peak amplitude dropped by 42 ± 22%.

Down: Individual values measured for each cell in the three classes. A considerable variability is to be noticed among the narrow- and wide-field cells, which responded very differently to application of GYKI 52466. The values measured for the AII group were rather similar among different cells.

The data illustrated in the *Figures 3.18.* and *3.19.* include only the experiments using KA as an agonist and are separately analysed for GYKI 52466 and GYKI 53655. As mentioned above, in the AII cells the overall current (integral) showed an average reduction by circa 50 ± 4% with GYKI 52466 (n = 5) and 60 ± 12% with GYKI 53655 (n = 3). The parameters measured for individual cells did not vary much in the sample. On

the contrary, the responses to GYKI 52466 among the narrow-field (n = 15) and wide-field (n = 11) cell populations were rather heterogeneous, ranging from extreme inhibition to no effect at all (*Figure 3.18., lower panels*). Variable responses were obtained also for the 4 glycinergic and 4 GABAergic cells tested (not illustrated).

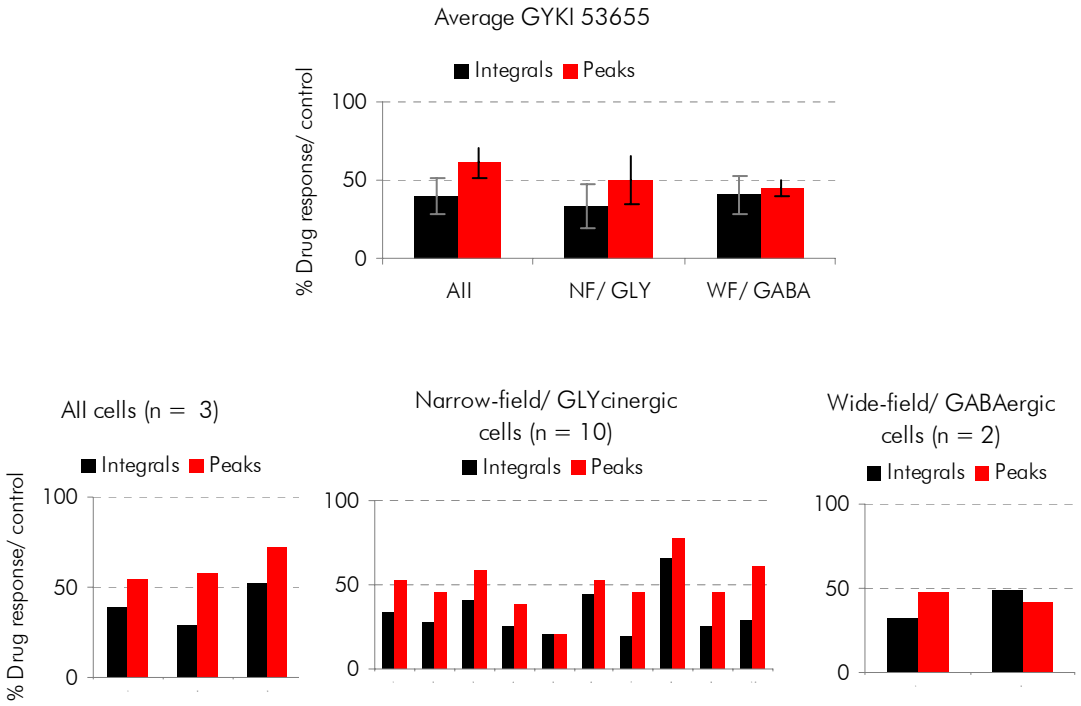

Figure 3.19. **Quantitative analysis of GYKI 53655 effects in narrow-field/ glycinergic (NF/ GLY) vs. wide-field/ GABAergic (WF/ GABA) amacrine cells.** Integrals (black) and peak amplitudes (red) have been measured for the drug response and the control current. The values obtained for the drug were divided by those obtained for the control and the ratio is shown in percents.

Up: Mean values calculated for the different cell classes. In the AII cells (n = 3), the peak amplitude dropped by 39 ± 10% and the overall current by 60 ± 12% on average at application of GYKI 53655. Narrow-field cells (n = 10) responded on average with a 50 ± 15% decrease in peak amplitude and a 67 ± 14% decrease in the overall current. For the wide-field cells (n = 2), the mean reduction in the overall current was 59 ± 12% and the peak amplitude dropped by 55 ± 5%.

Down: Individual values measured for each cell in the three classes. Some variability is to be noticed among the narrow-/ GLYcinergic cells, which responded differently to application of GYKI 53655. The values measured for the AII group were rather similar among different cells.

All cells tested (n = 20) were responsive to GYKI 53655. GYKI 53655 experiments were done on GlyT2-EGFP mice. Following filling with LY or neurobiotin, glycinergic and GABAergic cells were identified as narrow-field and wide-filed cells, respectively, so that in this case the two cell populations overlap. GYKI 53655 decreased the KA-activated current by more than 50% in all narrow-field/ glycinergic and wide-field/ GABAergic amacrines in the sample (*Figure 3.19., lower panels*). In presence of GYKI 53655, the

peak amplitude of the response was on average half the value of the control current (± 15%) and the integral was reduced to 33 ± 14% in the narrow-field/ glycinergic cells (n = 10) (*Figure 3.19., upper panel*). In the wide-field/ GABAergic cells tested (n = 2), the drug induced a reduction of 60 ± 12% on average of the control current (*Figure 3.19., upper panel*).

In all cells tested with both CTZ and GYKI (n = 40), a good correlation between the effects of the two AMPA-receptor selective drugs could be observed (*Figure 3.20.*). Thus, cells that responded with strong current increases to the application of CTZ responded also with strong decreases at application of GYKI. Cells in which CTZ had little or no effect showed at most a weak inhibition in the presence of GYKI. The predictions made after an experiment using one of the two drugs could be verified by the other drug, therefore cells tested with only one of the drugs were also included in the AMPA receptor expression statistics.

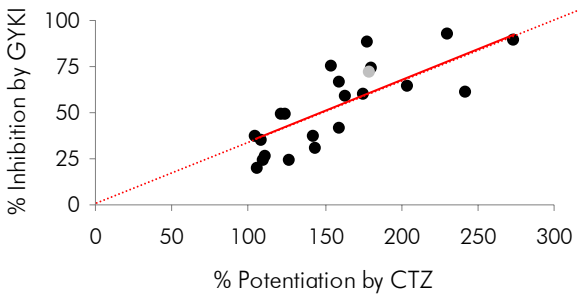

Figure 3.20. **Scatter diagram comparing the effects of GYKI and CTZ in the same cells (n = 22).** On the abscissa the amount of stimulation induced by CTZ is given; on the ordinate the amount of inhibition by GYKI is shown (in percents). For example, in the cell plotted in grey the AMPA-induced current was increased by 180% in the presence of CTZ, while GYKI led to a reduction by 72% of the control current. The calculated trendline (solid red) overlaps completely with the ideal trendline (dotted red line).

On basis of the CTZ and GYKI experiments, the expression of AMPA receptors among different cells could be evaluated. The results are summarised in the diagram of *Figure 3.21.*. 17 AII, 45 narrow-field, 21 wide-field, 20 glycinergic and 9 GABAergic cells were included in the statistics. Out of them, only 1 AII, 6 narrow-field and 3 glycinergic amacrines did not show convincing effects at application of CTZ and GYKI. Thus, AMPA receptors seem to be ubiquitously distributed on narrow-field and glycinergic cells. Positive results were also obtained for the majority of the wide-field and GABAergic cells tested for AMPA receptors. A slightly greater proportion of cells in these classes was found to express no AMPA receptor type.

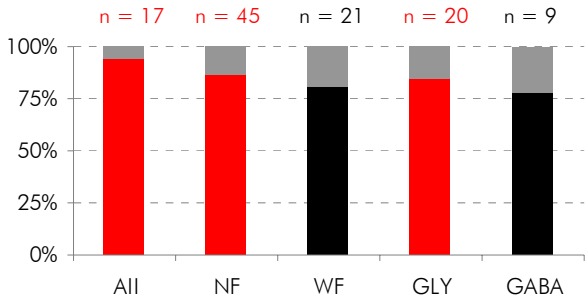

Figure 3.21. **Statistics of AMPA receptor expression in different cell classes.** Coloured bars indicate the proportion of cells that express AMPA receptors; grey domains stand for the percentage of cells that do not possess AMPA receptors. The great majority of AII cells (94%, n = 17), narrow-field (87%, n = 45) and glycinergic cells (85%, n = 20) were found to have AMPA receptors. Likewise, AMPA receptors were identified in 81% of the wide-field (n = 21) and 78% of the GABAergic cells tested (n = 9).

As AMPA or KA activate both AMPA and KA receptors, incomplete blocks by GYKI compounds in some cells can be attributed to activation of KA receptors. 20 μM GYKI 52466 or GYKI 53655 are usually enough to completely block activity at AMPA receptors, if applied in the bath shortly before the agonist. In our experiments, the drugs were co-applied with the agonist, so that some AMPA receptors might not have been blocked and a residual current might have been left. In order to be sure that the current remained unblocked is indeed generated at KA receptors, a security limit of 25% was imposed. Cells in which at least 25% of the control current was left at application of GYKI were considered to express KA receptors. The security limit was chosen by carefully comparing clear examples of cells that responded to both AMPA and KA receptor selective drugs. The

experience of the NBQX experiments was also useful – residual currents larger than 10% of the control have rarely been encountered.

For each cell, where possible, the results of the GYKI experiments were further confronted with the outcome of the SYM 2081 experiments, before including them in the statistics of KA receptor expression (provided in the next section).

3.2.3. Amacrine cell responses to KA receptor selective drugs

Although GYKI experiments can also provide information on expression of KA receptors, an additional drug was used. SYM 2081 is the only widely accepted drug thought to selectively act on all KA receptor subunits, while having no effect on AMPA receptor activity. SYM 2081 is a KA receptor agonist, which produces a brief activation followed by a maintained desensitization. Thus, its application induces a smaller and faster-deactivating current than the basic agonist KA.

SYM 2081 was tested both on its own and co-applied with 100 or 200 μM KA. A concentration of 20 μM SYM 2081was used for the concomitant and 50 or 100 μM for the separate application.

In combination with KA, SYM 2081 had varying effects in different cells. *Figure 3.22.* shows three examples of recordings in narrow-field cells, ordered by the strength of the drug effect. In some narrow-field cells tested, SYM 2081 had no effect at all (*panel A*), while in other cells it induced a potent desensitization resulting in a current significantly smaller than the control (*panel C*). However, the majority of the cells responded with a moderate decrease of the KA-triggered current, generated mainly by a faster deactivation (*panel B*). Several intermediary degrees of current reduction could be observed among the cells in the narrow-field group sample (n = 24).

Comparable effects were observed in wide-field cells (n = 16) (*Figure 3.23.*). Most of the cells were responsive to SYM 2081 when co-applied with the basic agonist (KA). The current generated in the presence of the drug generally had a smaller peak amplitude and showed a faster decay (*panel B*). Indeed, for the vast majority of all cells tested smaller decay time constants (τ) have been measured, accounting for as much as four-fold faster current deactivation at co-application of SYM 2081 and KA in comparison with KA alone.

Out of 34 cells for which the currents have been fitted, only 2 showed no modification of the τ constants, while 4 exhibited slightly slower ones.

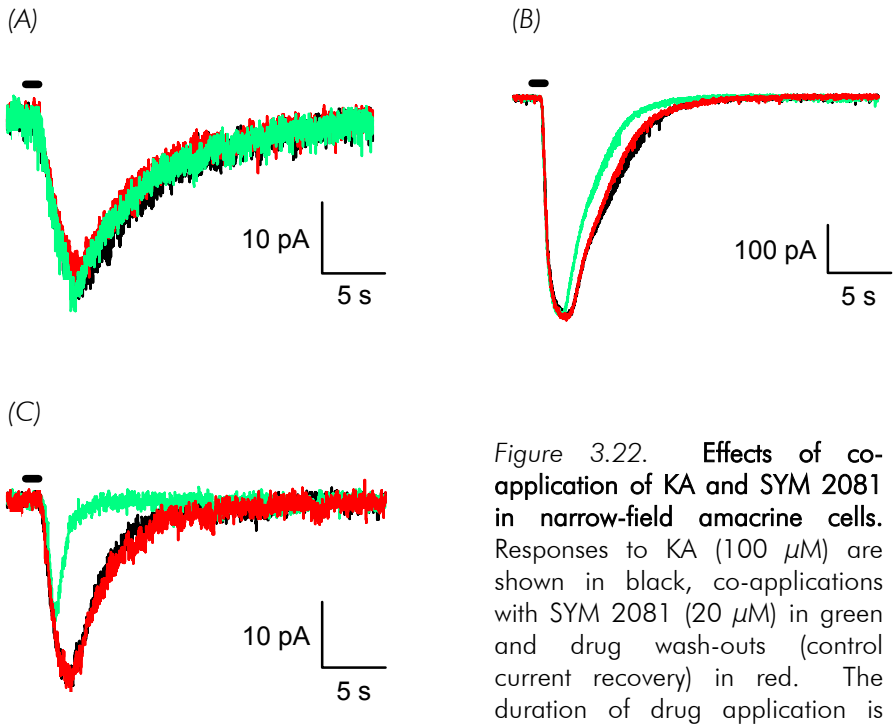

Figure 3.22. **Effects of co-application of KA and SYM 2081 in narrow-field amacrine cells.** Responses to KA (100 μM) are shown in black, co-applications with SYM 2081 (20 μM) in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

(A) No change in the KA-induced current can be noticed in the presence of SYM 2081.

