Trafficking of and Ethanol-Induced Inhibition of AMPA Receptors

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Education can make anyone a good scientist,
but great thinkers are forged in the eternal flame of gods.

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Abstract

AMPA receptors are an important class of ionotropic glutamate receptors which participate in fast excitatory synaptic transmission in most brain areas. They are tetrameric complexes composed of GluR-A, B, C and/or D subunits, and have a pivotal role in adjustment of cell membrane excitability as their cell membrane expression level is highly regulated in brain physiology. The subunit composition of AMPA receptors is developmentally and transiently regulated and differs between brain areas. Further, AMPA receptors have alternatively spliced variants with differences in ion channel function, pharmacology and localization of receptors. Recently, AMPA receptor trafficking and cellular localization have been found to be regulated by several proteins, such as transmembrane AMPA receptor regulatory proteins (TARPs). The interaction of TARPs and AMPA receptor subunits is required in normal AMPA receptor function in many, if not all, brain areas.

NMDA-type glutamate receptors are important target molecules of ethanol. They are inhibited by clinically relevant concentrations of ethanol. Whereas, the role of AMPA receptors in the actions of ethanol has not been clarified as thoroughly. Furthermore, the regulation of AMPA receptor synthesis and their possible adaptation in neurons with altered inhibitory mechanisms are poorly understood. In this thesis work AMPA receptor pharmacology, trafficking and synaptic localization was studied using patch-clamp electrophysiology. Mechanisms were clarified by studying functions of both native and recombinant, wild-type and mutant AMPA receptors in isolated neurons and HEK293 cells. In addition, hippocampal slices from transgenic Thy1α6 mouse line with altered $GABA_A$ receptor-mediated inhibition were used to study adaptation of AMPA receptor currents.

Ethanol (> 25 mM) was found to inhibit AMPA receptor function by increasing desensitization of the receptor, as the steady-state component of the current was inhibited more than the peak current. Ethanol inhibition was reduced when cyclothiazide was used to block desensitization in native neurons and when non-desensitizing point-mutated GluR-A and GluR-D receptors were studied. In GluR-D receptors, ethanol also accelerated the onset of desensitization, which was measured as a time constant of peak current decay. The coexpression of TARP-proteins stargazin and γ4 increased this effect of ethanol. The results suggest that ethanol inhibits AMPA receptors by increasing and accelerating their desensitization.

We found that the agonist binding capability is a feature of AMPA receptors that is important for trafficking them from endoplasmic reticulum to the cell membrane. Stargazin rescues the surface expression of non-binding AMPA receptor mutants in HEK293 cells, but not in native neurons. The state of synaptic AMPA receptor-mediated function was studied in Thy1α6 mice with lowered synaptic $GABA_A$ receptor-mediated function. Thy1α6 mice had decreased AMPA receptor-mediated function in hippocampal CA1 neurons, measured as reduced mEPSC amplitudes as compared to wild-type neurons. These transgenic mice also had lower sensitivity to maximal electroshock convulsions, presumably due to the decreased AMPA receptor function.

In conclusion, the results suggest that the AMPA receptor may be target for alcohol action at least during heavy intoxication, probably through accelerating onset and increasing the extent of desensitization. TARPs appear to enhance ethanol inhibition and to participate in trafficking of AMPA receptors upon their synthesis in the cell. AMPA receptors mediate also long-term adaptation to altered neuronal excitability, which adds to their well-known role in synaptic plasticity for e.g. in physiology of learning and in pathophysiology of drug addiction.

Tiivistelmä

AMPA reseptorit ovat tärkeä glutamaattisten reseptorien luokka, jotka osallistuvat nopeaan hermoviestinvälitykseen suurimmassa osassa aivoja. Ne koostuvat neljästä alayksiköstä ja niillä on keskeinen asema hermosolujen solukalvon toiminan säätelyssä, mm. synapsien voimakkuuden säätämisessä, sillä niitä kuljetetaan jatkuasti solukalvolle ja pois solukalvolta. AMPA reseptorien alayksikkökoostumus vaihtelee kehityksen kuluessa ja aivoalueiden välillä. Niillä on lisäksi vaihtoehtoisia silmikointimuotoja, jotka eroavat toisistaan ioniläpäisevyydeltään, farmakologialtaan ja sijainniltaan. Viime vuosina on löytynyt lisäksi monia AMPA reseptoreita sääteleviä proteiineja kuten TARPit (engl. transmembrane AMPA receptor regulatory protein). AMPA reseptoreiden ja TARPien välisten vuorovaikutuksien on osoitettu olevan tarpeellisia normaalille AMPA reseptorien toiminnalle monilla, ehkä jopa kaikilla, aivoalueilla.

Lukuisat tutkimukset ovat osoittaneet, että kliinisesti merkittävät etanolipitoisuudet estävät NMDA-tyyppisiä glutamaattireseptoreita. Sen sijaan, etanolit vaikutukset AMPA reseptoreihin ei ole yhtä tarkasti tiedossa. Tämän lisäksi huonosti tunnetaan miten estävän hermoviestinvälityksen muutokset vaikuttavat AMPA reseptoreiden säätelyyn. Tässä väitöskirjassa on tutkittu miten etanoli vaikuttaa AMPA reseptoreihin, miten niitä kuljetetaan solukalvolle sekä miten niitä esiintyy synapseissa käyttäen hyväksi patch clamp sähköfysiologiaa. Työssä käytettiin sekä aivopreparaatteja että HEK-solu ekspressiosysteemiä, joiden avulla tutkittiin sekä villityyppisiä että mutatoituja AMPA reseptoreita. Tämän lisäksi Thy1α6 hiirimallia käytettiin hyväksi AMPA reseptoreiden tilan selvittämisessä tilanteessa, jossa $GABA_A$ reseptorien välittämä estävä viestinvälitys on muuttunut.

Etanoli (> 25 mM) esti AMPA reseptoreiden toimintaa lisäten reseptorin desensitisoitumista. Reseptorin tasannevirta oli herkempi etanolin estovaikutukselle kuin piikkivirta. Desensitisaation vähentäminen vähensi etanolin estovaikutusta, mikä todettiin kun desensitisaatiota estettiin syklotiatsidilla ja kun mitattiin ei-desensitisoituvia AMPA reseptorin mutatoituja muotoja. GluR-D AMPA reseptoreilla havaittiin myös, että etanoli nopeutti desensitisoituneeseen tilaan siirtymistä. Etanolin desensitisaatiota nopeuttava vaikutus oli suurempi, kun TARP proteiineja ilmennettiin AMPA reseptorien kanssa.

Tutkimuksissamme selvisi, että AMPA reseptorin agonistin sitomiskyvyllä on tärkeä merkitys reseptorin kuljettamisessa sisäsolukalvostolta solukalvolle. Stargazin-TARP palautti sitomiskyvyttömien AMPA reseptorimutanttien kuljetuksen normaalitasolle HEK-soluissa, muttei hermosoluissa. Thy1α6 hiirimallilla tehdyt kokeet osoittivat, että AMPA reseptorien toiminta oli vähentynyt hippokampuksen CA1 alueen hermosoluissa, mikä näkyi mEPSC amplitudin pienenemisenä verrattuna villityyppsiin hiiriin. Hiirikannalla oli myös alentunut sähköshokkiherkkyys, mikä todennäköisesti johtui vähentyneestä AMPA reseptorien toiminnasta.

Tulokset osoittavat, että suuret etanolin pitoisuudet estävät AMPA reseptoreita, millä voi olla merkitystä voimakkaassa humalatilassa. Etanolin estovaikutuksen mekanismina näyttää olevan reseptorin desensitisaation lisäys ja desensitisoituneeseen tilaan siirtymisen nopeutuminen. TARPit lisäävän etanolin estovaikutusta nopeuttaen desensitisoitumista entisestään. Ne myös osallistuvat AMPA reseptoreiden kuljetukseen solukalvolle. AMPA reseptorit toimintassa tapahtuu myös pitempi aikaista säätelyä, jolla sopeudutaan hermosolujen muuttuneeseen tasapainotilaan kiihdyttävän ja estävän hermoviestinvälityksen välillä.

Original Publications

This thesis is based on the following original publications, referred to in the text by the Roman numerals:

I Möykkynen T, Korpi ER, Lovinger DM (2003) Ethanol inhibits α-amino-3-hydyroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function in central nervous system neurons by stabilizing desensitization. J Pharmacol Exp Ther. 306(2):546-55.

II Möykkynen TP, Coleman SK, Keinänen K, Lovinger DM, Korpi ER. Ethanol increases desensitization of recombinant GluR-D AMPA receptor and TARP combinations. 2009, in press.

III Coleman SK, Möykkynen T, Jouppila A, Koskelainen S, Rivera C, Korpi ER, Keinänen K (2009) Agonist occupancy is essential for forward trafficking of AMPA receptors. J Neurosci. 29(2):303-12.

IV Möykkynen TP, Sinkkonen ST, Korpi ER (2007) Compensation by reduced L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor responses in a mouse model with reduced γ-aminobutyric acid type A receptor-mediated synaptic inhibition. J Neurosci Res. 85(3):668-72.

Abbreviations

AMPA	α-amino-3-hydyroxy-5-methyl-4-isoxazolepropionic acid
APV	(2*R*)-amino-5-phosphonovaleric acid; NMDA receptor antagonist
CA	cornu ammonis of the hippocampus
CAM	cell adhesion molecule
CNS	central nervous system
DMCM	methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; $GABA_A$ inverse agonist
EC_{50}	effective concentration 50 %; concentration producing 50 % of maximal effect; also effective current producing effect in 50% of animals (IV, MES-test)
EPSC	excitatory postsynaptic current
ER	endoplasmic reticulum
fEPSP	field excitatory postsynaptic potentials
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A receptor
GluR-D_i	AMPA receptor subunit (-D indicates that D subunit out of A to D is in question; $_i$ = flip splice variant; $_o$ = flop splice variant)
GRIP	glutamate receptor interacting protein
HEK293	human embryonic kidney cells
IC_{50}	concentration producing 50 % inhibition
LBD	ligand binding domain
LGIC	ligand gated ion channel
LTD	long term depression
LTP	long term potentiation
MAGUK	membrane associated guanylate kinase
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitiory post synaptic current
NMDA	N-methyl-D-aspartate
PDZ	**P**SD95-**D**rosophila disc large-**Z**onula occludens-1; protein domain, which mediate contacts between proteins
PICK1	protein interacting with protein kinase Cα 1
PKC	protein kinase C
PSD-95	postsynaptic density protein of 95 kDa
S1S2-LBD	ligand binding domain of glutamate receptors
TARP	transmembrane AMPA receptor regulatory protein
THE	tonic hindlimb extension
Thy1α6	transgenic mouse line overexpressing $GABA_A$ α6 subunit in forebrain areas
TM1	transmembrane spanning domain 1 (number indicates which TM-domain is in question)
VTA	ventral tegmental area
WT	wild type

1. Introduction

The importance of the brain as an organ controlling our life is clear to each of us. During the last hundred years, the research in the field of neuroscience has revealed the basic principals of how the brain works. One key feature of the brain at the cellular level is that a single nerve cell, neuron, receives inputs from hundreds or even from thousands of other neurons. This allows the brain to have computational functions, which is the basis of information processing. Therefore, the brain can be seen as a complicated biological computer, which works so that the neurons are excited by excitatory inputs and inhibited by inhibitory inputs, and if the summation of these two causes an excitation which is above a certain threshold, the neuron fires an action potential.

The main excitatory neurotransmitter in the mammalian nervous system is glutamate, which mediates the fast synaptic signalling in most of the brain. Glutamate has several groups and types of receptors, among which are α-amino-3-hydyroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type of glutamate receptors. AMPA receptors are important in many neural functions and pathological conditions. The number of them in synapses can change rapidly in many physiological processes and is now considered to be one of the most important mechanisms underlying synaptic plasticity. Plasticity of the brain, in other words, the ability to change, is an important mechanism in adaptation to varying environmental conditions. The most widely studied form of synaptic plasticity is unquestionably long term potentiation (LTP) which is thought to be one of "memory traces" of nervous system. There are many different initiation mechanisms for LTP, but the final result seems to be the trafficking of more AMPA receptors to the synapses.