(B) Although the peak amplitude remained unchanged, a faster deactivation of the current in the presence of SYM 2081 determined an overall current drop of 26% in comparison with the control (τ_{KA} = 4,6 s, $\tau_{KA+SYM2081}$ = 2,7 s).

(C) Strong effect of SYM 2081. In the presence of the drug, the peak amplitude was reduced to 66% and the overall current to 22% of the control value. The deactivation time of the KA-induced current decreased significantly (τ_{KA} = 2,6 s, $\tau_{KA+SYM2081}$ = 0,8 s).

In the case of SYM 2081 experiments with co-application, integrals were thought to best reflect the effects of the drug and were therefore chosen as only parameter for quantification of the results (*Figure 3.24.*). As in the previous sections, the data is given as drug response/ control current ratio and shown in percents. Values under 100% show a reduction of the current at the drug application, while 100% indicates no effect of the drug.

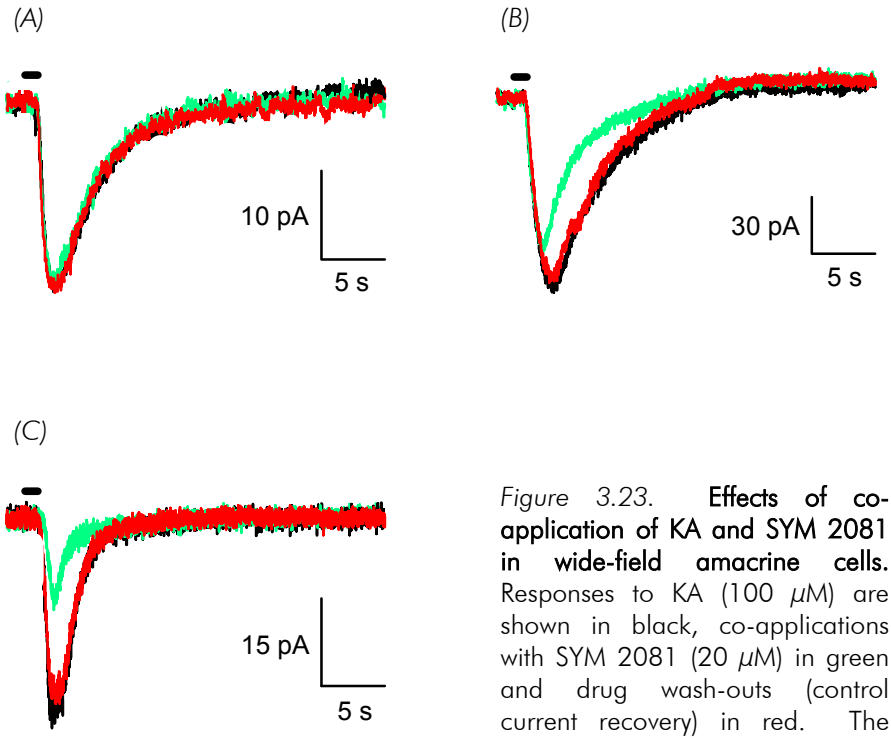

Figure 3.23. **Effects of co-application of KA and SYM 2081 in wide-field amacrine cells.** Responses to KA (100 μM) are shown in black, co-applications with SYM 2081 (20 μM) in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

(A) No change in the KA-induced current can be noticed in the presence of SYM 2081.

(B) Application of SYM 2081 determined a 20% drop in the peak amplitude and an overall current reduction of 48% in comparison with the control. In the presence of the drug, the deactivation time of the KA-induced current was also decreased (τ_{KA} = 5,5 s, $\tau_{KA+SYM2081}$ = 3,1 s).

(C) Strong effect of SYM 2081. The peak amplitude was reduced to 44% and the overall current to 33% of the control value, while the deactivation time of the KA-induced current did not change significantly (τ_{KA} = 1,9 s, $\tau_{KA+SYM2081}$ = 1,5 s).

The AII cells reacted to application of SYM 2081 with a 40 ± 4% decrease in the KA-activated current, on average. There was not much variation among the cells tested (n = 3) (*Figure 3.24., lower panels*). Not the same can be claimed about the narrow-field (n = 24) and wide-field groups (n = 16). A large individual variability could be observed among the cells of the two samples (*Figure 3.24., lower panels*). Their responses to SYM 2081 spanned all the way from no alteration to profound reduction of the KA-induced current. However, most narrow-field cell responses concentrated in the moderate range of effects.

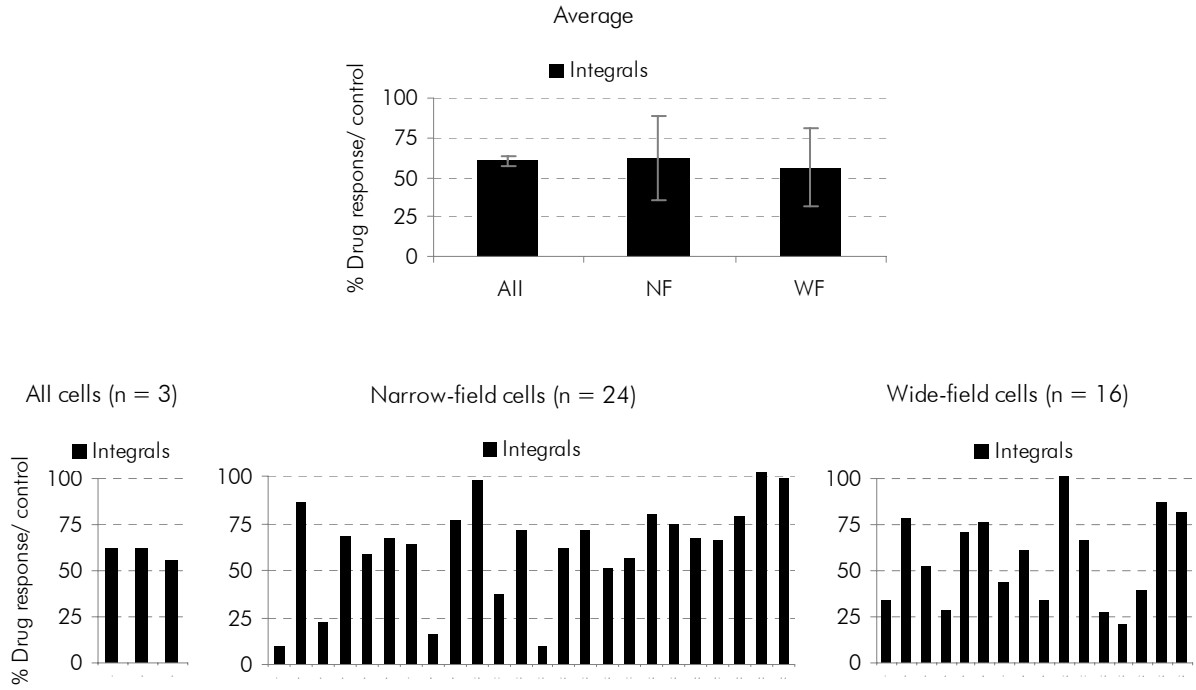

Figure 3.24. **Quantitative analysis of the effects of SYM 2081 co-applied with KA in narrow- (NF) *vs.* wide-field (WF) amacrine cells.** Integrals have been measured for the drug response and the control current. The values obtained for the drug were then divided by those obtained for the control and the ratio is shown in percents.

Up: Mean values calculated for the different cell classes. In the AII cells (n = 3), the overall current dropped by 40 ± 4% on average in the presence of SYM 2081. Narrow-field cells (n = 24) responded also with an average current reduction of 38 ± 27%. A slightly lower mean value, of 34 ± 25% was calculated for the wide-field cells (n = 16).

Down: Individual values measured for each cell in the three classes. The values measured for the AII group were rather similar among different cells. A considerable variability is to be noticed among the narrow- and wide-field cells, whose responses to SYM 2081 covered the whole spectrum of effects.

Various effects of SYM 2081 have been recorded also in the GlyT2-EGFP mouse (*Figure 3.25.*). 5 glycinergic and 2 GABAergic cells were tested with the drug co-applied with the basic agonist. 2 glycinergic cells did not react to application of SYM 2081, whereas the other showed a moderate reduction of the KA-activated current in the presence of the drug (*lower panels*). The 2 GABAergic cells tested reacted very differently, showing a current decrease of 20% and 60%, respectively (*lower panels*).

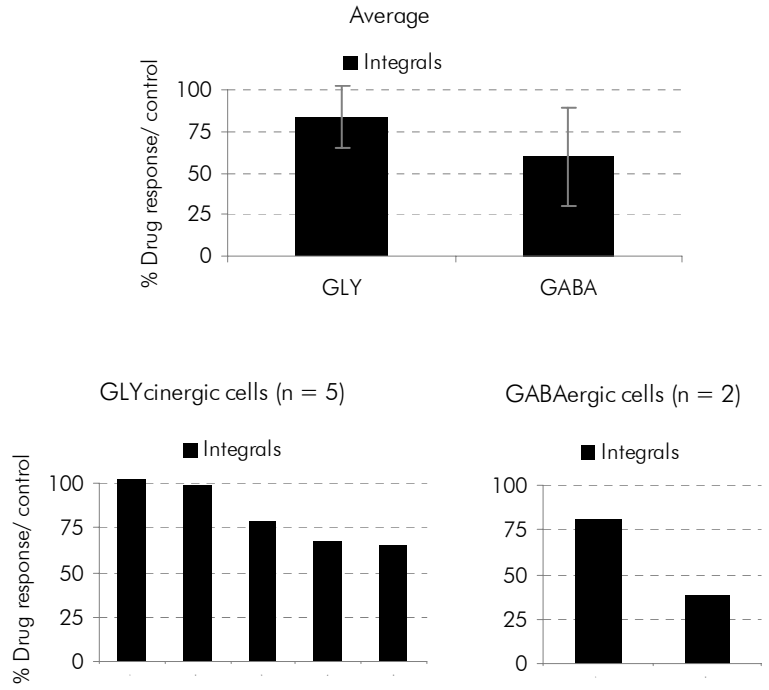

Figure 3.25. **Quantitative analysis of the effects of SYM 2081 co-applied with KA in glycinergic- (GLY) *vs.* GABAergic (GABA) amacrine cells.** Integrals have been measured for the drug response and the control current. The values obtained for the drug were then divided by those obtained for the control and the ratio is shown in percents.

Up: Mean values calculated for the two cell classes. In the glycinergic cells (n = 5), the overall current dropped on average to 83 ± 19% of the control value in the presence of SYM 2081. A mean value of 60 ± 30% was calculated for the GABAergic cells tested (n = 2).

Down: Individual values measured for each cell in the two classes. Some variability is to be noticed among the glycinergic cells, while the two GABAergic cells showed rather different responses in the presence of SYM 2081.

It was difficult to settle a clear limit defining the minimum amount of current reduction that would undoubtfully indicate presence of KA receptors in a cell. Following careful analysis of the modifications in the decay time constants and confrontation of KA plus SYM 2081 experiments with GYKI experiments, a value of 10% was found to be a fair estimation. 10% current reduction at application of SYM 2081 was thus considered evidence of a minimal KA activity in the cell.

A good correlation generally existed between the effects of SYM 2081 and GYKI compounds applied in the same cell (*Figure 3.26.*). Out of 25 cells tested for both drugs, only 4 showed slightly contradictory effects of SYM 2081 and GYKI and they were excluded from the further analysis. In all other cells, strong current reduction at application of SYM 2081 was correlated with weak or no inhibition by GYKI, while moderate effects of one drug were accompanied by moderate effects of the other.

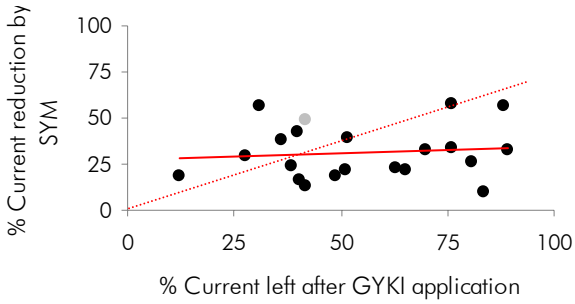

Figure 3.26. **Scatter diagram comparing the effects of SYM 2081 and GYKI in the same cell (n = 21).** On the abscissa the amount of current reduction by SYM is shown; on the ordinate the amount of current left after GYKI application is given (in percents). For example, in the cell plotted in grey the KA-evoked current was reduced by 48% in the presence of SYM, while 42% of the current was not affected by GYKI. The calculated trendline is shown as solid red line, while the ideal trendline as dotted red line.

Experiments were done also with SYM 2081 applied alone. SYM 2081 is able to open KA receptors on its own, the induced desensitization is however fast and profound. This accounts for the small activated currents, whenever currents could be at all seen. Currents could be elicited in many cells (n = 42), while still in a significant proportion of the sample no effect of the drug could be detected (n = 36).

Figure 3.27. shows examples of SYM 2081 responses in two narrow-field and two wide-field cells. The small amplitudes of the SYM 2081-induced currents (right side) reflect the average value of the currents measured among the cells of the two populations. Currents ranging from a few to 50 pA amplitude have been recorded in different cells. Currents of different magnitudes do not reflect different amounts of KA receptors expressed by the cells. They correlate with the amplitudes of the other agonist-induced currents (AMPA- and KA-) and are all the result of the quality of the recording and the time elapsed from the

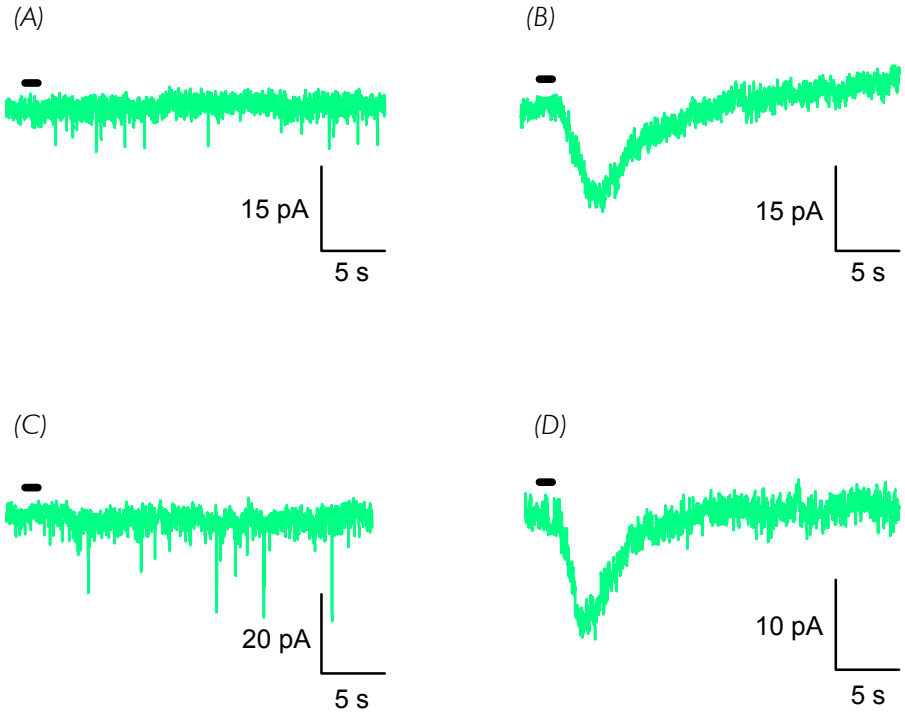

Figure 3.27. **Effects of SYM 2081 in narrow-field and wide-field amacrine cells.** Responses to application of 100 μM SYM 2081 are shown in green. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).
(A) Example of a narrow-field cell where application of SYM 2081 alone had no effect.
(B) Example of a narrow-field cell in which SYM 2081 induced a 15 pA large current.
(C) Example of a wide-field cell where SYM 2081 had no effect.
(D) Example of a wide-field cell in which SYM 2081 induced a 12 pA large current.

beginning of the experiment until the agonist application (rundown has been discussed in one of the previous sections).

Because of the fast and potent desensitization that SYM 2081 causes at KA receptors, there is a risk of accumulating false negative results when interpreting the experiments where the drug triggered no visible current. No detectable current can mean indeed no effect of the drug, but it might be possible in some cases that the amount of SYM 2081 applied was enough to completely desensitize all KA receptors expressed in the cell. Thus, many cells where SYM 2081 had no apparent effect when applied alone, showed a clear reduction of the KA-induced current when the drug was tested by co-application with the basic agonist. In order to exclude possible false negatives, only those negative results

were considered, which could be confirmed by other experiments (GYKI or KA plus SYM 2081). All positive results were included in the analysis.

Figure 3.28. summarises the results of the SYM 2081 experiments for the different cell groups analysed. In coloured bars, the percentage of the tested cells which responded to the drug is shown, while the grey domains correspond to the cells in which no effect of the drug was seen. Thus, less than half of the AII cells tested (n = 18), almost 70% of the narrow-field (n = 25) and 60% of the glycinergic cells (n = 7) exhibited SYM 2081-induced currents. In a slightly higher proportion, circa 75% of the wide-field (n = 19) and 70% of the GABAergic cells recorded (n = 6) were also responsive to the application of the drug.

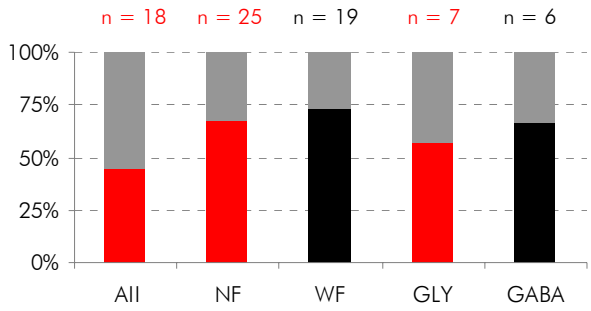

Figure 3.28. **Statistics of the SYM 2081 experiments.** Coloured bars indicate the percentage of cells responsive to SYM 2081, while grey domains stand for the cells where the drug had no effect. 44% of the AII (n = 18), 68% of the narrow-field (n = 25) and 57% of the glycinergic cells showed responses following application of SYM 2081. A majority of 74% wide-field (n = 19) and 67% GABAergic cells (n = 6) were also responsive to the drug.

On basis of the GYKI and SYM 2081 experiments, the expression of KA receptors among different cells could be evaluated. The results are summarised in the diagram of *Figure 3.29*. 14 AII, 43 narrow-field, 26 wide-field, 16 glycinergic and 6 GABAergic cells were included in the statistics. Out of them, 3 AII cells and only 3 narrow-fields and 1 glycinergic amacrine did not show KA receptor activity when tested with GYKI and SYM 2081. Thus, not only AMPA, but also KA receptors seem to be ubiquitously distributed on narrow-field and glycinergic cells. Positive results were also obtained for the majority of the wide-field (85%) and all GABAergic cells tested for KA receptors.

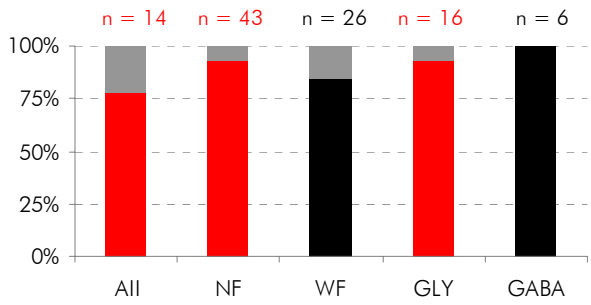

Figure 3.29. **Statistics of KA receptor expression among different cell classes.** Coloured bars indicate the proportion of cells that express AMPA receptors; grey domains stand for the percentage of cells that do not possess AMPA receptors. Most AII cells (79%, n = 14) and the great majority of narrow-field (93%, n = 43) and glycinergic cells (94%, n = 16) were found to express KA receptors. Likewise, KA receptors were identified in 85% of the wide-field (n = 26) and all GABAergic cells tested (n = 6).

3.2.4. AMPA and KA receptor co-expression

AMPA and KA receptors have been found in almost all cells tested for AMPA and KA receptors, respectively. The data sets accounting for expression of AMPA or KA receptors in different cell groups are not identical and the question arises whether the two receptor types are co-expressed in a cell or rather distributed on different cells making up a population.

Both cases have been encountered among the cells analysed (n = 65). *Figure 3.30.* shows an example of a cell expressing only AMPA receptors. The cell was tested with both AMPA receptor and KA receptor selective drugs. CTZ enhanced the overall AMPA-induced current by more than 30%, while the peak amplitude increased by 67% in the presence of the drug (*panel A*). Application of GYKI 52466 led to a strong inhibition of the agonist-

induced current, which was reduced to 8% of the initial magnitude (*panel B*). SYM 2081 had no effect on the KA-activated current (*panel C*) and was also not able to elicit any current when applied on its own (*panel D*). Thus, all the results support presence of AMPA, but not KA receptors in the cell.

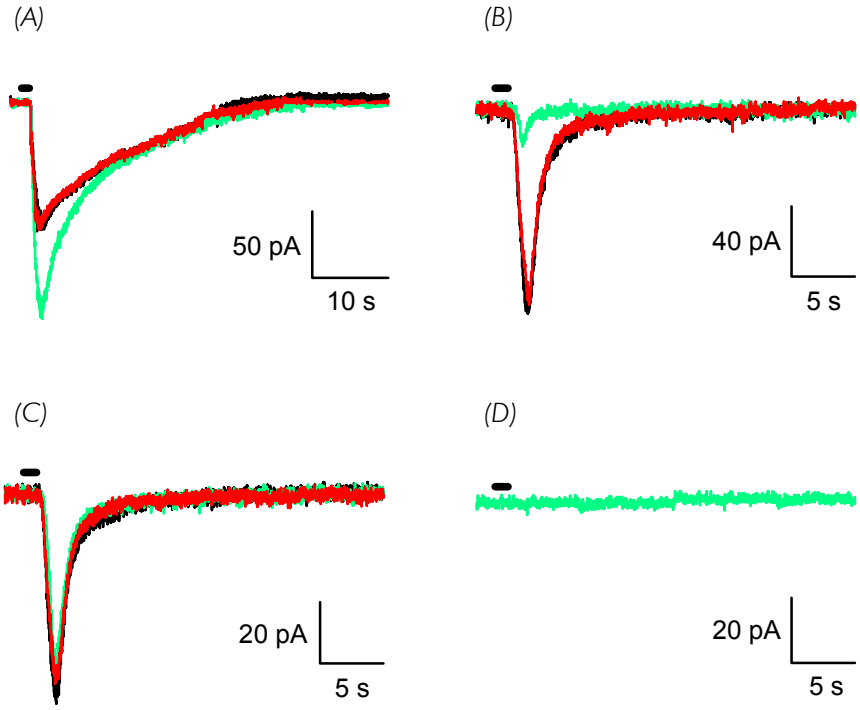

Figure 3.30. **Example of a cell expressing only AMPA receptors.** Responses to basic agonists (AMPA and KA) are shown in black, drug applications in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).
(A) 100 μM CTZ produced an increase of 68% in the peak amplitude and 30% in the overall AMPA-induced current.
(B) 20 μM GYKI 52466 almost completely inhibited the KA-induced current. The peak amplitude was reduced to 18% and the overall current to 8% of the control value.
(C) Co-application of 100 μM KA and 20 μM SYM 2081 had no effect on the control current.
(D) 50 μM SYM 2081 could not elicit any current in this cell.

Cells expressing KA receptors only have also been recorded. Such an example is illustrated in *Figure 3.31.*. No effect of the AMPA receptor selective drugs could be observed in this cell. AMPA- and KA-induced currents remained unaffected at the application of CTZ (*panel A*) and GYKI 52466 (*panel B*). SYM 2081 caused a significant reduction of the KA-induced current, when co-applied with the agonist (*panel C*). The response in the presence of the drug showed an almost two fold faster deactivation and

was overall 70% smaller than the control current. SYM 2081 could also elicit a small current when applied alone (*panel D*).

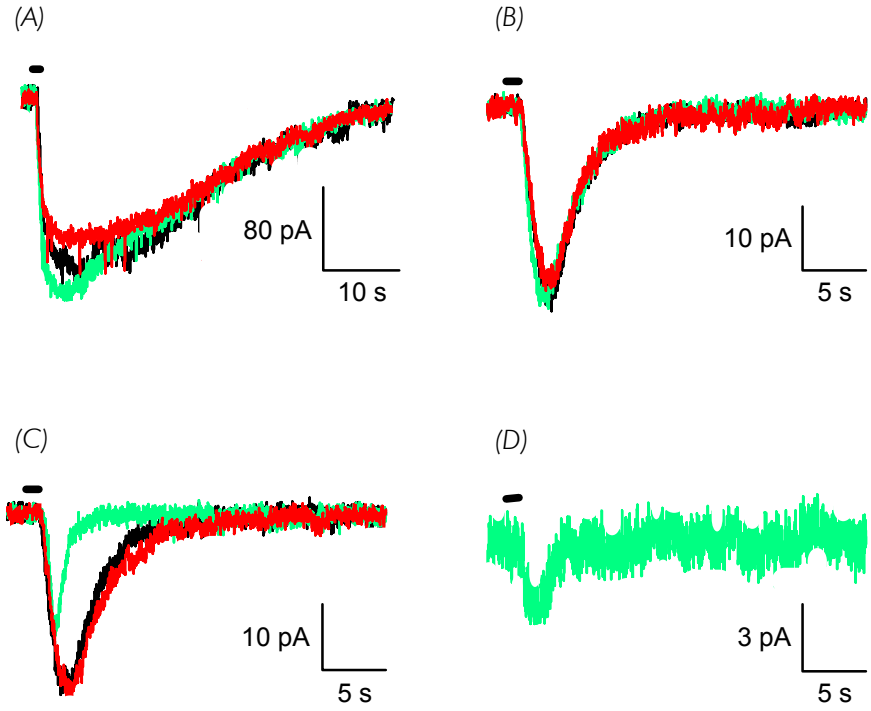

Figure 3.31. **Example of a cell expressing only KA receptors.** Responses to basic agonists (AMPA and KA) are shown in black, drug applications in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).
(A) 100 μM CTZ produced only a slightly increase the peak amplitude of the AMPA-induced current.
(B) 20 μM GYKI 52466 had no effect on the KA-induced current.
(C) Co-application of 100 μM KA and 20 μM SYM 2081 led to a clear reduction of the control current. The peak amplitude was reduced to 66% and the overall current to 22% of the initial value. The deactivation time of the KA-induced current decreased significantly (τ_{KA} = 2,6 s, $\tau_{KA+SYM2081}$ = 0,8 s).
(D) 50 μM SYM 2081 could elicit a small current in this cell.