AMPA receptors are tetramers composed of products of four genes. The expression of different AMPA receptor subtypes depends on developmental stage, brain regions and physiological situations. The expression of AMPA receptors in the cell membrane is precisely regulated in order to ensure that only certain type of receptors occur in synapses. In addition, transmembrane AMPA receptor regulatory proteins (TARPs) control trafficking, synaptic location, kinetics and pharmacology of AMPA receptors increasing thus the functional variation of the receptors. Since the neural function in many brain areas rely heavily on AMPA receptors, studies of the trafficking, function and pharmacology of the AMPA receptor is one of the key areas of neuroscience, to help understand how nervous systems work and modulate their function.

2. Review of the Literature

2.1 Synaptic Transmission

The function of neurons in the body, whether it is transduction of sensory information in the peripheral nervous system or information processing in the brain, is based on electrochemical changes in the cell membrane, which can travel from neuron to another. It consists basically of two aspects (1) short pulses of voltage changes which travel via axons and dendrites and (2) chemical communication between the neurons based on release and reception of the chemical messenger molecules. The latter is called synaptic transmission, and is one of the main themes of this thesis.

The electrical function of neurons is based on ion movement through the cell membrane, which leads to the change in voltage differential across the cell membrane. Ions can not pass the cell membrane freely, because they carry electrical charge with them which makes them hydrophilic. Therefore, the neural membranes are equipped with various ion channels. When an ion channel opens, specific ions move rapidly across the cell membrane, shifting the cell membrane potential rapidly towards the reversal potential of the ion in question. The driving force of the ion movement is called the electrochemical gradient, which is a combination of the ion's concentration and the voltage difference across the cell membrane. Under normal conditions there is, for instance, a strong driving force for sodium to flow inside the cell, because the concentration of Na^+ is about ten times greater extracellularly and the cell is charged negatively intracellularly. Positively charged sodium ions tend to depolarize the cell. Sodium current is important in the action potential fast depolarizing phase. If a channel is permeable to more than one species of ions, the membrane potential shifts towards an average of the reversal potentials of the ions passing through the channel. AMPA type glutamate receptors, which are studied in this thesis work, have a non-selective cation channel as part of their structure, which makes them permeable to Na^+ and K^+. In normal conditions AMPA receptor channel opening leads to strong sodium influx, which depolarizes the cell.

Essential parts of synaptic transmission consist of presynaptic transmitter release machinery and postsynaptic receptors. In neurotransmission, a chemical signal molecule, a neurotransmitter, is released from a presynaptic neuron and bound by the receptors of the receiving, postsynaptic neuron. Neurotransmitter is released from the synaptic vesicles when the calcium ions flow in to the cell through voltage sensitive calcium channels, which open as a response to the cell membrane depolarization traveling via presynaptic nerve fiber, axon. Presynaptic release machinery is very complicated and highly organized structure involving multible different proteins (Rizo and Rosenmund, 2008). Molecules affecting the neurotransmitter release machinery are often very potent toxins, some of them having marginal use as drugs, as botulinum toxin. It is used to treat painful muscle spasms, and as a cosmetic treatment. Neurotransmitter is released in to the highly organized narrow space between the nerve cells called the synaptic cleft and it diffuses rapidly (in milliseconds) to the postsynaptic side where it binds to its specific neurotransmitter receptors. Receptors can also be located in presynaptic side of synapse or in astrocytes. There are basically two types of receptors, ionotropic and metabotropic receptors. Ionotropic have an ion channel as a

part of their structure, which allows the ions flow through the cell membrane, causing the change in the membrane potential. Metabotropic receptors, on the other hand, are coupled to G-proteins, which mediate the effects to ion channels and intracellular signaling cascades. Ionotropic receptors usually participate in the signal delivery between the neurons, whereas metabotropic receptors modulate the actual neurotransmission, such as neurotransmitter release. Usually, each neurotransmitter has both ionotropic and metabotropic receptors, as is the case with the most abundant excitatory neurotransmitter, glutamate, and with the most abundant inhibitory neurotransmitter, γ-aminobuturic acid (GABA). Many medical drugs, as well recreational drugs, used nowadays are agonists or antagonists of neurotransmitter receptors.

2.2 Excitatory and Inhibitory Neurotransmission

Neurotransmitter receptors can be divided functionally into two groups, excitatory (depolarizing) and inhibitory receptors. The ion flow through the channel of receptor determines whether the neurotransmission is exciting or inhibiting. Ion flow depends on ion gradient, which is created by ion pumps and transporters. Transporter activity may change, which may affect the direction of ion flow and the type of neurotransmission. For example, early in development GABA is excitatory neurotransmitter, but later in more mature neurons it is inhibitory due to the activity of potassium chloride co-transporter KCC2 (Rivera et al., 1999). In excitation positively charged ions flow into the cell or negatively charged ions out from the cells, where as in inhibition positive ions flow out from the cell and negative ions into the cell. Excitatory ionotropic receptors are AMPA-, N-methyl-D-aspartate (NMDA)- and kainate-type of glutamate receptors, nicotinic acetylcholine receptors, 5-HT_3-receptors and P2X receptors and inhibitory receptors are GABA-A/C and glycine receptors.

In excitation, ion flow depolarizes the neurons. Neuronal membrane potential in resting conditions is normally around –70 mV. Excitation, therefore, changes the membrane potential towards the lower voltage values, towards the action potential firing threshold. Excitatory synapses are located in dendrites from where the depolarizing pulses must travel to the soma and reach the action potential firing zone at the axon hillock where an action potential is initiated, which is the ultimate goal of excitation. In dendrites, excitatory and inhibitory inputs coming from different neurons can sum up or cancel each other out, which is the basis of computational information processes of neural networks. The majority of excitation in the brain is mediated by the neurotransmitter glutamate. All ionotropic glutamate receptors are cation selective ion channels, which are permeable to Na^+ and K^+ and in the case of NMDA receptors and AMPA receptors lacking GluR-B subunit also to Ca^{2+}. The net movement of these ions results to depolarization. In addition, the Ca^{2+} entry has many roles in the regulation of cell function.

Inhibition makes the cell less likely to fire an action potential. Inhibition can be hyperpolarizing or shunting. In hyperpolarizing inhibition the cell membrane shifts to more negative potential from the resting membrane potential, like in the case of $GABA_A$-receptor transmission in mature brain. In shunting inhibition, the cell membrane potential shifts to less negative potential, but not to the action potential firing threshold and, in addition, the cells input

conductance raises, which means that the cell needs more excitation to reach the firing threshold. Inhibition is also divided into phasic and tonic inhibition on the basis of receptor's synaptic or extrasynaptic localization, respectively. Phasic inhibition occurs in synapses and prevents the neurons from being excited too much during excitatory transmission and is also used to generation of rhythmic activities in neuronal networks. Tonic inhibition is carried out by $GABA_A$ receptors that are activated by spill-over of synaptically released GABA and is important, in some brain areas, in fine tuning the neuronal nets to respond only to a certain size and type of excitatory inputs (Farrant and Nusser, 2005).

2.3. Balance of Neurotransmission

In the brain, excitation and inhibition are often mediated by two neurotransmitter systems, glutamate and GABA, respectively. Neuronal information processing is thought to be controlled by integration and summation of both of them in synaptic function (Li et al., 2006; Schummers et al., 2002). Therefore, a tight control between them must be maintained all the time. The relative contribution of molecular and cellular components to the balance of the excitation and inhibition may vary between the brain areas. For example, in the cerebellum, the high level of intrinsic inhibition is balanced by mainly excitatory afferents from various brain regions (Bower, 2002). Whereas various excitatory feedback loops in the hippocampus are balanced by several classes of interneurons to avoid hypersynchronicity and epileptiform activity (Levinson and El-Husseini, 2005; Mann et al., 2005). It is not currently clear how neurons balance the excitation and inhibition at a molecular level. However, overexpression and knock down experiments have revealed important proteins controlling synapse formation. Recent evidences suggest that two main groups in the regulation of formation of excitatory synapses are postsynaptic scaffolding proteins and cell adhesion molecules (CAMs). Postsynaptic density proteins include members of the PSD-95 family, shank and homer, which have been shown to promote excitatory synapse maturation (reviewed in Kim and Sheng, 2004). Important cell adhesion molecules in excitatory synapse formation are postsynaptic neuroligins and predominantly presynaptically expressed neurexins (Dean and Dresbach, 2006).

The most abundant postsynaptic scaffold molecule that is enriched at glutamatergic synapses is Postsynaptic density protein of 95 kDa, (PSD-95; also known as synapse associated protein 90, SAP90), a member of membrane associated guanylate kinase (MAGUK) family. PSD-95 keeps AMPA receptors at synapses by connecting to stargazin, or other members of transmembrane AMPA receptor regulatory proteins family (TARPs), which associate directly with AMPA receptors (Bats et al., 2007; Chen et al., 2000; Tomita et al., 2005). A role for PSD-95 in regulation of AMPA receptor trafficking and retention at the synapses has been reported in several studies. The first evidence that PSD-95 increases AMPA receptor recruitment and function at the synapses, came from cultured hippocampal neurons overexpressing PSD-95 (El-Husseini et al., 2000). This effect of PSD-95 was specific to AMPA receptors, since NMDA receptors were not affected. These results were surprising, since PSD-95 was not known to link to AMPA receptors,

but to directly interact with NMDA receptors. This discrepancy was solved later when the role of stargazin and other TARPs as auxiliary AMPA receptor subunits was established (Chen et al., 2000). In addition, knocking down PSD-95 reduced excitation as compared to inhibition (Prange et al., 2004).

Several studies have established that postsynaptically expressed CAM family neuroligins (neurogilin-1, 2 and 3 are expressed in brain) are important mediators of the formation of excitatory synapses in synaptogenesis and that they bind to PSD-95 protein with their cytoplasmic tail. Neuroligins seem to influence the maturation of both excitatory and inhibitory synapses (Prange et al., 2004; Levinson et al., 2005), but they show type specificity, since neuroligins 1 and 3 are predominantly localized to excitatory synapses, while neuroligin 2 is localized to inhibitory synapses (Chih et al., 2006; Graf et al., 2004; Levinson et al., 2005; Prange et al., 2004; Song et al., 1999). The binding partners of neuroligins are mainly presynaptically expressed neurexins. Several studies suggest that neurexins act as the presynaptic receptor for neuroligins to induce presynaptic protein recruitment and formation of presynaptic terminals (Dean et al., 2003; Scheiffele et al., 2000). Neurexins bind with their intracellular tail to presynaptic proteins, like CASK, which is a PDZ domain-containing protein related to PSD-95. Through this interaction, neurexins are coupled to vesicle fusion machinery (Biederer and Sudhof, 2000; Butz et al., 1998; Hata et al., 1996). Thus, the interaction between neuroligins and neurexins may activate the structural formation of presynaptic protein complexes.

Currently, the formation of inhibitory synapses is not as well studied as the formation of excitatory synapses, which itself is still poorly understood. In inhibitory synapses, a key scaffold protein seems to be gephyrin. Gephyrin plays a significant role in the development of both GABA and glycine synapses. In hippocampal cultures made from gephyrin knockout mice, the clustering of glycine receptors to the synapses was completely abolished and the clustering of $GABA_A$ receptors was decreased significantly as compared to wild type cultures (Levi et al., 2004). Although gephyrin is not needed for the formation of GABA synapses, overexpression of kimeric gephyrin containing bacterial and vertebrate domains induces inhibitory synapse formation and inhibits the formation of excitatory synapses (Lardi-Studler et al., 2007).

Synapses may compete for CAMs to regulate the type and strength of synapses formed. For instance, when GABA activity is absent in isolated hippocampal cells a mismatch of presynaptic and postsynaptic components occurs (Rao et al., 2000). Gephyrin is then mistargeted to glutamatergic synapses, possibly due to lack of appropriate inhibitory synapse scaffolding and adhesion molecules. Furthermore, overexpression of gephyrin increases $GABA_A$ receptor clustering while reducing the number of excitatory PSD-95 clusters (Lardi-Studler et al., 2007).