Although clear cases of cells expressing only one type of non-NMDA receptor could be constantly seen during the experiments, the majority of the cells tested seemed to express both. *Figure 3.32.* shows an example of a cell expressing both AMPA and KA receptors. Application of CTZ in this case triggered a current of almost double peak amplitude, which was overall 78% larger than the control (*panel A*). GYKI 52466 had a strong effect on the KA-induced current, however with an incomplete block leaving a residual current equal to 20% of the control current (*panel B*). The assumption that KA receptors might be

responsible for the remaining current was confirmed by the SYM 2081 experiments. In the presence of SYM 2081, the KA-induced current was 30% smaller and deactivated 1,5 times faster (*panel C*). A small current could be detected also at the application of SYM 2081 without the basic agonist (*panel D*).

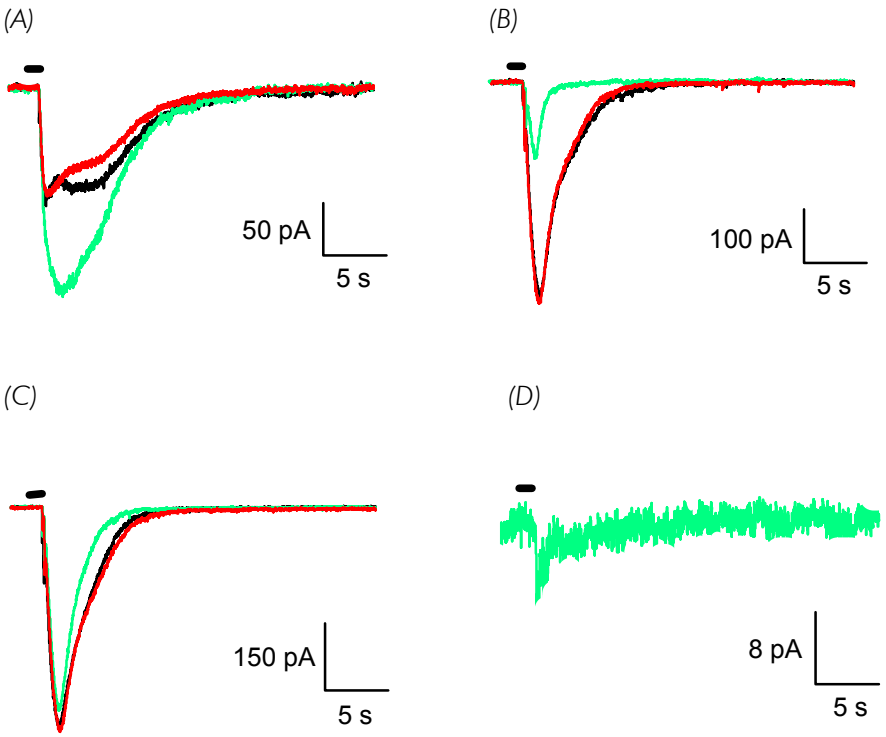

Figure 3.32. **Example of a cell expressing both AMPA and KA receptors.** Responses to basic agonists (AMPA and KA) are shown in black, drug applications in green and drug wash-outs (control current recovery) in red. The duration of drug application is indicated by the horizontal bar above the recordings (1 s).

(A) In the presence of 100 μM CTZ, the peak amplitude was increased by 86% and the overall current by 78%. The drug had an effect also on the kinetics of AMPA-induced current.

(B) 20 μM GYKI 52466 caused the peak amplitude to drop to 36% and the overall agonist-induced current to 20% of the control value.

(C) Co-application of 100 μM KA and 20 μM SYM 2081 did not change the peak amplitude much. However, a faster deactivation of the current in the presence of the drug caused an overall current drop of 23% in comparison with the control (τ_{KA} = 2,4 s, $\tau_{KA+SYM2081}$ = 1,6 s).

(D) 50 μM SYM 2081 could elicit a small current in this cell.

A total of 65 cells have been tested for both AMPA and KA receptors, out of which 43 were found to posses both types. The other 22 were equally split between expression of exclusively AMPA or KA receptors. The distribution of the non-NMDA receptor expression patterns among the different cell groups analysed is shown in *Figure 3.33.*.

Only less than one third of the AII amacrine cells tested (n = 13) showed no indication of KA receptors, most of them seemed to possess both receptor types. The same holds true for the other narrow-field cells tested. In this group (n = 27), 2 cells were found to have AMPA receptors only and 6 to express exclusively KA receptors, while the majority of the cells seemed to possess both. On the contrary, in most of the wide-field amacrines (almost 60%) only one type of non-NMDA receptors could be identified. Thus, out of 14 cells 5 were found to express AMPA and 3 KA receptors only.

In the GlyT2-EGFP mouse, no cells have been recorded that showed just AMPA receptors. In both glycinergic (n = 16) and GABAergic cells tested (n = 6), mostly double expression of both non-NMDA receptors has been found. Almost 20% of the glycinergic cells and one third of the GABAergic cells seemed to possess solely KA receptors.

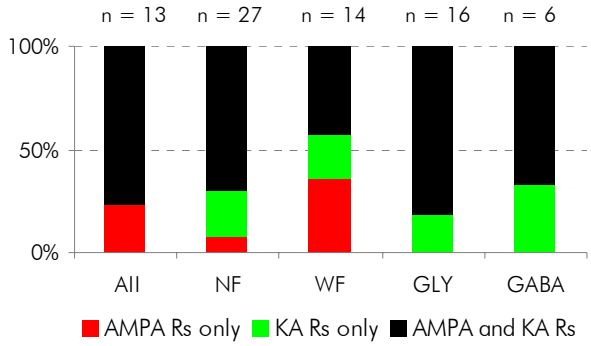

Figure 3.33. **Statistics of AMPA and KA receptor co-expression among cells of different classes.** Red bars indicate the proportion of cells tested for both non-NMDA receptor types that were found to express AMPA receptors only; green bars stand for the percentage of cells that possess KA receptors exclusively; black bars indicate the proportion of cells where AMPA and KA receptors are co-expressed. Most AII cells (77%, n = 13), as well as most narrow-field (70%, n = 27) and glycinergic cells (81%, n = 16) were found to express both AMPA and KA receptors. Only in 3 AII cells (23%) and 2 narrow-field amacrines (7%) exclusively AMPA receptors could be identified. 22% of the narrow-field and 19% of the glycinergic cells tested were found to express KA receptors only. The non-NMDA receptor expression profile of GABAergic cells (n = 6) was similar to that of the glycinergic cells; a majority of 67% of the cells tested possessed both AMPA and KA receptors, while 33% showed only KA activity. On the contrary, only less than half of the wide-field amacrines (43%, n = 14) were found to co-express both receptor types, most of them having solely AMPA (36%) or KA receptors (21%).

4. DISCUSSION

4.1. Isolation of currents mediated by different glutamate receptors. A study of qualitative nature.

The expression patterns of ionotropic glutamate receptor among different amacrine cells in the mouse retina have been investigated in this study. Expression of NMDA and non-NMDA receptors was evaluated pharmacologically, using the patch-clamp technique in the whole-cell configuration. Selective agonists and antagonists of NMDA, AMPA and KA receptors have been used in order to determine which of the different receptor types might mediate the responses to the endogenous neurotransmitter glutamate.

However, experiments testing the effects of various drugs on whole-cell currents evoked by basic receptor agonists do not allow discrimination between activity of synaptic and extrasynaptic receptors (refer to *Outlook*). Such experiments are useful to identify *all* receptors expressed by a cell, independent of their localization. Moreover, pharmacology does not permit precise quantification of the contribution of each receptor type to the agonist-evoked current (see below).

Experiments with application of L-glutamate, the endogenous agonist, were not performed in this study. Expression of NMDA and non-NMDA receptors was directly tested with the selective agonists NMDA and AMPA/ KA, respectively. It was therefore not possible to estimate the ratio of NMDA to non-NMDA receptors expressed by one cell. The expression of NMDA receptors was tested by application of the selective agonist NMDA, in the presence of small amounts of the co-agonist glycine. Magnesium ions were excluded from the solution, in order to prevent blockage of the NMDA receptors, and strychnine was added for preventing activation of glycine receptors. All cells in which application of NMDA under these conditions evoked a response were considered to possess glutamate receptors of NMDA type.

Expression of non-NMDA receptors was tested by application of AMPA and KA. Although AMPA receptors show a higher affinity for AMPA and KA receptors for KA, each of the two agonists is able to activate both receptor types. Receptor selective drugs were therefore employed to discriminate between AMPA and KA receptor-mediated responses. Presence of AMPA receptors was tested using cyclothiazide and two benzodiazepine

compounds (GYKI 52466 and GYKI 53655), while presence of KA receptors was investigated using (2R,4S)-4-methylglutamic acid (SYM 2081).

Cyclothiazide: Initially described as a diuretic agent (*Julius et al. 1962*), cyclothiazide (CTZ) was shown in the early 90s to modulate AMPA receptors by removing their fast desensitization (*Yamada and Rothman 1992*). Application of CTZ leads to an increase in the AMPA receptor-mediated current, accompanied by a prolongation of its decay time. 100 μM CTZ were used in our experiments, co-applied with the basic agonist AMPA (100 or 200 μM). The effects obtained in different cells ranged from no modification to more than 3-fold increase of the AMPA-evoked current in the presence of CTZ. Reports on the strength of the CTZ effect on AMPA receptors vary largely among studies using different methods and systems. Zorumski et al. (*1993*) were the first to test the drug, in whole-cell patch-clamp recordings on cultured rat hippocampal neurons. They found that 10 μM locally applied CTZ produced a more than 5-fold increase in the steady-state currents evoked by 1 mM glutamate. Using the same experimental set-up, Mayer and colleagues (*Wong and Mayer 1993, Patneau et al. 1993*) found that 100 μM CTZ increased KA-evoked currents by 3 times at high agonist concentrations (1 mM), but by 29,5 times at low concentrations (20 μM). Another study (*Bertolino et al. 1993*) reported that bath application of 100 μM CTZ produced a 16-fold potentiation of the glutamate-evoked current in rat hippocampal slices, at an agonist concentration of 1 mM. Partin et al. (*1993*) investigated the effects of CTZ on recombinant receptors expressed in *Xenopus* oocytes. They found that co-application of 100 μM KA and 100 μM CTZ produced a 8- to 12-fold potentiation of the control current in homo- and heteromeric receptors assembled from *flip* AMPA subunits, whereas *flop* variants showed much smaller values (4-fold). CTZ preference for *flip* variants was confirmed by Sekiguchi et al. (*1998*), who also showed that 4-[2-(phenylsulphonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide (PEPA) preferentially potentiates responses of *flop* variants (*Sekiguchi et al. 1997, 1998*) and that the ratio of PEPA to CTZ effects is a good parameter for estimating the relative abundance of the two splice variants expressed in a cell. In another study performed on *Xenopus* oocytes, Blanschke et al. (1997) found that neuronal and recombinant glutamate receptors differ markedly in their pharmacological properties (6,5 to 14-fold potentiation by CTZ in recombinant receptors, *flop* variants, *vs.* only 1,4 to 6-fold in neuronal receptors).

Thus, the results of our CTZ experiments indicated whether the cells tested possessed AMPA receptors or not, but could not provide quantitative information about the amount of receptors expressed.

GYKI 52466 and GYKI 53655: We further tested the cells with GYKI 52466 or GYKI 53655, selective blockers of AMPA receptors (*Tarnawa et al. 1989, 1993*). The effects obtained in different cells ranged from no modification to virtually complete block of the agonist-evoked current in the presence of the drug. It is known that GYKI 53655 is a more potent blocker of AMPA receptors than GYKI 52466. Both compounds block AMPA receptors in a non-competitive manner, independent of the agonist concentration. IC_{50} values were calculated for the two drugs and found to be 1 μM for GYKI 53655 and 18 μM for GYKI 52466 in cultured neurons from the rat cerebral cortex and dorsal root ganglions (*Wilding and Huettner 1995*). Earlier studies on cultured rat hippocampal neurons reported an IC_{50} value of 11 μM for GYKI 52466, when used with KA as an agonist (*Donevan and Rogawski 1993*) and more than 70% inhibition of KA-evoked currents by 25 μM drug (*Zorumski et al. 1993*). 20 μM of each of the two compounds were used in our experiments, co-applied with the basic agonists AMPA or KA (100 or 200 μM). While at this concentration GYKI 53655 completely blocks transmission at AMPA receptors, the weaker form 52466 could leave some residual current. Currents left after application of AMPA receptor blockers can be interpreted as being mediated by KA receptors. In order to exclude the possibility of false positive results, a security limit was imposed for GYKI 52466, so that only cells in which at least 25% of the control current was left at the drug application were considered to express KA receptors. Moreover, there was always a good correlation between the effects of CTZ and GYKI in the same cell.