In the healthy brain, a balance of excitation and inhibition is essential for the proper function, including representation of sensory information, cognitive processes such as decision making, sleep and motor control. An imbalance of excitation and inhibition is suggested to contribute to the development of several diseases including epilepsy (Brenner, 2004; Clancy and Kass, 2004; McCormick and Contreras, 2001; Stafstrom, 2006), schizophrenia (Konradi and Heckers, 2003; Lewis and Levitt, 2002; Lewis and Moghaddam, 2006), Fragile X syndrome (Huber et al., 2002; Weiler et al., 1997) and autism (Andres, 2002; Rubenstein and Merzenich, 2003).

2.3.1 Synaptic Scaling

Neurons tend to balance their excitatory and inhibitory synaptic inputs to a appropriate functional level in a process called synaptic scaling. The strength of individual synapses is modulated to compensate for changes occurring in other synapses in the same neuron that would otherwise affect the cellular activity of whole neuron. Synaptic scaling can be seen as a homeostatic counter force for the synaptic plasticity. The relative strengths of individual synapses appear to be maintained, thus preserving the plastic changes in synaptic strength. Without this counter force, LTP would cause a domino-like effect which would feed forward stronger changes in the next neurons in the line of information flow. On the other hand, LTD would silence the subsequent neurons.

Synaptic scaling has been shown to occur in studies, where either inhibition or excitation has been manipulated in various ways. For instance, a block of neuronal activity by tetrodotoxin (TTX) for 48 h in cultured cortical neurons caused an increase in miniature excitatory postsynaptic currents (mEPSC), which is probably a compensatory sensitization for decreased glutamatergic activity (Turrigiano et al., 1998). On the other hand, the increase of the function of AMPA receptors by ampakines caused a decrease in AMPA receptor subunit expression accompanied by the decrease of synaptic scaffolding proteins SAP97 and GRIP1 (Jourdi et al., 2005). Chronic ethanol, which causes increased $GABA_A$ receptor function, and thus inhibits neuronal activity, is compensated by an increased NMDA receptor expression accompanied by increase in PSD-95 expression and dendritic spine size (Carpenter-Hyland and Chandler, 2006). Elevation of the $GABA_A$ receptor- mediated inhibition by chronically administering a benzodiazepine agonist, flurazepam, for one week has been shown also to elevate AMPA receptor function in CA1 neurons in rat hippocampus (Van Sickle and Tietz, 2002). Logically, chronic flurazepam treatment decreased the $GABA_A$ receptors expression, but surprisingly, NMDA receptor expression was also decreased (Van Sickle et al., 2002).

As seen in the examples mentioned above, synaptic scaling often seems to involve changes in the number of AMPARs (and NMDARs) at individual synapses (Perez-Otano and Ehlers, 2005; Turrigiano and Nelson, 2004; Watt et al., 2000) and likely presynaptic changes as well (Burrone et al., 2002; Burrone and Murthy, 2003). Molecular mechanisms underlying these changes are not well understood. Recent evidence suggest that secreted factors have a role in the induction of homeostatic plasticity, suggesting that key triggers for this form of plasticity may come from outside of the neuron. Specifically, proinflammatory cytokine tumor necrosis factor-α from glial cells appears to be necessary for the increase in the level of synaptic AMPARs during extended periods of activity blockade (Beattie et al., 2002; Stellwagen and Malenka, 2006; Stellwagen et al., 2005). In addition, there is evidence suggesting a role for secreted brain-derived neurotrophic factor (BDNF) in the opposite form of synaptic scaling, the decrease in synaptic strengths caused by extended periods of increased network acitivity (Rutherford et al., 1998; Turrigiano, 2006).

2.4. Phasic and Tonic Inhibition

Phasic inhibition refers to synaptic inhibition and tonic inhibition to extrasynaptic inhibition. Phasic inhibition occurs when inhibitory synaptic receptors are activated as a response to presynaptic vesicle release, which is precisely timed event. Tonic inhibition, on the other hand, is mediated by extrasynaptic receptors, which are activated by minute concentration of ambient neurotransmitter leaking out of the synapses and possibly released by astrocytes. Most of the research focusing on tonic inhibition has concentrated on extrasynaptic $GABA_A$ receptors. Substantial amount of tonic conductance exists in the cerebellar granule cells (Brickley et al., 1996), granule cells of the hippocampal dentate gyrus (Nusser and Mody, 2002), thalamocortical relay neurons of the ventral basal complex, (Porcello et al., 2003), CA1 pyramidal cells (Bai et al., 2001) and in certain inhibitory interneurons in CA1 of the hippocampus (Semyanov et al., 2003).

2.4.1 Function of Phasic Inhibition

The central function of synaptic inhibition is the fine tuning of excitation and to prevent the neurons from being excited too much, which might lead to the development of pathological conditions, such as epilepsy. In addition to this, GABAergic interneurons generate and maintain rhythmic activities, like theta and gamma oscillations, in the hippocampus (Cobb et al., 1995; Jonas et al., 2004; Somogyi and Klausberger, 2005), thalamus (Huntsman et al., 1999) and olfactory bulb (Laurent, 2002). The impact of phasic inhibition on excitation depends of the location of GABAergic synapses along the somatodentritic axis. Inhibition is most effective when the synapses are located near the soma region, whereas synapses located more distally in dendrites allow more precise fine tuning of specific excitatory inputs.

2.4.2 Function of Tonic Inhibition

The function of tonic inhibition manifests most clearly in the electrical properties of the cell membrane. Tonic inhibition increases the input conductance of the cell. This affects the magnitude and duration of the voltage responses that the cell receives, and increases the attenuation of the depolarizing voltage pulses with distance. When the tonic inhibition is high, the EPSCs that are received by the neuron cause shorter and smaller potential changes which shortens the window of time during which the summation of excitation coming from different synapses can happen. This reduces the likelihood of the action potential firing, and increases the need for synchronous excitation. In cerebellar granule cells tonic inhibition has been shown to shift the input current intensity vs. firing output curve to the right, which means that more current is needed to achieve a given firing rate (Brickley et al., 1996; Chadderton et al., 2004; Hamann et al., 2002).

2.4.3. $GABA_A$ Receptor Localization Depends on Subunit Composition

The location of $GABA_A$ receptors in either synapses or extrasynaptic cell membrane depends on the subunit composition. To date nineteen mammalian ionotropic GABA receptor subunits have been cloned: α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3. Native pentameric $GABA_A$ receptors are usually formed such that they have two α and two β subunits with one γ, δ, ε, θ or π (reviewed in Sieghart and Sperk, 2002). The ρ1-3 form $GABA_C$ receptors. In theory, there are numerous ways of how $GABA_A$ receptors can assembly, but in practice only a few dozen have been shown to exist suggesting that there are brain region-specific expression patterns for subunits and that some sort of rules of assembly exist (Fritschy and Mohler, 1995; Pirker et al., 2000; Wisden et al., 1992). All $GABA_A$ Receptor subtypes are found in large numbers in extrasynaptic membranes, but only a certain subtypes are located synaptically (Nusser et al., 1995). The γ2 subunit seems to be important for synaptic clustering of $GABA_A$ receptors, since the deletion of γ2 leads to a marked reduction in the clustering of both $GABA_A$ receptors and inhibitory synapse related protein gephyrin (Essrich et al., 1998). A subunit that is exclusively related to the extrasynaptic localization of $GABA_A$ receptors is δ (Nusser et al., 1998). In addition, α4, α5 and α6 containing receptors seem to be predominantly extrasynaptic (Brickley et al., 2001; Caraiscos et al., 2004a; Peng et al., 2002).

The subunit composition of $GABA_A$ receptors profoundly affects the binding and gating properties of the receptor. The agonist binding sensitivity is mostly affected by α subunit, with α3 conferring the highest and α6 to the lowest EC_{50} values. The δ subunit seems to increase GABA sensitivity in some cases, such as when γ2 is changed to δ in α4β3γ2 receptor (Brown et al., 2002), but it does not make any difference within α1β3γ2 and α6β3γ2 assemblies (Feng and Macdonald, 2004). In addition, extrasynaptic $GABA_A$ receptors activate after a delay during the GABA exposure and the single channel conductance increses gradually indicating that some still unknown mechanism increases GABA sensitivity (Lindquist and Birnir, 2006). The increase in conductance is seen in cell-attach recordings but not in outside-out patches indicating that a soluble intracellular factor or process, such as kinases, phosphatases or binding proteins are required for the phenomenon (reviewed in Birnir and Korpi, 2007).

Single channel conductance of $GABA_A$ receptors depends on subunit composition. Receptors formed only from α and β subunits have a low single channel conductance of around 15 pS, but when either γ or δ is incorporated to receptors, the conductance raises to around 25-28 pS (Angelotti and Macdonald, 1993; Fisher and Macdonald, 1997). Most $GABA_A$ receptors in mature neurons probably contain either γ or δ subunit, so there is no difference between extrasynaptic and synaptic receptors in single channel conductance. However, synaptic receptors seem to have higher mean open time and duration of burst channel opening and replacing γ2 with δ reduces both (Fisher and Macdonald, 1997).

$GABA_A$ receptor desensitization is also affected by subunit composition. Adding γ2 to αβ receptors slows the macroscopic desensitization (Benkwitz et al., 2004; Boileau et al., 2003). This decreases the possibility that synaptic receptors are partially desensitized and not in fully prepared state to receive signals. Extrasynaptic δ-containing receptors desensitize more slowly than synaptic γ2-containing receptors (Haas and Macdonald, 1999). For both αβγ and αβδ, the rate of

desensitization is dependent on the type of α subunit. α1βγ types desensitize more rapidly than α5βγ (Caraiscos et al., 2004a) or α6βγ types (Tia et al., 1996). However, when α1 is substituted by α6 in αβδ receptors the desensitization becomes faster and more extensive (Bianchi et al., 2002).

2.4.4. Modulation of Phasic and Tonic Inhibition

Phasic and tonic inhibition are differently affected by endogenous modulators and drugs. The differences are due to differences in subunit content between synaptic and extrasynaptic cell membranes. For instance, benzodiazepines have an effect on $GABA_A$ receptors if the subtypes contain α1, α2, α3 or α5 together with two β and γ2. Therefore, phasic inhibition is generally affected by benzodiazepines, whereas tonic inhibition is affected only in the brain areas where substantial number of extrasynaptic receptors are composed of benzodiazepine-sensitive α and γ2 subunits (Bai et al., 2001; Caraiscos et al., 2004a; Hamann et al., 2002; Nusser and Mody, 2002; Semyanov et al., 2003; Yeung et al., 2003). An agonist that activates more extrasynaptic than synaptic receptors is 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridin-3-ol (gaboxadol, THIP), which is a partial agonist of α4β3γ2 receptors, but full or "super" agonist of α4β3δ (Belelli et al., 2005; Chandra et al., 2006; Cope et al., 2005).

Tonic inhibition seems to be highly sensitive to various clinically relevant agents, for example, endogenous neuroactive steroids, intravenous and inhalation anaesthetics and ethanol. The function of $GABA_A$ receptors containing δ subunit are selectively enhanced by neurosteroids (Belelli et al., 2002; Brown et al., 2002). In brain slice experiments low concentrations of 3α,21-dihydroxy-5α-pregnan-20-one (THDOC) increases the tonic conductance without affecting the phasic currents in granule cells of dentate gyrus and cerebellum (Stell et al., 2003). In cultured hippocampal neurons the tonic conductance has been shown to be enhanced by anaesthetics isoflurane (Caraiscos et al., 2004b) and propofol (Bai et al., 2001). Ethanol has been long suggested to enhance the function of $GABA_A$ receptors, but there has been great controversy about the subject: The results have not been reproducible between laboratories (Korpi et al., 2007). A few studies show that ethanol might affect extrasynaptic receptors. The mainly extrasynaptically located α4/α6β3δ-containing $GABA_A$ receptors have been reported to be sensitive to low concentrations of ethanol when expressed in oocytes (Wallner et al., 2003; Sundstrom-Poromaa et al., 2002). There are also some studies showing that native $GABA_A$ receptors are sensitive to ethanol. Low concentrations of ethanol have been shown to enhance the tonic inhibition in hippocampal slices (Wei et al., 2004) and GABA-evoked currents in isolated hippocampal neurons (Sundstrom-Poromaa et al., 2002).

2.4.5. Thy1α6 Mice as a Model of Increased Tonic Inhibition

Tonic conductance is very demanding to measure electrophysiologically, because its amplitude is generally very small. Tonic inhibition appears in patch clamp recordings as a higher cell membrane conductance, which shows itself as higher holding current and current trace noise (Kaneda et al.,

1995). Tonic conductance can be blocked by saturating concentration of $GABA_A$ antagonist and the difference in current levels before and after antagonist application is the amplitude of the tonic conductance. In some brain areas, like CA1 of the hippocampus, the measurement of tonic conductance requires the usage of GABA uptake blockers or addition of low concentration (100 nM to 300nM) of GABA in the recording solution (Wallner et al., 2006).

Thy1α6 is a mouse model of increased tonic conductance. The mice overexpress $GABA_A$ receptor α6 subunit under the thy1.2 promoter in various forebrain brain areas including CA1 of the hippocampus. This leads to an increase of extrasynaptic $GABA_A$ receptors. Pyramidal neurons of CA1 area of the hippocampus of these mice have a fivefold increase in tonic conductance as compared to wild types and, surprisingly, about 30 % decreased synaptic $GABA_A$ activity measured as mIPSC frequency and amplitude (Wisden et al., 2002). This animal model has been used to study the effect of gaboxadol on anxiolysis and sedation (Saarelainen et al., 2008). Gaboxadol has a strong anxiolytic effect in Thy1α6 mice for the light : dark exploration and elevated plus-maze tests at doses which did not show any effect with wild types. In addition, gaboxadol also had a stronger effect on loss of the righting reflex in Thy1α6 mice than wild type controls. The results suggest that tonic inhibition can play a role in anxiolysis and in sedation, offering a possible target for medical treatment of anxiety and sleep disorder.

Thy1α6 mice have altered convulsion sensitivity. They are more prone to convulsions, which are induced by $GABA_A$ receptor inverse agonist DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) and $GABA_A$ receptor antagonist picrotoxinin (Sinkkonen et al., 2004). On the other hand, GABA uptake inhibitor tiagapine delayed the onset of DMCM and picrotoxinin induced convulsions in Thy1α6 mice but not in wild types (Sinkkonen et al., 2004). The results suggest that synaptic inhibition is more efficient in protecting the mice from convulsions induced by $GABA_A$ receptor blocking drugs. However, are these mice also more sensitive to electroshock convulsions, which probably involve AMPA receptor-mediated function? AMPA receptors have been shown to have a role in electroshock convulsions, since these type of convulsions are inhibited by administration of AMPA receptor inhibitors (Micale et al., 2002; Yamashita et al., 2004). Another interesting question about Thy1α6 mice is: How is AMPA receptor-mediated synaptic function as compared to wild type mice? Are synaptic scaling processes scaling down the AMPA receptor function to match the level of decreased synaptic $GABA_A$ function? One aim of our research was to clarify the status of AMPA receptor driven excitation in the hippocampus of Thy1α6 mice.

Summary. Phasic inhibition is mediated by synaptic $GABA_A$ receptors and it participates in fast synaptic neurotransmission counterbalancing the fast synaptic excitation. Phasic inhibition also has roles of its own in the creation of oscillatory activity in some brain areas. Tonic inhibition is mediated by extrasynaptic receptors and is activated by low ambient concentration of GABA spilling over from synapses. Tonic inhibition increases the input conductance of neurons, thus increasing the currents that are needed to generate action potentials. Subunit composition primarily defines mostly which receptors are located in synapses, with certain subtypes being exclusively extrasynaptic. The different subunit composition between the extrasynaptic and synaptic receptors

also confers different drug sensitivities. The functional properties of synaptic and extrasynaptic receptors correlate well with their physiological roles. Synaptic receptors have lower GABA sensitivity, as compared to extrasynaptic receptors, and GABA has a high efficacy on them. On the other hand, extrasynaptic receptors have a high binding affinity for GABA, but GABA has a low efficacy on them. In addition, extrasynaptic receptors are modulated by endogenous signal molecules, like neurosteroids, which allows the modulation of large neural networks on a relatively fast time scale, in other words without the synthesis of new proteins. Synaptic scaling is an important process that balances the alterations in synaptic strength, keeping the overall activity of the neuron at the functionally relevant level.

2.5 Ligand-gated Ion Channels

In this thesis work, the function of one type of ligand-gated ion channels, AMPA receptors, are studied in various pharmacological and physiological situations. Ligand-gated ion channels (LGICs) a.k.a. ionotropic receptors, mediate fast postsynaptic potential changes. LGICs are found both in peripheral and central nervous systems, where they mediate various functions. For instance, they function to get the skeletal muscles to contract and allow signals to travel from neuron to neuron. They are found on the cell membrane, usually in close proximity to the release site of neurotransmitter, and are opened when agonist binds to them permitting the movement of ions through the cell membrane. LGICs are divided into three ligand-gated ion channel superfamilies. The Cys-loop LGIC-superfamily is comprised of nicotinic acetylcholine, $GABA_A$, $GABA_C$, glycine and $5\text{-}HT_3$ receptors. They are composed of five protein subunits and possess a characteristic loop formed by a disulfide bond between two cysteine residues. Ionotropic glutamate receptors form the second LGIC-superfamily, which includes AMPA, kainate and NMDA receptors that are composed of four subunits (Rosenmund et al., 1998). Each subunit has three membrane-spanning α-helixes and one re-entrant membrane loop. The third family, ATP-gated channels (P2X receptors), are composed of three subunits, each subunit having two plasma membrane spanning domains, a large extracellular loop and intracellular carboxyl and amino termini.

2.5.1 Desensitization of LGICs

One key feature of LGICs is desensitization. Desensitization is defined as the state of the receptor where the ion channel is closed in the presence of agonist molecule in the binding pocket of the receptor. Desensitization of LGICs is seen in electrophysiological recordings as a peak current decay during relatively long agonist applications (**Fig. 1A**). In this type of recording the current first has a clear peak component followed by a steady state current. The peak component results from a large number of receptors opening simultaneously due to rapid application of high concentration of agonist. Shortly after the current peaks, receptors start to desensitize leading to decay phase of the peak current. Single or polynomial fit of peak current decay can be used to determine a time constant of desensitization, τ-value, which is a measure of the rate of desensitization. During steady

state current the individual receptors are either open or desensitized. The proportion of open channels can be estimated by comparing the steady state current amplitude to peak current amplitude, which produces the SS/peak-ratio, a measurement of extent of desensitization (**Fig. 1B**). SS/peak ration and τ-value of desensitization varies greatly between different receptors and differing subunit compositions. It should be mentioned that the measurement of exact peak current by electrophysiological recording techniques is very demanding and requires conditions where the agonist application occurs extremely fast. If the agonist application is slower, the peak current results from a receptor pool containing some desensitized receptors. Molecular mechanisms of AMPA receptor desensitization have been clarified with X-ray crystallography and are discussed in chapter 2.8.6. Mechanisms of AMPA Receptor Activation and Desensitization and illustrated in figure 5.

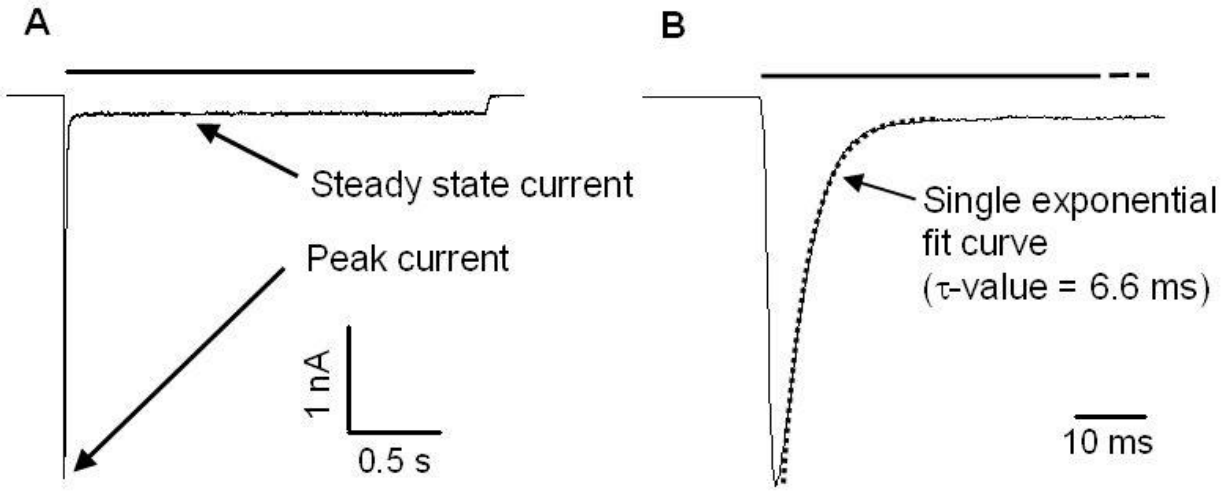

Figure 1. desensitization of LGIC; GluR-D_i receptors. **A)** example trace of patch clamp recording of GluR-D_i AMPA receptors expressed in HEK293 cells. In the figure the different current components, peak and steady state current are shown. Horizontal bar indicates application of 10 mM glutamate. **B)** shows the peak component of the current trace enlarged in order to illustrate the determination of the time constant of desensitization (τ-value) from peak current decay with single exponential fit (dashed line). The SS/peak ratio, a measure of the extent of desensitization, is the relationship of steady state current amplitude to peak current amplitude, in this case 170 pA / 3696 pA = 0.046.

2.6 Glutamate as a Neurotransmitter

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) acting through both ligand-gated ion channels and G-protein coupled receptors. Major glutamatergic pathways in the brain are the cortico-cortical pathways, the pathways between the thalamus and the cortex, and the extrapyramidal pathway (the projections between the cortex and striatum). Important glutamatergic projections exist also between the cortex, substantia nigra, subthalamic nucleus and pallidum (Carlsson, 1995).

L-Glutamate can be termed a work-horse of the vertebrate central nervous system, since probably every central neuron receives glutamatergic excitation. Glutamate receptors participate in fast synaptic neurotransmission, but they also have a major role in the plastic changes in the efficacy of synaptic transmission thought to underlying learning and memory, and the formation of neuronal networks during development. For instance, AMPA receptors are constitutively recycled in and out of cell membrane, and their number can be rapidly changed to produce alterations in synaptic strength like, long term potentiation (LTP) and long term depression (LTD) (Esteban, 2003). Metabotropic glutamate receptors modulate the function of glutamatergic and also other synapses (Ferraguti and Shigemoto, 2006; Swanson et al., 2005).

Neuronal glutamate is synthesised by astrocytes and neurons in a metabolic cycle called glutamate/GABA- glutamine cycle (**Fig. 2**) (Bak et al., 2006). Neurons are unable to synthesize glutamate *de novo* from glucose and need help from astrocytes to get their glutamate in the form of glutamine. In the synthesis cycle astrocytes take up the glutamate that is released from the glutamatergic neurons during neurotransmission and transform it to glutamine by astrosyte-specific glutamine synthetase enzyme. Glutamine is then released into the extracellular space from where it is taken up by glutamatergic neurons and transformed back to glutamate by phosphate activated glutaminase. Interestingly, astrocytes and glutamine have also a similar role in synthesis pathway of GABA. Astrocytes take up GABA from extracellular space and cycle that through tricarboxylic acid cycle to produce glutamate and then glutamine. Glutamine is released back to extracellular space and taken up by GABAergic neurons, where it is converted first to glutamate and then to GABA by glutamate decarboxylase. Astrocytes are the cells predominantly responsible for extrasynaptic glutamate clearance. The most important glutamate uptake molecules are astroglial excitatory amino acid transporter 1 and 2 (EAAT1 and 2), which are considered to be largely responsible for glutamate up-take throughout the brain (Danbolt, 2001). The other uptake transporters are EAAT3-5, which have regional significance in glutamate clearance. Glutamatergic neurons are dependent on astrocytes to get their glutamate, since they do not express enough glutamate transporters of their own to supply the demand of glutamate.