The results of our GYKI experiments indicated whether the cells tested possessed AMPA receptors, KA receptors, or both, but could not provide quantitative information on the relative expression of the two receptor types. Depending on subunit composition, which underlies the receptor functional properties, different activation patterns and reactions to drugs can be expected. Very recently, Shen et al. (*2004*) investigated the glutamate receptors expressed in human retinal horizontal cells. They found that, at a concentration of 25 μM, GYKI 52466 totally blocks the current evoked by AMPA (100 μM), but produces only 20% inhibition of the KA-evoked (100 μM) current. On the contrary, in all cells that we tested with both agonists, the KA-activated current was more sensitive to inhibition by GYKI than the AMPA-activated current. This may reflect a different glutamate receptor

expression pattern in the two systems, as well as a different proportion of AMPA to KA receptor subtypes.

SYM 2081: We also tested the cells with a KA-receptor selective drug. SYM 2081 is an agonist which specifically inhibits activity at KA receptors (*Gu et al. 1995, Donevan et al. 1998*), by inducing fast and profound desensitization (*Zhou et al. 1997*). Zhou and co-workers (*1997*) tested SYM 2081 on recombinant GluR6 receptors and found that 30 nM drug, pre-applied in the bath, reversibly blocked KA-induced currents, whereas higher concentrations (3 μM) were able to elicit rapidly desensitizing currents. In cultured rat hippocampal neurons, Wilding and Huettner (*1997*) found that 1 μM SYM 2081 inhibited KA receptor-mediated currents by almost 90%, while higher concentrations evoked rapidly desensitizing currents ($EC_{50} \sim 50$ μM). We used 50 μM SYM 2081 for separate application and 20 μM for co-application with the basic agonist (100 or 200 μM KA). Just like for the AMPA-receptor selective drugs, SYM 2081 had various effects in different amacrine cells, ranging from no modification to profound reduction of KA-evoked responses. In some cells, the drug was able to elicit small currents when applied on its own. Generally, the results of the SYM 2081 experiments correlated well with the GYKI experiments.

SYM 2081 experiments indicated whether the cells tested possessed or not KA receptors, but could not provide quantitative information about the amount of receptors expressed.

Taken together, the pharmacological experiments allowed us to identify the ionotropic glutamate receptor types expressed in different cells. Due to the various effects that drugs can have on different receptor subtypes and the different pharmacological properties of neuronal *vs.* recombinant receptors, accurate quantitative evaluation is not yet possible in *in-vivo* pharmacology.

4.2. Differential expression of ionotropic glutamate receptors among amacrine cells in the mouse retina.

Although glutamate receptors play a key role in filtering the information that amacrine cells feed into the retinal network, few studies have been dedicated to the investigation of the receptor types expressed by different subclasses of these important interneurons. The great diversity of amacrine cell types and the lack of specific markers that could be used to

selectively label them make this pursuit difficult. Here we investigated and identified the ionotropic glutamate receptors expressed by AII, narrow-field, wide-field, glycinergic and GABAergic amacrine cells in the mouse retina.

4.2.1. Ionotropic glutamate receptors of AII amacrine cells

The most intensively studied amacrine cell type has been for a long time the AII, the cell linking rod and cone pathways in the retina. Thanks to their characteristic morphology (*Figure 3.1. A, Results*), AII cells can be easily identified even in unstained preparations and could be described as a group of their own in the present study.

It has been previously shown that AII amacrine cells of different species express NMDA and AMPA receptors. Hartveit and Veruki (*1997*) were the first to report presence of NMDA receptors on AII cells of the rat retina, after an earlier study (*Boos et al. 1993*) reported the opposite. Following application of large amounts of NMDA (500 and 1000 μM) at depolarizing holding potentials (20 to 60 mV), they were able to evoke currents in 13 out of 15 cells tested. Another group (*Shen et al. 1999*) reported that NMDA could induce no current in isolated carp AII-like amacrine cells. Bloomfield and Xin (*2000*) found that blocking NMDA receptors with the antagonist MK-801 reduces the amplitude of the rabbit AII amacrine cell on-center light response by 50 to 70%. Expression of NMDA receptors by AII cells of the rabbit retina was then confirmed by Zhou and Dacheux (*2004*), who showed clear NMDA-mediated currents in these cells.

We could clearly identify non-NMDA-mediated currents in all AII cells tested, but only few cells responded to application of NMDA. Although we eliminated magnesium ions and always added the co-agonist glycine, we were able to evoke NMDA-responses only in 3 out of 22 AII cells. The 3 positive results were obtained in a special series of experiments, in which the cells (n = 12) were tested immediately after establishing the whole-cell configuration, to exclude any run-down of intracellular compounds, with high agonist concentrations (500 μM) and at depolarizing holding potentials.

With regard to non-NMDA glutamate receptors, the evidence in the literature supports expression of only AMPA receptors in the AII amacrine cells. Several immunocytochemical studies showed GluR2/3 and GluR4 antibody staining associated with AII amacrine cells in different retinae (*Qin and Pourcho 1999b*, cat; *Ghosh et al. 2001*, monkey and rabbit; *Li et al. 2002*, rabbit). A study on isolated carp AII-like cells (*Shen et al. 1999*) reported

that glutamate-induced responses in these cells could be completely blocked by 10 μM GYKI 53655. Hartveit and co-workers also reported that GYKI 53655 (100 μM) blocked the steady-state component of AMPA- and KA-evoked currents in rat AII amacrine cells (*Mørkve et al. 2002*). The same group found that concanavalin A had no effect on the KA-evoked currents (*Mørkve et al. 2002*) and SYM 2081 (2 μM) was unable to block spontaneous postsynaptic currents in these cells (*Veruki et al. 2003*). EPSCs could be instead blocked by 25 μM GYKI 52466 (*Singer and Diamond 2003*). Very recently, Zhou and Dacheux (*2004*) obtained the same results for the AII cells in the rabbit retina. AMPA- and KA-activated currents could be blocked by GYKI 53655 (40 and 200 μM, respectively), while 40 μM SYM could not elicit any response.

We found both AMPA and KA receptors in the AII cells of the mouse retina. In all cells tested (n = 8), CTZ induced a clear potentiation of the AMPA-evoked current, of 240 ± 34%, even larger than previously found in the rat (134 ± 28%, *Mørkve et al. 2002*). GYKI 52466 (20 μM) only partially blocked KA-evoked responses, leaving 52 ± 4% of the control current unaffected (n = 5). Even in the presence of the more potent variant, GYKI 53655 (20 μM), 40 ± 12% of the KA-evoked current remained unblocked (n = 3). In fact, Mørkve et al. (*2002*) also observed that even at high concentrations of GYKI 53655 (100 μM), which strongly reduced or blocked the steady-state response to AMPA or KA, the agonist evoked an initial transient peak, possibly mediated by GYKI-insensitive receptors. When tested with SYM 2081 (20 μM) co-applied with KA, the cells (n = 3) responded with 40 ± 4% inhibition of the KA-evoked currents. Also application of 50 μM SYM 2081 could elicit responses in 8 out of 18 AII cells tested. Taken together, our experiments provided evidence of AMPA receptor expression in virtually all AII cells (94%, n = 17), as well as presence of KA receptors in most of the cells tested (79%, n = 14).

4.2.1. Ionotropic glutamate receptors of other amacrine cells

Many immunocytochemical and *in-situ* hybridization studies have reported expression of virtually all ionotropic glutamate receptor subunits in the inner plexiform layer (IPL) of different retinae (*Brandstätter et al. 1998*, *Thoreson and Witkovsky 1999* vertebrate; *Haverkamp and Wässle 2000* mouse; Qin *and Pourcho 1999 and 2000* cat; *Grünert et al. 2002* primate retina) and NMDA-, AMPA- and KA-receptor subunits have all been

identified on amacrine cells. Many electrophysiological studies also demonstrated presence of both NMDA and non-NMDA receptors in amacrine cells of a large variety of species (*Slaughter and Miller 1983*, *Coleman and Miller 1988,* mudpuppy; *Massey and Miller 1988*, *Zhou and Dacheux 2004,* rabbit; *Dixon and Copenhagen 1992*, *Tran et al. 1999*, salamander; *Boos et al. 1993*, *Menger and Wässle 2000,* rat; *Shen et al. 1999,* carp).

Amacrine cells make up the most heterogeneous group of all retinal neurons, both morphologically and physiologically. We wondered whether this diversity is based on a differential expression of glutamate receptors and took a comparative look at narrow-field *vs.* wide-field and glycinergic *vs.* GABAergic amacrine populations. As morphology is often a good predictor of function, our initial expectations were that different receptor expression patterns may exist between these cell groups.

An evident trend in our data relates to the expression of NMDA receptors. Out of 15 narrow-field and 2 glycinergic amacrine cells tested, only 7 narrow-field cells showed NMDA-mediated responses. In contrast, 10 out of 13 wide-field and 2 out of 3 GABAergic cells were found to express NMDA receptors. It has been previously shown in the rat retina that glycinergic amacrines have small dendritic trees (*Menger et al. 1998*), and it is believed that GABAergic amacrines are mostly wide-field cells. Thus, a good correlation exists between the results obtained for the two population categories and more wide-field/ GABAergic than narrow-field/ glycinergic cells tend to express NMDA receptors. This finding is in agreement with previous studies on amacrine cell light-evoked excitatory responses. In the tiger salamander retina, Dixon and Copenhagen (*1992*) found that transient amacrine cells express NMDA receptors, while sustained amacrine cells do not. In the same species, Maguire (*1999*) noted that the two functional cell types have a characteristic morphology, with transient amacrine cells having a wide dendritic field (χ = 375 μm) and sustained cells a narrow dendritic field (χ = 85 μm). Transient ON-OFF amacrine cells in the turtle retina also seem to possess NMDA receptors (*Vigh and Witkovsky 2004*).

Immunocytochemical results also support this notion. In the rat retina, Brandstätter et al. (*1994*) found that NR2A, NR2B and NR2C subunits are expressed by subsets of amacrine cells, while in the cat Goebel et al. (*1998*) found that all GABAergic and some other amacrines express NR2A. However, in the primate retina, Grünert at el. (*2002*) found that NR2A and NR1C2' subunits are concentrated in IPL sublaminae 2 and 4, where

glycinergic markers are prominently expressed. An early study on chick retinal cultures reported that GABAergic amacrine cells possess NMDA receptors (*Huba and Hofmann 1991*), while in the rabbit retina the most sensitive amacrine cells to NMDA were found to be GABAergic cells also (*Sun et al. 2003*).

Although NMDA receptors do seem to be expressed by certain amacrine cells, non-NMDA agonists are more potent, and while NMDA receptor blockers can only modify light-evoked responses of amacrine cells, non-NMDA receptor antagonists block them completely (*Slaughter and Miller 1983*, *Massey and Miller 1988*, *Dixon and Copenhagen 1992*). Our main finding concerning non-NMDA glutamate receptors is that they are ubiquitously, but heterogeneously expressed among individual cells making up different amacrine populations.

Most amacrine cells expressed AMPA receptors. Thus, 87% of the narrow-field (n = 45), 85% of the glycinergic (n = 20), 81% of the wide-field (n = 21) and 78% of the GABAergic cells tested (n = 9) showed AMPA receptor-mediated responses. AMPA receptor expression in amacrine cells of different retinae is well established, both by immunocytochemical (see *Introduction*) and physiological studies (*Maguire 1999, Tran et al. 1999,* salamander; *Shen et al. 1999*, carp; *Singer and Diamond 2003,* rat; *Firth et al. 2003,* rabbit; *Vigh and Witkovsky 2004,* turtle). In fact, most of these studies also reported that AMPA receptors are the dominant glutamate receptors of amacrine cells. In our sample, a great variability existed among the individual cell responses to AMPA receptor selective drugs. In each group we recorded cells where CTZ strongly potentiated the AMPA-evoked current and GYKI strongly inhibited it, cells in which the two drugs had very little effect, and many intermediary cases.

We found that most amacrine cells express KA receptors as well. Thus, 93% of the narrow-field (n = 43), 94% of the glycinergic (n = 16), 85% of the wide-field (n = 26) and all of the GABAergic cells tested (n = 6) showed KA receptor-mediated responses. Although KA receptor subunits have been found by immunocytochemistry throughout the IPL (*Haverkamp and Wässle 2000*, mouse) and have also been identified on some amacrine cells (*Brandstätter et al. 1994, 1997*, rat), to our knowledge, this is the first study to report KA receptor activity in amacrine cells. A big diversity of responses among cells of one population existed also in this case. Not only GYKI had different effects, also SYM 2081 showed a heterogeneous pattern, ranging from strong to no inhibition of KA-evoked currents in different cells.

The big individual variability among cells in narrow-field/ wide-field and glycinergic/ GABAergic groups indicates that the grouping applied is still too coarse and several different types of amacrine cells are grouped together, which express different sets of glutamate receptors. In the same direction points the work of Brandstätter and colleagues, who found that many glutamate receptor subunits, both ionotropic and metabotropic, are exclusively expressed by only one element at the bipolar cell dyads (*Hartveit et al. 1994, Koulen et al. 1996, 1997, Brandstätter et al. 1997*).