2.7 Glutamate Receptors

2.7.1 AMPA Receptors

AMPA receptors have four subunits, which are transcribed from genes which are located in humans in 4, 5, 11 and X chromosomes (**Fig. 3**). Alternative splicing and RNA editing increase the variation of subunits. A functional receptor is composed of four subunits and is usually heteromeric, although GluR-A homomers are thought to exist during the synaptic plasticity (Derkach et al., 2007; Wenthold et al., 1996). Molecular domains of receptor consist of a large extracellular N-terminus, an extracellular loop, three transmembrane spanning helixes (TM1, 3 and 4), a membrane re-entrant loop (TM2), and intracellular C-terminus. Glutamate binding domain is formed as a combination of part of the extracellular N-terminus, also called as ligand binding domain 1 (S1) and an extracellular

loop between TM3 and TM4, called as ligand binding domain 2 (S2). AMPA receptor subunits have several unique features, which alter their function and physiological properties, which are discussed below in the chapter 2.8 Characteristics of AMPA receptors. Figure 4 shows a schematic presentation of AMPA receptor structure.

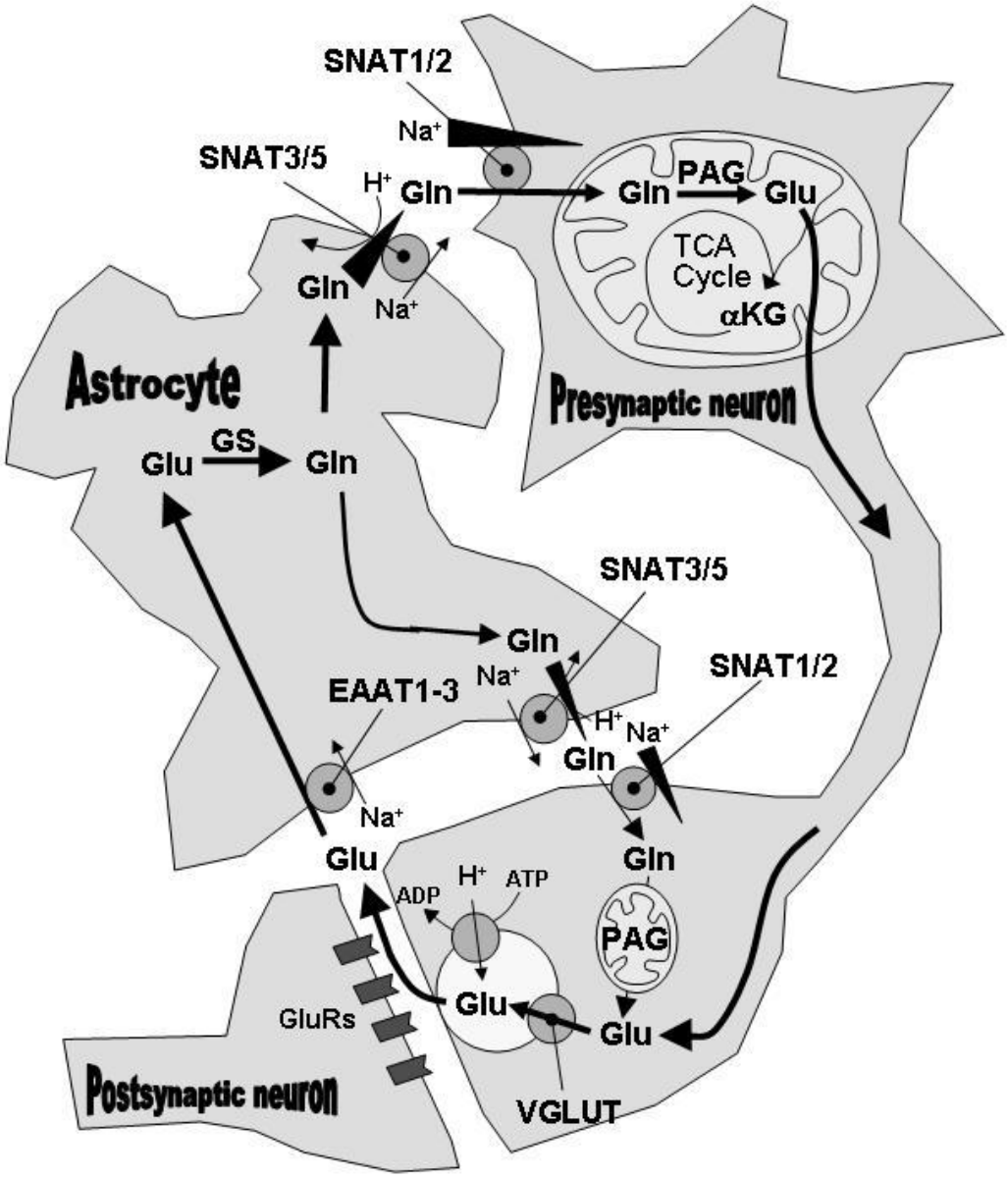

Figure 2. Glutamate-glutamine cycle. Astrocytes take up the glutamate (Glu) released from neurons during the neurotransmission. The uptake is done by excitatory amino acid transporters (EAATs), which also clear glutamate from extracellular space. In the astrocytes glutamate is converted to glutamine (Gln) by enzyme glutamine synthetase (GS). Glutamine is then transported to extracellular space and then to neuron by the activity of sodium-coupled neutral amino acid (System N/A) transporters (SNAT) (Mackenzie and Erickson, 2004). Glutamine is converted back to glutamate by mitochondrial phosphate-activated glutaminase (PAG). Glutamate is then packed in the synaptic vesicles by vesicular glutamate transporters, (VGLUTs) after which glutamate is ready for release. TCA = tricarboxylic acid; GluRs = postsynaptic glutamate receptors.

2.7.2 NMDA Receptors

NMDA is a full agonist of ionotropic glutamate receptor subgroup, NMDA receptors. They are abundantly distributed through the CNS in excitatory synapses along with AMPA receptors. The NMDA receptors assemble as a combination of two mandatory NR1 subunits and two NR2A-NR2D subunits (**Fig. 3**). In order for the NMDA receptor channel to open both glutamate and co-agonist glycine (or D-serine) are required to bind to NR2 subunits and NR1 subunits, respectively. NMDA receptors also possess binding sites for polyamines and protons (McBain and Mayer, 1994). NMDA receptors have also NR3A and B subunits, which lower the receptor currents, reduce Mg^{2+} block and reduce the inflow of Ca^{2+}(Cavara and Hollmann, 2008).

The function of NMDA receptors differs from AMPA receptors in two ways which have important physiological consequences. First, during normal neurotransmission, they are silent, because Mg^{2+} blocks the channel. Mg^{2+}-block is removed during strong synaptic activation (depolarization), making NMDA receptors a postsynaptic activity sensor able to detect neurotransmission which is above of certain strength. Secondly, NMDA receptors let Ca^{2+} flow into the cell, which activates downstream intracellular Ca^{2+} sensing machinery, which may lead to alteration of the strength of synapse, in other words to LTP or LTD. The role of NMDA receptors is well characterized in the prototypic form of synaptic plasticity, NMDA receptor-dependent LTP. It is initiated after strong Ca^{2+} entry and the subsequent activation of intracellular kinases, most importantly calmodulin-dependent kinase II (CaMKII). NMDA receptor-dependent LTD, on the other hand, follows after moderate elevation of intracellular Ca^{2+} leading to preferential activation of protein phosphatases, which leads to dephosphorylation of proteins in postsynaptic density (PSD) leading to removal of AMPA receptors from the synapses (Dudek and Bear, 1992).

2.7.3 Kainate Receptors

Kainic acid, or kainate, is a partial agonist of AMPA receptors, but a full agonist of the closely related ionotropic glutamate receptor subgroup, called kainate receptors. Kainate receptors are abundantly expressed throughout the CNS, located in both pre- and postsynaptic compartments. The physiological role of kainate receptors is not as well understood as that of AMPA and NMDA receptors. Presynaptic kainate receptors can rapidly regulate the glutamate release and therefore affect the short-term dynamics of synaptic transmission. Kainate receptors have been found to increase glutamate release in mossy fiber synapses in the hippocampus and function as a source for calcium entry initiating LTP formation (Bortolotto et al., 2003). During early development of CA3-CA1 synapses in the hippocampus, kainate receptors lower the glutamate release probability and therefore set the synapses to respond to high frequency burst activity typical to developing synaptic connection (Lauri et al., 2006).

Native kainate receptors are composed either homomeric or heteromeric KA1, 2, GluR-5, -6 and -7 subunits. GluR5-7 have 75-80 % amino acid identity with each other and 40 % with GluR-A-D AMPA receptors. KA1 and 2 share 70 % amino acid identity with each other and 40 % with GluR5-7 kainate receptor subunit and GluR-A-D AMPA receptor subunits. Similarly to

GluR-B (see below), GluR5 and GluR6, but not GluR7, have a Q/R-RNA editing site in the M2 segment. In contrast to GluR-B, the Q/R site editing is incomplete during development, and significant amounts of both edited and unedited versions coexist in adult brain. There are two additional editing sites in M1 of GluR6 subunit, [I(isoleucine)/V(valine) and Y(tyrosine)/ C(cysteine)]. Alternative splicing of GluR5 further adds to receptor diversity (reviewed in Seeburg, 1993).

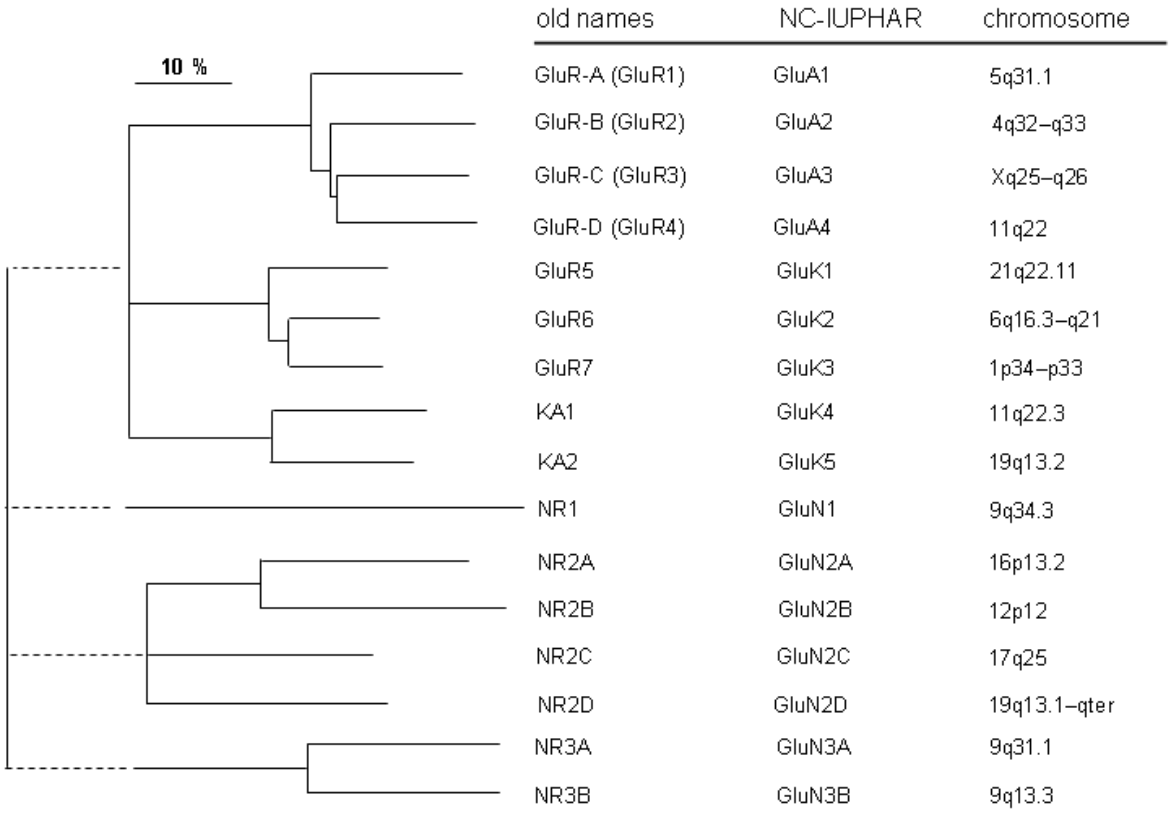

Figure 3. Dendrogram of the members of the ionotropic glutamate receptor family. Amino acid identity between the subunits can be calculated subtracting 100% minus the sum of the length of the horizontal solid lines between the two subunits. For example, the % identity in the amino-acid sequence between GluR-A and GluR-B is the distance of GluR-A and GluR-B to each nearest node (15% and 14%), respectively on the dendrogram, and the distance between these two nodes is 2%, resulting in a total of 15%+ 14%+ 2%=31%. Therefore, the identity in the amino-acid sequence between them is (100% - 31%)= 69%. Dashed line indicates low (ca. 20 %) amino-acid identity between subunit groups which are not used for estimating the distance. NC-IUPHAR stands for nomenclature committee of The International Union of Basic and Clinical Pharmacology, which have formulated and suggested new names for glutamate receptors (Collingridge, et al., 2009). Figure modified from Ozawa (1998).

2.7.4. Metabotropic Glutamate Receptors

There are eight metabotropic glutamate receptors (mGluR's), which are divided into three subgroups based on their amino acid sequence homology and pharmacological characteristics called group I (contains mGluR1 and 5), II (mGluR2 and 3) and III (mGluR4, 6, 7 and 8) (Conn and Pin,

1997). Group I mGluRs are coupled to phospholipase C, which hydrolyze phosphoinositide producing inositol triphosphatases, which leads to increase in intracellular calcium (Yuzaki and Mikoshiba, 1992). They are also reported to inhibit voltage dependent calcium channels in hippocampus by straight interaction between the activated G-protein and the calcium channel (Lester and Jahr, 1990; Swartz and Bean, 1992). Group II of mGluRs are coupled to the inhibition of synthesis of cyclic AMP by activation of a G-protein that inhibits adenylyl cyclase enzyme (Baba et al., 1993; Prezeau et al., 1994), and they also inhibit calcium channels (Choi and Lovinger, 1996) and activate a G-protein coupled inwardly rectifying K^+-channel (GIRK) (Knoflach and Kemp, 1998). Group III of mGluRs also couple negatively to cAMP production and they also inhibit the neurotransmission by inhibition of presynaptic voltage-dependent calcium channels (Takahashi et al., 1996).

The main function of mGluRs is the control and adjustment of glutamate transmission. They are located in pre- and postsynaptic side of both excitatory and inhibitory synapses, sometimes far away from release sites, and also in astrocytes (illustrated in Swanson et al., 2005). They also participate in plastic changes of synaptic strength, such as mGlu dependent LTP in the hippocampus (Bellone and Luscher, 2005) and LTD in the cerebellum (Lante et al., 2006) to name a couple of examples. Activation of mGluRs by synaptically released glutamate may sometimes require high-frequency or repetitive stimulation, because receptors are located extrasynaptically on axons and dendrites. Receptors might be activated by the glutamate spilling over from release sites from far away making possible the cross talk between different synapsies, even between the different pathways. The location of mGluRs differs between the subunits.

Metabotropic glutamate receptors are not currently target molecules of any drugs, but they are studies as a potential targets to treat of several diseases. Blockage of group I mGluRs and activation of group II mGluRs are studied as a treatment for Parkinson's disease, as they control glutamatergic and cholinergic neurotransmission in striatum (Bonsi et al., 2007). mGluR receptor acting drugs are also studied as a treatment for stress and anxiety disorder. Agonists for group II mGlu receptors and antagonists for group I (in particular mGluR5) receptors have shown activity in animal and/or human conditions of fear, anxiety or stress (Swanson et al., 2005). In addition, a growing body of evidence suggests that metabotropic glutamate receptors also play important roles in the behavioural responses to drugs of abuse and participate in producing synaptic plasticity at glutamate synapses. Many studies show that group I receptors have a role in cocaine-related behaviours (Backstrom and Hyytia, 2006; Chiamulera et al., 2001; Dudek and Bear, 1992; Herzig and Schmidt, 2004; Hu et al., 1999). mGluR5 specific antagonists, MPEP and MTEP, attenuate cocaine-related behaviors such as cue-induced relapse to cocaine seeking (Backstrom and Hyytia, 2006; Chiamulera et al., 2001; Herzig and Schmidt, 2004;). These antagonists also disrupt reinforcing properties of alcohol (Lominac et al., 2006).

Summary. Glutamate is the major excitatory neurotransmitter in the brain. It has three classes of ionotropic receptors, AMPA, NMDA and kainate receptors and eight metabotropic receptors, mGluR1-8. AMPA receptors mediate the fast excitatory neurotransmission between the neurons. The number of synaptic AMPA receptors can be altered rapidly making it possible to adjust the

synaptic strength. NMDA receptors are normally closed by Mg^{2+}-block, but can open during a strong AMPA receptor-mediated depolarization. NMDA receptors are permeable to calcium, which leads to the plastic changes of synaptic strength, like LTP and LTD. Kainate receptors control the activity of glutamatergic cells in some brain areas. Metabotropic receptors modulate the glutamate release and participate also in the induction of LTP and LTD.

2.8 Characteristics of the AMPA Receptors

2.8.1. Splice Variants of AMPA Receptors

All AMPA receptor subunits have two splice variants called "flip and flop", the alternative splicing site being in the extracellular loop near the ligand binding domain S2 (**Fig. 4**). During early development, the flip forms of AMPA receptor subunits are more prominent, whereas in adolescence, the "flip" subunits are replaced by "flop"-containing subunits (Monyer et al., 1991). AMPA receptors containing "flip" subunits generally desensitize with slower kinetics than those with "flop" subunits (Mosbacher et al., 1994).

The second alternative splicing site is the intracellular C-terminus of the GluR-B and GluR-D subunits, which can be expressed either as "long" or as "short" isoforms. The C-terminal splicing pattern is developmentally controlled (Köhler et al., 1994). The amino acid sequence of C-terminal domain of GluR-B_{long} and GluR-D_{long} are related to that of GluR-A. Early in the development of the central nervous system, GluR-B_{long} and GluR-D_{long} might have a similar function in AMPA receptor trafficking as GluR-A in adults (Kolleker et al., 2003). In adults, the prevalent GluR-B transcript codes for the short C-terminal tail (Kolleker et al., 2003). Long and short forms of C-tails bind to different synaptic scaffold proteins and protein kinases. C-tail splice variants have therefore important role in regulation of the appearance and function AMPA receptors in synapses (Barry and Ziff, 2002; Braithwaite et al., 2000; Sheng and Lee, 2001; Song and Huganir, 2002).

2.8.2 Nuclear RNA Editing

Nuclear RNA editing increases the diversity of AMPA receptors in the TM2 region in GluR-B and ligand binding domain 2 in GluR-B, GluR-C and GluR-D (**Fig. 4**). The RNA editing of GluR-B M2 leading to a change of glutamine (Q) to arginine (R) at the protein level, has very important functional consequences since the receptor containing this subunit becomes calcium impermeable. The mechanism of editing is by a nuclear adenosine deaminase, ADAR2 that causes a conversion in the RNA from a codon for glutamine to that for an arginine (Sommer et al., 1991). In the adult brain, most excitatory neurons express AMPA receptors that contain R/Q-edited GluR-B, making them impermeable to calcium (Seeburg et al., 1998). Calcium permeable AMPA receptors are still found in glia cells and GABAergic interneurons (Geiger et al., 1995; Muller et al., 1992). The editing of the other AMPA receptor RNA-editing site at the third extracellular loop, just upstream of the flip/flop cassette, speeds up the recovery from desensitization (**Fig. 4**) (Lomeli et al., 1994).

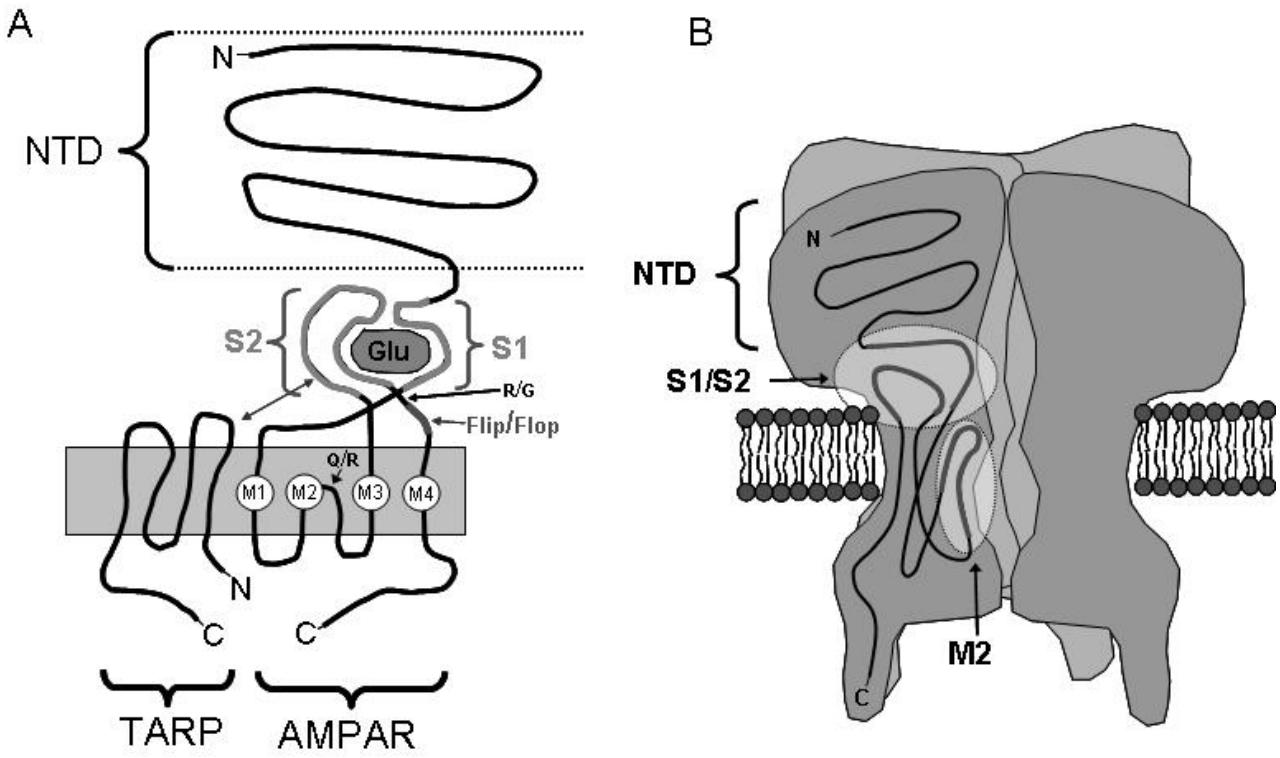

Figure 4 Topology of the AMPA receptor. **(A)** Scheme of AMPA receptor subunit in the plasma membrane in association with a transmembrane AMPA receptor protein (TARP). N-terminal domain (NTD), extracellular ligand binding domains (S1 and S2), transmembrane domains (M1-4), the flip/flop alternative splicing site and the RNA editing sites (Q/R and R/G) are also shown. Two headed arrow indicates the interacting domains between AMPA receptor and TARP. **(B)** Three-dimensional representation of the tetrameric AMPA receptor depicting the arrangement of one subunit within the complex. M2 region, which lines the channel and S1/S2 ligand binding domain are highlighted. Figure modified from Ashby et al. (2007).

2.8.3. AMPA Receptor Distribution

AMPA receptors are distributed ubiquitously throughout the CNS, although regional differences in the distribution are evident. Brain areas rich in AMPA receptors include CA1 and CA3 areas of hippocampus, dentate gyrus and superficial layer of cerebral cortex. Lower levels are found in the diencephalon, midbrain, brainstem and cerebellum. The expression of AMPA receptor subunits differ between the brain areas and developmental stages. GluR-A and GluR-B subunits are ubiquitously expressed in the brain and therefore are probably present in most AMPA receptors in the adult mammalian CNS (Keinänen et al., 1990). GluR-C mRNA is found in hippocampal and cortical cell layers and in the Purkinje cell layer of the cerebellum (Keinänen et al., 1990). GluR-D subunit is expressed mainly in the early development (Zhu et al., 2000) and is a predominant

AMPA subunit in a few brain areas such as the granule cells of the olfactory bulb and in the molecular layer of the cerebellar cortex (Keinänen et al., 1990).