Few studies have been conducted to identify the non-NMDA glutamate receptors in particular amacrine cell types, with the exception of the AII cells. AMPA receptors are believed to mediate acetylcholine release from starburst amacrine cells in the rabbit retina (*Firth et al. 2003*), while they have been found to express NMDA receptors as well (*Zhou and Fain 1995*). In the rat retina, EPSCs recorded in AI amacrine cells could be potentiated by CTZ, pleading for involvement of AMPA receptors (*Singer and Diamond 2003*). AI amacrine cells are also suspected to express GluR6/7 and KA2 kainate receptor subunits (*Brandstätter et al. 1997*, cat), while ∂1/2 subunits were also found on their processes (*Ghosh et al. 2001,* monkey*; Li et al. 2002,* rabbit).

We found AMPA and KA receptors in almost all cells tested for AMPA and KA receptors, respectively. We were interested to know whether the two receptor types are co-expressed in individual cells of the same type or rather distributed on different cells making up a population. Among the cells tested for the two receptors (n = 65), we encountered both exclusive expression of AMPA or KA receptors and co-expression of the two types. Most narrow-field (70%, n = 27) and glycinergic cells (81%, n = 16) were found to have both AMPA and KA receptors. 22% of the narrow-field and 19% of the glycinergic cells were found to express KA receptors only, while 2 cases of narrow-field cells with just AMPA receptors have been encountered. The small sample of GABAergic cells (n = 6) showed a similar pattern to the glycinergic group: a majority of 67% of the cells had both AMPA and KA receptors, while the rest showed only KA receptor activity. Wide-field amacrine cells were different from all other groups, as only less than half of the cells (43%, n = 14) were found to co-express AMPA and KA receptors. Most wide-field cells were found to possess exclusively AMPA (36%) or KA receptors (21%).

4.3. Functional significance of heterogeneous expression of glutamate receptors among amacrine cells.

NMDA, AMPA and KA receptors differ not only in their pharmacology, but also in ionic selectivity and kinetic properties. NMDA receptors show high affinity for glutamate and extremely slow kinetics in comparison with AMPA and KA receptors, which share many common features, such as low binding affinity, fast activation, deactivation and desensitization. It is well established that many glutamatergic synapses express postsynaptically both AMPA and NMDA receptors. The relative roles of the two types in synaptic transmission depend on the relative rates of release and uptake of glutamate from the synapse. If the release is faster than uptake, than glutamate may remain in the synaptic cleft long enough to desensitize most of the AMPA receptors, while activating the slower NMDA receptors, which might then induce larger and longer lasting responses in the postsynaptic cell. Under normal conditions, AMPA receptors are activated and used for fast synaptic transmission. Due to their voltage sensitivity, NMDA receptors play an important role during coincident pre- and postsynaptic activity, which creates the basis for synaptic plasticity. In the retina, the fast AMPA receptor kinetics might be involved in the conversion of sustained light-evoked responses of bipolar cells into transient signals of amacrine cells. There is evidence that glutamate release at bipolar cell terminals is a sustained event (*Lagnado et al. 1996, Hartveit 1999*). Slow EPSCs are recorded in sustained amacrine cells, while very brief events are found in transient neurons (*Maguire 1999*). The brief light responses of transient amacrine cells are generated by the fast EPSCs, mediated by fast desensitizing AMPA receptors (*Maguire 1999*). On the other hand, NMDA receptors have been found on sustained amacrine cells (*Dixon and Copenhagen 1992*), findings also supported by our data.

KA receptors are also found at postsynaptic sites (reviewed by *Frerking and Nicoll 2000*), however their function in synaptic transmission is not clear. It is also not clear why AMPA should be preferred to KA receptors, as long as the two receptors share many functional properties. The only significant difference between the two types is the 10-fold faster recovery from desensitization of AMPA *vs.* KA receptors, however it is not known whether this difference is used by the synapses co-expressing the two receptors. EPSCs mediated by KA receptors have generally a slower kinetics than those mediated by AMPA receptors

(an exception however are the rapidly decaying EPSCs in the bipolar cells recorded by DeVries and Schwartz *1999*), which suggests different roles in temporal integration. A modelling study investigating the potential functions of AMPA and KA receptors on hippocampal interneurons (*Frerking and Ohlinger-Frerking 2002*) concluded that the two receptors differ indeed in their ability to encode temporal information. In the retina, only one case of KA receptor-mediated transmission has been reported. In the ground squirrel retina, DeVries (*2000*) showed that b3 OFF-bipolar cells express KA receptors, in contrast to b2 and b7 types which express AMPA receptors. B2 and b7 cells are therefore thought to transmit transient components of the cone signal, while b3 cells emphasize the steady components.

We found only a minority of cells exclusively expressing either AMPA or KA receptors. Most of the amacrine cells we recorded showed both AMPA and KA receptor-mediated activity. Since AMPA and KA receptors are not generally clustered at the same post-synaptic sites, a single amacrine cell which expresses both receptor types may provide inhibition with different temporal characteristics to individual synaptic partners.

AMPA receptor expression in AII amacrine cells is well established and strong evidence supports their localization on the arboreal dendrites, where the AII receives input for the rod bipolar cell. Mørkve and co-workers (*2002*) found Ca^{2+}-permeable AMPA receptors on AII amacrine cells, while Habermann (*2000*) reported that application of non-NMDA agonists evoke an increase of Ca^{2+} in the arboreal dendrites, but not in the lobular appendages of the cells. Meanwhile, GluR3 and GluR4 subunits were also found on the arboreal dendrites of AII amacrine cells in different retinae (*Qin and Pourcho 1999b*, cat; *Ghosh et al. 2001*, monkey and rabbit; *Li et al. 2002*, rabbit).

For the first time, we report the presence of KA receptors in AII cells of the mouse retina. Purely speculative, they might be localized on the lobular appendages, in the outer part of the IPL (*Brandstätter et al. 1994*), where AII amacrine cells receive input from OFF cone bipolar cells. AII cells receive here also input from dopaminergic amacrines and there is evidence that AII cells express D1 receptors coupled to adenylate cyclase (*Hampson et al. 1992*). Dopamine might modulate glutamate receptors by protein-kinase A (PKA) phosphorylation (reviewed by *Dowling 1991*). PKA can be activated by adenylate cyclase and the GluR6 subunit possesses a PKA phosphorylation site (*Raymond et al. 1993, Wang et al. 1993*). However, there is still no evidence that AII amacrine cells express GluR6,

although *in-situ* hybridization studies reported GluR6 staining in the outer part of the IPL (*Brandstätter et al. 1994*).

Several groups also reported NMDA receptor expression in AII amacrine cells in some species (*Hartveit and Veruki 1997,* rat*; Bloomfield and Xin 2000, Zhou and Dacheux 2004,* rabbit), while we could see NMDA receptor activity in only few cells and outside the physiological voltage range. It has been for some time suspected that these receptors are extrasynaptic. Very recently, it has been shown that NR1 subunit clusters are associated with connexin36 in some regions of the central nervous system in rat (*Rash et al. 2004*). It is well known that AII amacrine cells are coupled with each other and with ON-cone bipolar cells via gap junctions expressing connexin36 (*Massey et al. 2003*), it is therefore plausible that NMDA receptors of AII cells are involved in modulation of gap junction activity.

4.4. Outlook

Differential expression of glutamate receptors among individual amacrine cell types supports the idea that each of the numerous varieties of these neurons has a role of its own in the transmission of information through the inner retina. Further studies should be therefore targeted to specific amacrine types, trying to identify *individual* glutamate receptor expression patterns. While this is at present still difficult, we can hope that new markers will be soon found, which will allow selective labelling of different amacrine cells in immunocytochemistry or expression of the green fluorescent protein (GFP) under the control of specific promoters in transgenic animals.

Functional studies of glutamate receptors should also try to focus on identifying the synaptic receptors, which actually contribute to processing of glutamatergic input in the cells. Agonist-evoked responses do not allow such discrimination, but analysis of spontaneous or evoked synaptic activity is necessary in this case.

As in a single cell co-expressing several receptor types different receptors can mediate transmission at different synapses, receptor localization is important for understanding the circuitry and finally the function of the different amacrine cells. Immunostainings, calcium-imaging techniques (for identifying Ca^{2+}-permeable glutamate receptors) and pair recordings from pre- and postsynaptic neurons are useful approaches in this direction.

5. ABSTRACT

The mammalian retina contains around 30 morphological varieties of amacrine cell types. These interneurons receive excitatory glutamatergic input from bipolar cells and provide GABA- and glycinergic inhibition to other cells in the retina. Amacrine cells exhibit widely varying light evoked responses, in large part defined by their presynaptic partners. We wondered whether amacrine functional diversity is based on a differential expression of glutamate receptors among cell populations and types.

In whole cell patch-clamp experiments on mouse retinal slices, we used selective agonists and antagonists to discriminate responses mediated by NMDA/ non-NMDA (NBQX) and AMPA/ KA receptors (cyclothiazide, GYKI 52466, GYKI 53655, SYM 2081). We sampled a large variety of individual cell types, which were classified by their dendritic field size into either narrow-field or wide-field cells after filling with Lucifer yellow or neurobiotin. In addition, we used transgenic GlyT2-EGFP mice, whose glycinergic neurons express EGFP. This allowed us to classify amacrines on basis of their neurotransmitter into either glycinergic or GABAergic cells.

All cells (n = 300) had good responses to non-NMDA agonists. Specific AMPA receptor responses could be obtained from almost all cells recorded: 94% of the AII (n = 17), 87% of the narrow-field (n = 45), 81% of the wide-field (n = 21), 85% of the glycinergic (n = 20) and 78% of the GABAergic cells (n = 9). KA receptor selective drugs were also effective on the majority of the AII (79%, n = 14), narrow-field (93%, n = 43), wide-field (85%, n = 26), glycinergic (94%, n = 16) and GABAergic amacrine cells (100%, n = 6). Among the cells tested for the two receptors (n = 65), we encountered both exclusive expression of AMPA or KA receptors and co-expression of the two types. Most narrow-field (70%, n = 27), glycinergic (81%, n = 16) and GABAergic cells (67%, n = 6) were found to have both AMPA and KA receptors. In contrast, only less than half of the wide-field cells (43%, n = 14) were found to co-express AMPA and KA receptors, most of them expressing exclusively AMPA (36%) or KA receptors (21%).

We could elicit small NMDA responses from most of the wide-field (75%, n = 13) and GABAergic cells (67%, n = 3), whereas only 47% of the narrow-field (n = 15), 14% of the AII (n = 22) and no glycinergic cell (n = 2) reacted to NMDA.

Our data suggest that AMPA, KA and NMDA receptors are differentially expressed among different types of amacrine cells rather than among populations with different neurotransmitters or different dendritic coverage of the retina. Selective expression of kinetically different glutamate receptors among amacrine types may be involved in generating transient and sustained inhibitory pathways in the retina. Since AMPA and KA receptors are not generally clustered at the same postsynaptic sites, a single amacrine cell expressing both AMPA and KA receptors may provide inhibition with different temporal characteristics to individual synaptic partners.

6. ZUSAMMENFASSUNG

6.1. Ziel und Methode dieser Studie

Amakrinzellen sind, morphologisch wie physiologisch, die heterogenste Klasse aller retinalen Neurone. Sie erhalten glutamaterge Synapsen von Bipolarzellen, mit denen sie eine Rückkoppelungsschleife bilden (reziproke Synapsen). Weitere Ausgangssynapsen bilden sie mit Ganglienzellen, die die Lichtsignale der Retina aufsummieren und im optischen Nerv an die höheren visuellen Zentren weiterleiten. Die vorliegende Studie sollte klären, ob die funktionelle Vielfalt der verschiedenen Amakrinzelltypen durch eine spezifische Expression unterschiedlicher Glutamatrezeptoren zustande kommt. Die drei Hauptgruppen der ionotropen Glutamatrezeptoren werden nach ihren Liganden AMPA, KA und NMDA genannt. In einem vertikalen Schnittpräparat („slice") der Mausretina wurden elektrophysiologische Ableitungen durchgeführt und die Expression von AMPA, KA und NMDA Rezeptoren wurde pharmakologisch untersucht. Hierfür wurden selektive Agonisten und Antagonisten verschiedener Rezeptortypen, wie etwa Cyclothiazide (CTZ), GYKI 52466, GYKI 53655, SYM 2081 und NBQX, verwendet und deren Auswirkungen auf die Applikation der Glutamatrezeptor-Agonisten AMPA, KA und NMDA untersucht.

6.2. Klassifizierung der Amakrinzellen

Mindestens 30 verschiedene Typen von Amakrinzellen sind von Untersuchungen an der Kaninchen- (*MacNeil and Masland 1998*) und der Ratten-Netzhaut (*Menger et. al. 1998, Perry and Walker 1980*) bekannt. Die genaue Homologie der Amakrinzelltypen in verschiedenen Tierspezies ist nicht vollständig geklärt (*MacNeil et. al. 1999*). In dieser Studie wurden die abgeleiteten Amakrinzellen anhand der Größe ihrer dendritischen Felder in „narrow-field" (Durchmesser des dendritischen Feldes >100 μm) und „wide-field" Zellen (Durchmesser <100 μm) eingeteilt (*Abbildung 3.1., Seite 35*). Aufgrund ihrer charakteristischen Morphologie, nämlich dem verlängerten Soma und den dicken Hauptdendriten, konnten AII-Amakrinzellen als eigener Typ erkannt und charakterisiert

werden (*Abbildung 3.1., Seite 35*). Sie wurden nicht in die Gruppe der „narrow-field" Zellen eingeschlossen. Zusätzlich wurde eine transgene Mauslinie verwendet (GlyT2-EGFP), deren glyzinerge Neurone das grün fluoreszierende Protein EGFP unter der Kontrolle des GlyT2-Promotors exprimieren. Dies erlaubte die Unterscheidung zwischen glyzinergen und GABAergen Amakrinzellen.

6.3. Elektrophysiologie

6.3.1. Expression von NMDA und nicht-NMDA Rezeptoren in Amakrinzellen

In der vorliegenden Arbeit wurde von mehr als 300 Amakrinzellen abgeleitet und 130 Zellen davon wurden in die Auswertung einbezogen. Die Dauer der Ableitungen betrug zwischen 10 und 25 Minuten. Während dieser Zeit wurde das Membranpotential bei -75 mV geklemmt. Alle Zellen (n = 300) reagierten auf Applikation der nicht-NMDA Rezeptor Agonisten AMPA und KA, wobei die Reaktionen der „wide-field" Zellen geringer ausfielen als die der „narrow-field" Zellen. Dies könnte teilweise damit erklärt werden, dass an den distalen Dendriten eine niedrigere Wirkstoffkonzentration vorlag. Ferner bedingt die Schnittdicke von nur 200-300 μm den Verlust einiger Teile des Dendritenbaumes. In ein und derselben Zelle rief die Applikation von KA üblicherweise einen schneller deaktivierenden Stromfluß hervor als die Applikation von AMPA (*Abbildung 3.6., Seite 40*). Das berechnete Umkehrpotential für Ströme, die durch AMPA und KA evoziert wurden, lag bei 0,7 $\pm$ 5 mV (*Abbildung 3.7., Seite 41*), was den Ionenfluß durch nicht-NMDA Rezeptoren bestätigt. AMPA- und KA-Ströme konnten durch NBQX, einen nicht-selektiven nicht-NMDA Rezeptor Blocker, unterdrückt werden. Die Applikation von 10 μM NBQX führte in allen Zellen zu einer starken Reduktion (90-100%) der durch Applikation der Agonisten AMPA und KA bedingten Ströme (*Abbildung 3.8., Seite 41*).

Im Gegensatz dazu löste die NMDA Applikation nur bei einigen wenigen Zellen einen einwärts gerichteten Kationenstrom aus (*Abbildung 3.9., Seite 43*). Das Membranpotential der Zellen wurde wie bereits beschrieben bei -75 mV gehalten und 100 μM NMDA wurden in einer magnesiumfreien Lösung zusammen mit dem Co-agonisten Glyzin (2 μM) und Strychnin (1 μM), welches die Aktivierung von Glyzin Rezeptoren hemmen sollte, appliziert. Unter diesen Umständen konnten bei den meisten „wide-field" (6 von 8) und bei der Hälfte der „narrow-field" Amakrinzellen (4 von 8) niedrige Kationenströme

gemessen werden. Unter dieselben Bedienungen löste NMDA in keiner der 10 getesteten AII-Amakrinzellen einen Stromfluß aus. Durch die Erhöhung der NMDA-Konzentration auf 500 μM und des Membranpotentials (-35 bis +45 mV) konnte in 4 von 5 „wide-field" und in 3 von 7 „narrow-field" Zellen eine NMDA-Antwort ausgelöst werden. Weiterhin konnte man in 3 von 12 getesteten AII-Amakrinzellen eine NMDA-Antwort beobachten. In der GlyT2-EGFP Maus wurden lediglich zwei glyzinerge Amakrinzellen bzgl. ihrer Reaktion auf NMDA getestet, wobei keine von ihnen eine solche zeigte. In 2 von 3 GABAergen Zellen konnte eine durch NMDA ausgelöste Zellantwort bei unterschiedlichen Membranpotentialen und Wirkstoffkonzentrationen beobachtet werden.

Folglich scheinen mehr „wide-field" und GABAerge Amakrinzellen NMDA Rezeptoren zu exprimieren als „narrow-field" und glyzinerge Zellen. Nur ein kleiner Teil der AII-Amakrinzellen reagierte auf NMDA, allerdings nicht im physiologischen Spannungsbereich (*Abbildung 3.10., Seite 44*).

AMPA und KA aktivieren beide nicht-NMDA Rezeptortypen. Aus Applikationsexperimenten mit AMPA und KA lässt sich nicht ableiten, ob die Zellen AMPA-, KA- oder beide Typen von Rezeptoren exprimieren. Dazu bedarf es des Einsatzes rezeptorselektiver Pharmaka.

6.3.2. Expression von AMPA Rezeptoren in Amakrinzellen

Die Expression der AMPA Rezeptoren wurde mithilfe des selektiven AMPA-Rezeptor-Agonisten Cyclothiazid (CTZ) und der selektiven AMPA-Rezeptor-Antagonisten GYKI 52466 und GYKI 53655 untersucht. Diese wurden in Kombination mit den Agonisten AMPA und KA appliziert und deren Effekt im Vergleich zur Kontrolle ausgewertet.

CTZ. 100 μM CTZ wurden in Kombination mit 100 oder 200 μM AMPA appliziert. Diese Applikationen hatten in den verschiedenen Zellen unterschiedliche Effekte – von keiner bis hin zu starker Reaktion. Dieses Phänomen war bei allen Zelltypen zu beobachten. Zur Analyse des CTZ-Effektes wurden sowohl die Stromamplitude als auch das Integral (die Ladung integriert über die Antwortdauer) der Zellantwort herangezogen. Die Parameter des CTZ-induzierten Stromes wurden gemessen und durch den Kontrollwert (der durch den Agonisten allein hervorgerufene Effekt) dividiert. Der relative Effekt von

CTZ im Vergleich zur Kontrolle ist in Prozent angeben. Folglich zeigt ein Wert von 100% keine Änderung der Amplitude bzw. des Integrals des Stromflusses im Vergleich zur Kontrolle an. Werte über 100% stellen eine Erhöhung dar, Werte unter 100% reflektieren eine Erniedrigung. Anstiege von mindestens 25% (sowohl bei der Amplitude als auch beim Integral des durch CTZ hervorgerufen Kationeneinstromes) wurden als AMPA Rezeptor-Aktivität interpretiert. Wenn möglich, wurden die CTZ Experimente mit den Ergebnissen der GYKI Experimente verglichen, bevor sie in die Statistik der AMPA Rezeptor Expression einbezogen wurden.

Bei AII-Amakrinzellen (n = 8) erhöhte CTZ die Amplitude des durch AMPA hervorgerufenen Stromflusses um durchschnittlich 50%, während das Integral um 140% anstieg. „Narrow-field" Zellantworten waren während der Applikation von CTZ 60% größer (n = 31), „wide-field" Zellen hingegen zeigten einen Anstieg um 40% (n = 13). Während der CTZ Effekt bei AII-Zellen einheitlich ist, findet man bei „narrow-field" und „wide-field" Zellen eine große Variabilität (*Abbildung 3.13., Seite 48*). Die gleiche Untersuchung, durchgeführt an glyzinergen Amakrinzellen von GlyT2-EGFP-Mäusen, ergab eine Erhöhung des AMPA-induzierten Stromes durch CTZ um 22% (n = 11) (*Abbildung 3.14., Seite 49*). Sowohl für die GABAergen als auch für die glyzinergen Amakrinzelltypgruppen wurde eine gewisse Variabilität beobachtet, wenn auch nicht so stark ausgeprägt wie bei den „narrow-field" und „wide-field" Zellen. Bei 3 der glyzinergen Zellen und bei einer der zwei GABAergen Zellen zeigte CTZ keine Wirkung.

GYKI 52466 und GYKI 53655. Die Expression von AMPA Rezeptoren wurde auch durch Applikation eines selektiven Antagonisten überprüft. 20 μM GYKI 52466 oder GYKI 53655 wurden zusammen mit entweder AMPA oder KA (100 oder 200 μM) appliziert. Wie im Falle von CTZ wurden unterschiedliche Wirkungen von GYKI bei verschiedenen Zellen festgestellt. Sowohl „narrow-field" als auch „wide-field" Zellen zeigten das komplette Spektrum von Effekten, angefangen bei starker Inhibition über eine Reduktion der Zellantwort bis hin zum Ausbleiben von Hemmung. Das gleiche gilt für glyzinerge und GABAerge Zellen, von denen in GlyT2-EGFP Mäusen abgeleitet wurden.

Für die Quantifizierung des GYKI Effekts bei den verschiedenen Zellen wurden sowohl die Amplitude als auch das Integral der Zellantwort verwendet. Die Daten wurden in gleicher Weise wie im CTZ Experiment dargestellt. Zellen, die eine Reduktion der Zellantwort von mindestens 25% (GYKI 52466) oder 15% (GYKI 53655) bei der

Amplitude und beim Integral der Zellantwort zeigten, wurden als Zellen mit AMPA Rezeptoren gewertet. Wenn möglich, wurden die Werte mit den Ergebnissen der CTZ Experimente verglichen, bevor sie in die Statistik aufgenommen wurden. Bei AII-Amakrinzellen reduzierten GYKI 52466 den gesamten Stromfluss um ca. 50% (n = 5) und GYKI 53655 um ca. 60% (n = 3). Hierbei bestand innerhalb der Zelltypklassen keine große Variabilität (*Abbildungen 3.18. und 3.19., Seiten 53, 54*). Dagegen waren die Zellantworten von „narrow-field" (n = 15) und „wide-field" Zellen (n = 11) auf GYKI 52466 sehr unterschiedlich und reichten von starker Inhibition bis hin zu keinem Effekt des Wirkstoffes (*Abbildungen 3.18. und 3.19., Seiten 53, 54*).

Alle getestete Zellen (n = 20) reagierten auf die Applikation von GYKI 53655. GYKI 53655 reduzierte den durch KA induzierten Strom um mehr als 50% in „narrow-field" und „wide-field" Amakrinzellen (*Abbildung 3.19., Seite 54*). In Anwesenheit von GYKI 53655 war in „narrow-field" Amakrinzellen (n = 10) die Amplitude der Zellantworten um die Hälfte reduziert, das Integral betrug nur noch 33% der Kontrollantwort. In „wide-field" Amakrinzellen (n = 2) führte GYKI 53655 zu einer Reduktion um durchschnittlich 60% verglichen mit der Kontrollantwort.

Bei allen Zellen, bei denen die Wirkung von CTZ und GYKI untersucht wurde (n = 40), konnte eine gute Korrelation zwischen den Wirkungen der zwei AMPA Rezeptor selektiven Pharmaka beobachtet werden. Zellen, die mit einer sehr starken Erhöhung der Stromantwort auf die Applikation von CTZ reagierten, zeigten eine starke Inhibition durch GYKI. Umgekehrt zeigten Zellen, in denen CTZ nur einen geringen oder keinen Effekt hatte, meist auch eine schwache Inhibition durch GYKI. Da die Wirkung des einen Pharmakons durch die des anderen bestätigt werden konnte, wurden auch Zellen in die Statistik der AMPA Rezeptor Expression miteinbezogen, bei denen lediglich eine der beiden Wirkstoffe appliziert worden war.

Aufgrund der CTZ und GYKI Experimente konnte die Expression von AMPA Rezeptoren in verschiedenen Zellen gemessen werden (*Abbildung 3.21., Seite 56*). Die große Mehrheit von AII-Amakrinzellen (94%, n = 17), „narrow-field" (87%, n = 45) und glyzinergen Zellen (85%, n = 20) besitzen AMPA Rezeptoren. Auch bei 81% der „wide-field" (n = 21) und bei 78% der GABAergen Zellen (n = 9) wurden AMPA Rezeptoren gefunden.

Da AMPA und KA jeweils sowohl AMPA als auch KA Rezeptoren aktivieren, kann die unvollständige Hemmung der Zellantwort durch GYKI in einigen Zellen auf die Aktivierung von KA Rezeptoren zurückgeführt werden. 20 µM GYKI 52466 bzw. GYKI 53655 reichen üblicherweise aus, um AMPA Rezeptoren vollständig zu blockieren, wenn sie kurz vor dem Agonisten in die Badlösung appliziert werden. In unseren Experimenten wurden die Pharmaka zusammen mit den Agonisten appliziert, so dass möglicherweise einige AMPA Rezeptoren nicht blockiert wurden und somit ein Reststrom verblieb. Um diese Möglichkeit auszuschließen und sicher zu sein, dass der Strom durch KA Rezeptoren verursacht wird, wurde ein Sicherheitslimit von 25% gewählt. Zellen, die in Anwesenheit von GYKI immer noch 25% der Kontrollantwort zeigten, wurden als KA Rezeptor exprimierende Zellen gewertet. Das Sicherheitslimit wurde sorgfältig gewählt, indem Beispiele für Zellen, die eindeutig sowohl auf AMPA als auch auf KA ansprachen, verglichen wurden. Außerdem waren die Experimente mit NBQX nützlich - selten wurde ein Reststrom von über 10% des Kontrollstromes beobachtet. Wenn möglich, wurden die Ergebnisse der GYKI mit denen der SYM 2081 Experimente verglichen, bevor sie in die Statistik der KA Rezeptor Expression aufgenommen wurden (siehe unten).