2.8.4. Proteins Interacting with AMPA Receptors

AMPA receptors are assembled in ER (Tichelaar et al., 2004). After the assembly, the receptors undergo tightly regulated quality checking for agonist binding and gating. After this the receptors are escorted to dendritic synaptic and extrasynaptic cell membranes. The studies during past decades have revealed several groups of proteins that participate in the formation, trafficking and synaptic targeting and maintenance of AMPA receptors.

TARPs (Letts et al., 1998) have been found to control the trafficking of AMPA receptors from the endoplastic reticulum and connect them in synapses. Nowadays, TARPs may be considered as auxillary AMPA receptors subunits (Fukata et al., 2005) that assist receptor folding, affect their kinetics (Bedoukian et al., 2006; Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005) and rectification (Soto et al., 2007). TARP γ2, also known as stargazin, is a prototypical TARP. It was found by a spontaneous mutation in the stargazer mouse line that completely lacked surface expression of AMPA receptors in cerebellar granule cells (Chen et al., 2000). Stargazin affects the AMPA receptor function by decreasing the rate and extent of desensitization and increasing the efficacy of partial agonist kainate (Turetsky et al., 2005). According to a recent study, TARPs can be divided into two groups, Type I and Type II TARPs (Kato et al., 2008). Type I TARPS, stargazin, γ3, γ4, and γ8, have a similar effect on AMPA receptors, albeit the extent of the effects differs between the TARP subunits (Kott et al., 2007). Type II TARPs, γ5 and γ7, do not seem to participate in the trafficking of AMPA receptors, but regulate AMPA receptor function in subunit specific manner (Kato et al., 2008; Kato et al., 2007). Important domains of TARP structure for their regulation of AMPA receptors are the first extracellular loop which makes contact with the glutamate-binding module in AMPA receptors (Tomita et al., 2007) and thereby modulates channel gating and pharmacology **(Fig 4)**, and the C-terminal tail which associates with postsynaptic density-95 and related membrane-associated proteins, which cluster AMPA receptors at synapses (Schnell et al., 2002). TARPs contain a PDZ-interacting motif in the C terminus, which can bind the PDZ domain of membrane-associated proteins like membrane associated guanylate kinase (MAGUK) scaffolding proteins (Chen et al., 2000).

Very recent study has revealed that members of family of transmembrane proteins, chornichon homologs CNIH-2 and CHIN-3, associate with AMPA receptors, and like TARPs, also affect the trafficking and kinetics of AMPA receptors (Schwenk et al., 2009). Surprisingly, the study showed stronger association of chornichons with AMPA receptors than TARPs, as majority (ca. 70 %) of AMPA receptors were found in a complex with CNIH-2 and CHIN-3, only a minor portion associating with most widespread TARPs, γ2 and γ3.

PSD-95 is a PDZ domain-containing scaffolding protein, which belongs to the MAGUK-protein family. It is almost exclusively located in the postsynaptic density of neurons. PSD-95 controls synaptic AMPA receptor content and thereby synaptic strength. Acute

overexpression of PSD-95 increases AMPA receptor-mediated EPSCs, but not NMDA receptor EPSCs in hippocampal neurons, while acute knockdown of PSD-95 via RNA interference (RNAi) decreases AMPAR EPSCs, but not NMDA EPSCs (Ehrlich and Malinow, 2004; Ehrlich et al., 2007). PSD-95 also appears to have a role in synaptic plasticity, since hippocampal slices prepared from mutant mice lacking PSD-95 expressed greatly enhanced NMDA receptor-dependent LTP, but they did not express NMDA receptor-dependent LTD (Migaud et al., 1998). Overexpression of PSD-95, on the other hand, occluded LTP (Ehrlich and Malinow, 2004; Stein et al., 2003) and decreased the threshold for LTD induction (Beique and Andrade, 2003; Stein et al., 2003).

SAP97 (synapse-associated protein 97) is a member of the MAGUK family of proteins that has been implicated in AMPA receptor transport to and from the synapses. It binds directly only to long C-terminus containing GluR-A (Cai et al., 2002). SAP97 appears to predominantly associate with GluR-A subunits early in the secretory pathway and has been suggested to function during receptor maturation, not anchoring, of GluR-A at synapses (Sans et al., 2001). However, other studies localize SAP97 to excitatory synapses, and overexpression of SAP97 can enhance synaptic AMPA receptor function and promote dendritic spine growth (Rumbaugh et al., 2003). GluR-A and SAP97 together are reported to promote dendrite branching in an activity-dependent manner, although this does not seem to require physical association between them (Zhou et al., 2008).

N-ethylmaleimide-sensitive fusion protein (NSF) is a membrane fusion protein that has ATPase activity. Together with PICK1 (protein interacting with protein kinase Cα 1), it has a role in the delivery of GluR-B subunit containing AMPA receptors to the cell membrane during synaptic activation. Blocking either PICK1 or NSF with peptides added to pipette solution prevents the delivery of GluR-B containing AMPA receptors to the synapses (Gardner et al., 2005; Liu and Cull-Candy, 2005). PICK1 is a PDZ-domain containing protein that interacts with glutamate receptors, monoamine plasma membrane transporters and non-voltage gated sodium channels, and is a target of protein kinase C (Xu and Xia, 2006).

Glutamate receptor interacting protein (GRIP) is important in clustering of AMPA receptors at the synapses. GRIP binds to GluR-B subunit C-terminus with its PDZ domain. The phosphorylation of Ser880 in the intracellular C-terminal domain of GluR-B by PKC decreases the association of GluR-B and GRIP. It has been speculated that this phosphorylation has a specific role in the formation of LTD at parallel fiber-Purkinje cell synapses in the cerebellum (reviewed in Hirai, 2001).

2.8.5. AMPA Receptor Synthesis and Trafficking to the Cell Membrane

Glutamate receptor subunits are synthesized by endoplasmic reticulum (ER)-associated rhibosomes. Each of the glutamate receptor (except GluR7) contains an N-terminal signal sequence that targets insertion in the ER membrane. Tetrameric AMPA receptor assembly takes place in ER, where they are assembled as dimer of dimers (Ayalon and Stern-Bach, 2001; Tichelaar et al., 2004). Dimerization starts from N-terminal LIVBP-like domain (**Fig. 4**). It is suggested that the N-

terminus assures that the association happens between the subunits of only a given ionotropic glutamate receptor family, thereby preventing the association of AMPA receptor subunits to kainate receptor subunits (Ayalon and Stern-Bach, 2001; Kohda et al., 2003). This is probably based on differences in chemical properties of dimer interfaces in N-terminus between subunits families, in AMPA receptors being more hydrophopic and in kainate receptors more polar (Ayalon et al., 2005). However, large N-terminus is not mandatory for AMPA receptor folding and trafficking to the cell membrane, since GluR-D receptors lacking N-terminus are expressed as efficiently as wild type GluR-D receptors in HEK cells (Pasternack et al., 2002). Assembly of the dimers in the formation of tetramers probably happen step by step towards the C-terminus after the ligand binding domains and transmembrane domains have folded (reviewed in Greger and Esteban, 2007).

ER has quality control machinery that allows forward trafficking of certain glutamate receptors, while retaining others in ER. The purpose of this system is to assure that only appropriate and rightly formed glutamate receptors are trafficked further to the cell membrane. The exact molecular mechanisms of how the quality control works are not known to date, but studies with various point mutated glutamate receptor subunits have revealed what properties are checked. The quality control seems to especially target checking functional elements of receptors, such as ligand binding domain residues (Fleck et al., 2003; Grunwald and Kaplan, 2003; Mah et al., 2005; Valluru et al., 2005), dimer-interface residues (Fleck et al., 2003; Horning and Mayer, 2004) and channel pore residues at or near the Q/R editing site (Greger et al., 2002; Grunwald and Kaplan, 2003).

Desensitization seems to play a role in forward trafficking of AMPA receptos. For example, L483Y point-mutated GluR-B is retained largely in ER (Greger et al., 2006). L483Y mutation affects ligand binding domain such that the receptor does not desensitize properly. In addition, kainate receptor mutants with an engineered disulfide bridge across the ligand binding domain interface, which also does not desensitize, have drastically reduced surface expression (Priel et al., 2006). On the other hand, N754D GluR-B mutant that has accelerated desensitization, is trafficked to the neuronal cell membrane more efficiently than wild type GluR-B (Greger et al., 2006).

The exit of AMPA receptors from ER is facilitated by the interaction with TARPs. This is evident for at least the flop form of GluR-D that is expressed poorly in the cell membrane of HEK cells, but the surface expression raises to the level of GluR-D flip, when stargazin is coexpressed with it (Coleman et al., 2006).

AMPA receptors are trafficked at synapses in a subunit-dependent manner. The general rule, at least in the hippocampus, is that GluR-B/GluR-C receptors cycle constitutively in and out of synapses and GluR-A containing receptors are inserted to synapses in activity-dependent manner during LTP (Hayashi et al., 2000; Passafaro et al., 2001; Shi et al., 2001). The hypothesis for this difference in subunit specific membrane trafficking is that long C-tail splice variants containing receptors are added to synapses in activity dependent-manner and sort tail containing receptors are constitutively cycled. Receptors are delivered to synapses possibly via two different routes, directly to postsynaptic density or by lateral diffusion. Direct insertion to the synapses occurs via exocytosis (Gerges et al., 2006). Lateral diffusion involves trapping of the wandering AMPA receptors by PDZ-dependent interaction between TAPRs and PSD95 scaffold proteins.

Lateral diffusion has been observed almost exclusively in primary neuronal cultures, whereas evidence for intracellular trafficking has been seen in both brain slices and dissociated neurons (Greger and Esteban, 2007). It may be so that the lateral diffusion occurs in developing neurons and intracellular insertion in mature neurons. However, it should be mentioned that aforementioned difference might be artificial due to technical reasons, since in cortical cultures the lateral diffusion is easier to observe than in brain slice preparations.

2.8.6 Mechanisms of AMPA Receptor Activation and Desensitization

AMPA receptors undergo rapid and extensive desensitization. In AMPA receptors the τ-value of desensitization of homomeric receptors is fastest with GluR-D flop-splice variant (GluR-D_o) being 0.9 ms and slowest in GluR-C flip (Glur-D_i), 4.8 ms, when 1 mM glutamate is applied rapidly (Mosbacher et al., 1994). τ-value of desensitization of AMPA receptors in native neurons range from 1 to 16 ms (Barbour et al., 1994; Colquhoun et al., 1992; Geiger et al., 1995; Hestrin, 1992; Tang et al., 1989; Trussell and Fischbach, 1989). The SS/peak-ratio of AMPA receptors is usually around 0.1 or lower, meaning that 90 % or over of the receptors are desensitized in each time point during steady state current.

The X-ray crystal structure analysis of isolated ligand binding domain (LBD) of AMPA receptors, with and without the agonist, has revealed how ligand binding converts to channel pore opening. LBD of AMPA receptor crystalizes as dimers, so that the backsides of two LBDs form a dimer interface (**Fig. 5**). Closure of the clam shell shaped LBD, after agonist binding, leads to opening of the channel, with a full agonist causing more closure than partial agonists (Armstrong and Gouaux, 2000; Hogner et al., 2002). The LBD of an AMPA receptor connects to transmembrane domain M3 (**Fig. 4** and **5**) with a linker segment which is thought to stretch when the LBD closes. It has been suggested that stretching of the linker segment cause reorientation of TM-domains, which converts to channel opening (Sobolevsky et al., 2005). Agonist binding also decreases the stability of LBD interface of dimers, which results in desensitization by allowing the repositioning of transmembrane domains in more relaxed confirmation, in which the ion channel can close (Sun et al., 2002).