6.3.3. Expression von KA Rezeptoren in Amakrinzellen

SYM 2081 ist der einzige weitgehend akzeptierte Agonist, der selektiv auf alle KA Rezeptoruntereinheiten wirkt, während es keinen Effekt auf die Aktivität der AMPA Rezeptoren hat. SYM 2081 ruft eine kurze Aktivierung der KA Rezeptoren, gefolgt von einer lang anhaltenden Desensibilisierung, hervor. Folglich induziert seine Applikation einen kleineren und schneller deaktivierenden Strom als KA. SYM 2081 wurde sowohl alleine als auch zusammen mit 100 oder 200 µM KA angewendet. SYM 2081 wurde in einer Konzentration von 20 µM eingesetzt, wenn es gemeinsam mit KA appliziert wurde, bei separater Anwendung hingegen in Konzentrationen von 50 oder 100 µM.

In Kombination mit KA hatte SYM 2081 unterschiedliche Effekte in verschiedenen Zellen. In den SYM 2081 Ko-Applikationexperimenten wurden die Integrale der Zellantworten als beste Beschreibung der Wirkung der Pharmaka und als einziger Parameter für die Quantifizierung der Ergebnisse gewählt. Die AII-Amakrinzellen reagierten mit einer Reduktion des durch KA aktivierten Stromes um durchschnittlich 40% (*Abbildung 3.24., Seite 60*). Bei den 3 untersuchten Zellen fand sich nur eine geringe Variabilität. Dies lässt

sich über „narrow-field" (n = 24) und „wide-field" Amakrinzellen (n = 16) dagegen nicht behaupten. Hier offenbarte sich eine große individuelle Variabilität innerhalb der Zellen beider Gruppen. Ihre Antworten auf SYM 2081 reichten von keiner Änderung der Zellantwort bis hin zu einer deutlichen Reduktion derselben (*Abbildung 3.24., Seite 60*). Dennoch lag der überwiegende Teil der Antworten der „narrow-field" Zellen in einem moderaten Variationsbereich. Die Wirkung von SYM 2081 in Zellen, die in der GlyT2-EGFP Maus abgeleitet wurden, war wiederum sehr variabel (*Abbildung 3.25., Seite 61*). 5 glyzinerge und 2 GABAerge Zellen werden auf SYM 2081 bei gleichzeitiger Applikation von KA getestet. 2 glyzinerge Zellen reagierten nicht auf Applikation von SYM 2081, wogegen die dritte Zelle eine leichte Reduktion des durch KA induzierten Stromes zeigte. Die beiden GABAergen Zellen reagierten völlig verschieden, eine zeigte eine Reduktion des Stromflusses um 20%, die andere eine Reduktion um 60%.

Es erwies sich als schwierig, diejenige Reduktion der Stromantwort zu finden, die eindeutig KA Rezeptoren bei der untersuchten Zelle nachweist. Durch gründliche Analyse der Veränderungen der abfallenden Zeitkonstanten und den Vergleich der KA+SYM 2081 mit den GYKI Experimenten, wurde ein Wert von 10% als annehmbare Schätzung festgelegt. Somit wurde bei einer Reduktion der Zellantwort um 10% oder mehr, verursacht durch SYM 2081, von einer KA-Aktivität der Zelle ausgegangen.

Eine gute Korrelation bestand zwischen den Effekten von SYM 2081 und GYKI bei individuellen Zellen (*Abbildung 3.26., Seite 62*). Von 25 bezüglich ihrer Reaktion auf beide Pharmaka untersuchten Zellen zeigten nur 4 leicht widersprüchliche Wirkungen von SYM 2081 und GYKI. Sie wurden von der weiteren Analyse ausgeschlossen. Bei allen anderen Zellen korrelierte eine starke Reduktion der Stromantwort durch SYM 2081 mit einer schwachen Inhibition durch GYKI.

Bei einer weiteren Gruppe von Experimenten wurde lediglich SYM 2081 appliziert. SYM 2081 kann alleine KA Rezeptoren öffnen, wobei die induzierte Desensibilisierung schnell eintritt. So konnten, wenn überhaupt, nur kleine Stromantworten gemessen werden. Ströme konnten in 42 Zellen hervorgerufen werden, kein Effekt des Pharmakons wurde bei 36 Zellen beobachtet (*Abbildung 3.28., Seite 64*). Weniger als die Hälfte der AII-Amakrinzellen (n = 18), fast 70% der „narrow-field" (n = 25) und 60% der glyzinergen Zellen (n = 7) zeigten eine Stromantwort nach der Applikation von SYM 2081. Ein etwas höherer Anteil, circa 75% der „wide-field" (n = 19) und 70% der GABAergen Amakrinzellen reagierten ebenfalls auf die Applikation dieses Pharmakons.

Aufgrund der GYKI und SYM 2081 Experimente konnte die Expression von KA Rezeptoren in verschiedenen Zellen ausgewertet werden (*Abbildung 3.29., Seite 65*). Viele AII-Amakrinzellen (75%, n = 14) und fast alle „narrow-field" (93%, n = 49) und glyzinerge Zellen (94%, n = 16) exprimieren KA-Rezeptoren. Bei 85% der „wide-field" (n = 26) und bei allen getesteten GABAergen Amakrinzellen (n = 6) wurden ebenfalls KA Rezeptoren identifiziert.

6.3.4. AMPA und KA Rezeptor Ko-Expression

Bei fast allen untersuchten Zelltypen wurden sowohl AMPA als auch KA Rezeptoren gefunden. Wir waren daran interessiert herauszufinden, ob die zwei Rezeptoren in einzelnen Zellen eines Typs ko-exprimiert werden oder eher auf verschiedene Zellen einer Population verteilt sind. Bei den auf AMPA und KA Rezeptoren untersuchten Zellen (n = 65) wurde sowohl die Expression von AMPA oder KA Rezeptoren als auch die Ko-Expression der Rezeptoren gefunden (*Abbildung 3.33., Seite 69*). Die meisten „narrow-field" (70%, n = 27) und glyzinergen Zellen (81%, n = 16) besitzen beide Rezeptortypen. 22% der „narrow-field" und 19% der glyzinergen Zellen exprimierten ausschließlich KA Rezeptoren, während bei 2 „narrow-field" Zellen ausschließlich AMPA Rezeptoren gefunden wurden. Die wenigen abgeleiteten GABAergen Zellen (n = 6) zeigten eine ähnliche Rezeptorverteilung wie die glyzinergen Zellen: die Mehrheit (67%) der Zellen besaß sowohl AMPA als auch KA Rezeptoren, während der Rest nur KA Rezeptoraktivität aufwies. „Wide-field" Amakrinzellen verhielten sich anders als die anderen Zellgruppen. Weniger als die Hälfte dieser Zellen (43%, n = 14) exprimierte beide Rezeptortypen. Die meisten „wide-field" Zellen zeigten entweder AMPA (36%) oder KA Rezeptoraktivität (21%).

6.4. Die funktionelle Bedeutung heterogener Expression von Glutamatrezeptoren durch Amakrinzellen

Die Minderheit der untersuchten Zellen exprimierte nur AMPA oder nur KA Rezeptoren. Die meisten Zellen, von denen abgeleitet wurde, zeigten sowohl AMPA als KA Rezeptor-vermittelte Aktivität. Da AMPA und KA Rezeptoren nicht generell an denselben postsynaptischen Zonen exprimiert werden, ist es möglich, dass Amakrinzellen von

unterschiedlichen präsynaptischen Bipolarzellen über AMPA bzw. KA Rezeptoren unterschiedliche Signale erhalten.

Die Expression von AMPA Rezeptoren in AII-Amakrinzellen ist gut untersucht und es gibt deutliche Hinweise, dass sie auf den so genannten „arboreal dendrites" liegen, wo die Zellen synaptischen Eingang von Stäbchenbipolarzellen bekommen. Mørkve und Kollegen (*2002*) fanden Ca^{2+}-durchlässige AMPA-Rezeptoren auf AII-Amakrinzellen, während Habermann (*2000*) durch Applikation von nicht-NMDA-Agonisten eine Erhöhung der Ca^{2+}-Konzentrazion in den „arboreal dendrites", nicht aber in den „lobular appendages", festgestellte. Inzwischen wurden in verschiedenen Spezies GluR3 und GluR4 Untereinheiten an den „arboreal dendrites" von AII-Amakrinzellen identifiziert (*Qin and Pourcho 1999b*, Katze; *Ghosh et al. 2001*, Affe und Kaninchen; *Li et al. 2002*, Kaninchen), wo sie Synapsen von Stäbchenbipolarzellen erhalten.

Wir konnten an der Mausretina erstmalig KA Rezeptoren auf AII-Amakrinzellen nachweisen. Man kann spekulieren, diese seien an den „lobular appendages" lokalisiert, also in der äußeren Hälfte der IPL (*Brandstätter et al. 1994*), wo AII-Amakrinzellen Eingang von OFF-Zapfenbipolarzellen erhalten. Dort erhalten sie überdies Eingang von dopaminergen Amakrinzellen und es gibt Hinweise darauf, dass AII-Amakrinzellen D1 Rezeptoren, die über G-Proteine mit Adenylatcyclase gekoppelt sind, exprimieren (*Hampson et al. 1992*). Möglicherweise moduliert Dopamin Glutamatrezeptoren durch Phosphorylierung der Proteinkinase A (PKA) (rezensiert von *Dowling 1991*). PKA kann durch Adenylatcyclase aktiviert werden, und die GluR6 Untereinheit besitzt eine PKA-Phosphorylierungsstelle (*Raymond et al. 1993, Wang et al. 1993*). Allerdings gibt es noch keine Hinweise darauf, dass AII-Amakrinzellen die GluR6 Untereinheit exprimieren, wobei aber *in-situ* Hybridisierungsuntersuchungen von GluR6 Färbung in der äußeren IPL nachweisen (*Brandstätter et al. 1994*).

Mehrere Arbeitsgruppen berichteten über NMDA Rezeptor Expression in AII-Amakrinzellen in verschiedenen Spezies (*Hartveit and Veruki 1997,* Ratte*; Bloomfield and Xin 2000, Zhou and Dacheux 2004,* Kaninchen). Wir konnten NMDA Rezeptoraktivität nur in einigen wenigen Zellen feststellen und das auch nur unter nicht-physiologischen Bedingungen. Für einige Zeit wurde vermutet, die NMDA Rezeptoren seien extrasynaptisch lokalisiert. Aktuelle Untersuchungen an der Ratte zeigten eine Assoziation der NR1 Untereinheit mit Connexin36 in einigen Regionen des Zentralnervensystems (*Rash et al. 2004*). Gut belegt ist, dass AII-Amakrinzellen miteinander und mit ON-

Zapfenbipolarzellen über gap junctions gekoppelt sind und Connexin36 exprimieren (*Massey et al. 2003*). Daher scheint es plausibel, dass die NMDA Rezeptoren der AII-Amakrinzellen an der Modulation von gap junctions beteiligt sind.

6.5. Ausblick

Eine spezifische Expression von Glutamatrezeptoren bei einzelnen Amakrinzelltypen unterstützt die Hypothese, dass die verschiedenen Typen dieser Neurone eine spezifische Rolle bei der Signalübertragung in der inneren Retina spielen. Zukünftige Studien sollten deshalb zum Ziel haben, die individuelle Glutamatrezeptorexpression in bestimmten Zelltypen zu untersuchen. Während dies zur Zeit noch Schwierigkeiten bereitet, bleibt zu hoffen, dass in Zukunft Amakrinzellmarker gefunden werden, die es erlauben, bestimmte Typen von Amakrinzellen immunhistochemisch selektiv anzufärben. Eine weitere Möglichkeit bestünde darin, das grün fluoreszierenden Proteins (GFP) unter der Kontrolle amakrinzellspezifischer Promotoren in transgenen Tieren zu exprimieren.

Studien über Glutamatrezeptoren sollten außerdem versuchen, sich auf die Identifizierung synaptischer Rezeptoren zu konzentrieren, die den eigentlichen glutamatergen Eingang der Zellen vermitteln. Die Zellantworten, die durch Agonisten ausgelöst wurden, erlauben eine solche Diskriminierung nicht, hierfür sind Analysen spontaner oder evozierter synaptischer Aktivität nötig.

Da eine Zelle mehrere Rezeptortypen exprimieren kann, können unterschiedliche Rezeptoren die Transmission an verschiedenen Synapsen vermitteln. Deshalb ist die Lokalisierung der Rezeptoren wichtig für das Verständnis des Netzwerkes der Zellen und somit für die Funktion der einzelnen Amakrinzellen. Immunhistochemie, Ca^{2+}-imaging Techniken (um Ca^{2+}-permeable Glutamatrezeptoren zu identifizieren) und Doppelableitungen von prä- und postsynaptischen Neuronen sind erfolgversprechende Ansätze für künftige Experimente.

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