2.8.7. Genetically Modified AMPA Receptor Mouse Lines

In order to study the role of specific AMPA receptor subunits in synaptic plasticity and learning and memory behaviours, several genetically engineered AMPA receptor mouse lines have been generated. In GluR-A knock out mice, hippocampal LTP is absent in field excitatory postsynaptic potential (fEPSP) recordings (Zamanillo et al., 1999) and they have an impaired regulation of distance-dependent scaling of excitatory postsynaptic currents, EPSCs, along the dendrite (Andrasfalvy et al., 2003). In addition, they do not have LTP-like potentiation in ventral tegmental area (VTA) after an injection of cocaine (Dong et al., 2004). In behavioural studies, GluR-A knock

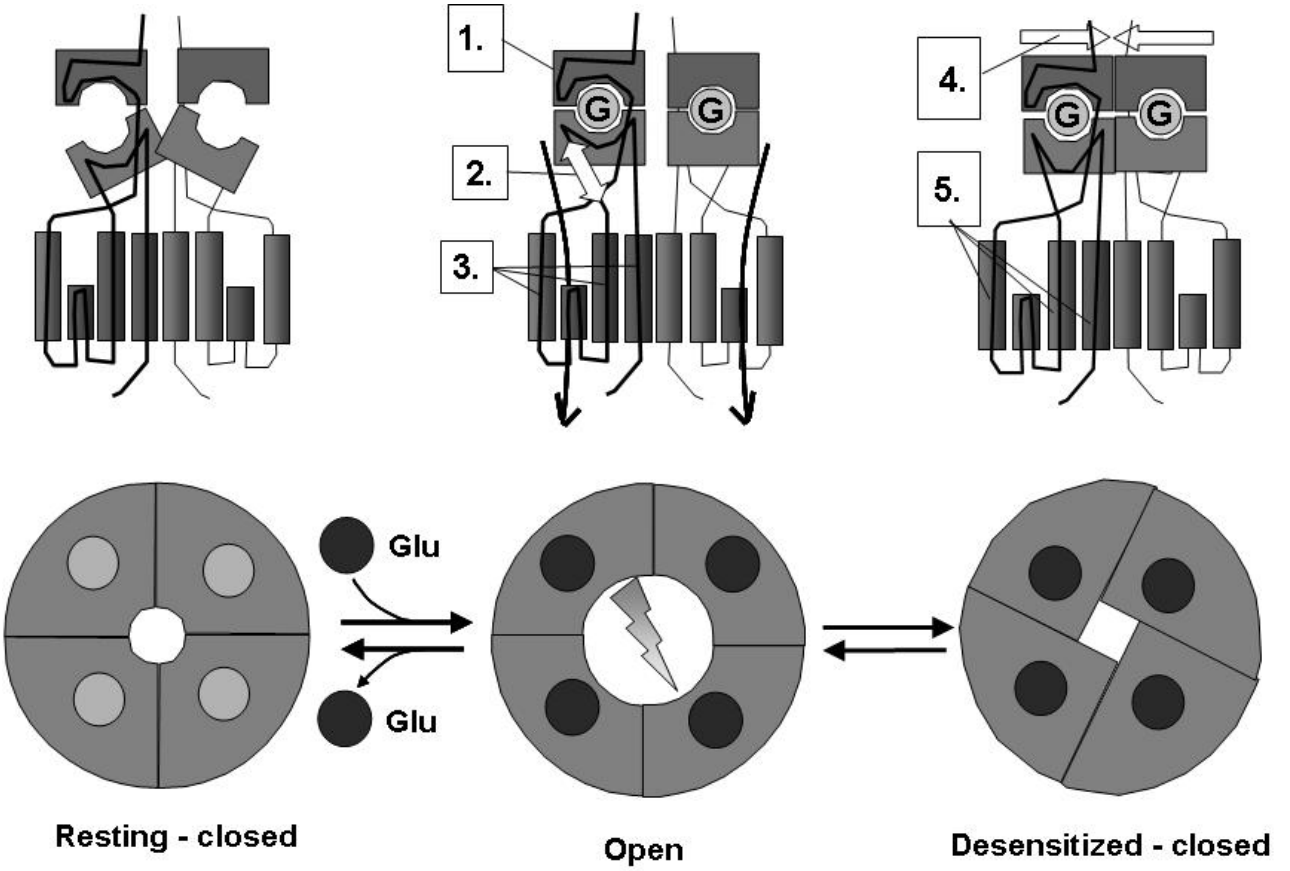

Figure 5. Activation and desensitization AMPA receptors. A very simplistic scheme of three states of AMPA receptor is depicted in the figure. Receptor can be open, closed or desensitized. The transition between these states does not require any energy, but is a stochastic process. The receptor constantly undergoes some fluctuations between the states even in the absence of agonist. However, agonist binding increases markedly the probability of receptor to be open and when agonist binding lasts longer, several milliseconds, probability is highest for the desensitized state. When agonist leaves the surrounding environment, the receptor closes in a process called deactivation. Movements of AMPA receptor domains involved in activation and desensitization of the receptor are marked with arrows and numbers and are: 1.) closure of LBD; 2.) stretching of the linker sequence which cause the movement of the transmembrane domains and channel opening (3.); 4.) decrease of the stability of LDB dimer interface which relaxes the conformation leading to (5.) the repositioning of transmembrane domains and channel closure. Modified from Madden 2002.

outs do not respond to cues that were conditioned to reward (Mead and Stephens, 2003b) and they have altered conditioned place preference behaviour towards cocaine (Dong et al., 2004).

AMPA receptor subunit GluR-B renders AMPA receptors Ca^{2+} impermeable. GluR-B knock outs exhibit increased mortality, and those surviving show reduced exploration and impaired motor coordination. GluR-B subunit knock outs have enhanced LTP in hippocampus, probably due to 9-fold increased Ca^{2+} permeability (Jia et al., 1996). GluR-B knock out mice have altered stimulus reward learning (Mead and Stephens, 2003a).

GluR-C knock out mice are viable and have normal locomotor activity. They also have normal CNS anatomy and synaptic structures (Meng et al., 2003). However, GluR-C knock outs do have enhanced LTP in the CA1 region of hippocampus. When GluR-C knock outs are bred with GluR-B knock outs to produce double knock outs, AMPA fEPSPs in the hippocampus are significantly smaller, being only 10-20 % of the level of wild types. However, synaptic plasticity processes, LTD, LTP, depotentiation and depression are present in these mice (Meng et al., 2003). These data suggest that GluR-A alone is sufficient to produce various forms of synaptic plasticity. This is probably because the primary mechanism for LTP (especially in the hippocampus) is likely the insertion of GluR-A containing AMPA receptors to the synapses, and LTD occurs as a result of removal of all types of AMPA receptors equally from the synapses.

Summary. AMPA receptors have many special features which have significance for their function. The expression of AMPA receptor subunits varies between the brain regions and is developmentally regulated. In brain AMPA receptors seem to be interacting with various proteins, which participate in synthesis and assembly of tetrameric receptors in ER, trafficking of receptors to the cell membrane and synapses and retention of them in synapses. The TARPs have especially emerged as an important family of AMPA receptor interacting proteins, because they affect trafficking, synaptic localization, gating and pharmacology of AMPA receptors. AMPA receptors are constitutively recycled in and out of synapses, which is mainly done by GluR-B/C AMPA receptors, and added to the synapses when required primarily in the form of GluR-A containing receptors.

2.9 Pharmacology of Ionotropic Glutamate Receptors

Glutamate receptors play the central role in signaling of neurons and neural plasticity in virtually all brain areas. In theory, this makes them tempting drug targets, but AMPA receptor subtypes do not differ enough from each other to make it possible to affect only a certain aspect of brain function. In addition, to date only a few molecules affecting glutamate receptors show subunit selectivity.

Drugs affecting the ionotropic glutamate receptors are only used in a small set of clinical indictions. Ketamine is an anesthetic, which blocks the channel of NMDA receptor. It is not used generally in anesthesia, but has been used in special cases, like veterinary medicine and animal anesthesia in research. Topiramate is a drug used in epilepsy and migraine. It has many molecular targets, e.g. it inhibits kainate and AMPA receptors. In Alzheimer's disease, memantine, a NMDA channel blocker is used to slow down the progress of the disease. Memantine is thought to decrease pathologically elevated glutamatergic function. Glutamate receptor potentiators, so called ampakines, are currently being researched as cognition enhancing drugs to treat diseases involving mental disability like Alzheimer's disease, Parkinson's disease, schizophrenia, depression and ADHD (Lynch, 2006).

In research, many NMDA, AMPA and kainate receptor agonist and antagonists have been used. Traditionally, ionotropic glutamate receptors have been divided into two crude groups: NMDA and non-NMDA receptors, mainly because of the drug selectivity. AMPA and kainate

receptor pharmacology is very similar, and it has not been possible to pharmacologically isolate only one of them until recently, when the commercially available selective compounds arrived. Most commonly used synthetic agonists to activate AMPA and NMDA receptors are AMPA and NMDA, respectively. Blockage of AMPA receptors can be accomplished by 6,7-dinitroquinoxaline-2,3-dione (DNQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline -7-sulfonamide (NBQX) and NMDA receptors are usually blocked by (2*R*)-amino-5-phosphonovaleric acid (APV) or channel blocker MK-801 (dizocilpine). In **figure 6** examples of glutamate receptor currents are shown to demonstrate the isolation of NMDA and AMPA currents using NMDA antagonist APV.

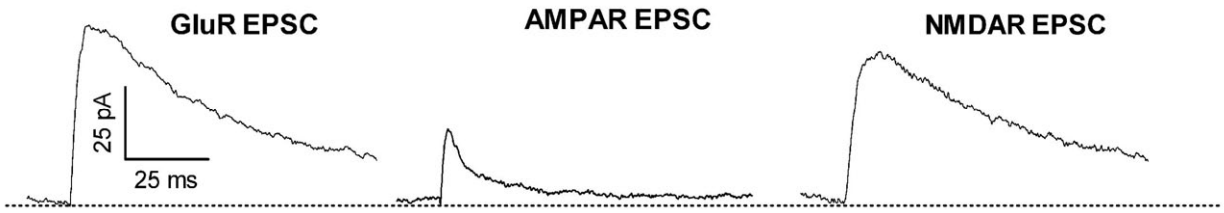

Figure 6. Representative traces of excitatory postsynaptic currents of glutamate receptors measured from VTA (ventral tegmantal area) dopamine neuron at the holding potential of +40 mV and in the presence of picrotoxn. GluR EPSC = baseline EPSC containing both AMPA and NMDA receptor conductances; AMPAR EPSC = AMPA receptor EPSC, recorded in the presence of APV; NMDAR EPSC, generated by subtracting GluR EPSC – AMPAR EPSC. The dotted line represents zero current level. The stimulus artefact is omitted for clarity. Note the typical short duration of AMPA receptor current and longer duration of NMDA receptor current.

2.10. Ethanol

Ethanol (ethyl alcohol) has a wide variety of effects on many type of proteins expressed in central nervous system. Even though several target molecules of ethanol have been identified during past decades, it is not still clear which of them are important in ethanol intoxication and the development of alcohol dependency. One clear difficulty in studying the effects of ethanol is that ethanol does not bind with high affinity to biomolecules. Therefore, binding assays are not possible with ethanol, which makes it difficult to prove that ethanol has a direct action on biomolecules. In the next few chapters the current knowledge of ethanol's central nervous system effects are reviewed starting from historical theories about lipid membrane action ending with effects on proteins.

2.10.1. Theories of How Ethanol Causes Its Effects

The first theory of how ethanol and general anesthetics produce their effect arose at the end of nineteenth century, when H. H. Meyer published a study suggesting that these compounds produce their action by interaction with lipid membranes. A couple of years later a similar theory was

published independently by Overton. Therefore, the theory is known as Meyer-Overton's lipid theory of anesthesia (reviewed in Lovinger, 1997). Meyer and Overton had discovered a most striking correlation between the physical properties, mainly lipid solubility, of general anaesthetic molecules and their potency. Meyer compared the potency of many agents required to induce anaesthesia in tadpoles, with their olive oil/water partition coefficient. He found a nearly linear relationship between potency and the partition coefficient for many types of anesthetic molecules such as alcohols, aldehydes, ketones and esters. From the correlation between lipid solubility and anesthetic potency, both Meyer and Overton proposed that anesthesia occurs when the anesthetic reaches a critical concentration in some lipid phase within the body. However, the logic is not flawless; general anesthetics could equally well be binding to hydrophobic target sites on proteins in the brain. In fact, the necessity for general anesthetics to cross the blood-brain barrier to have their effect is the main reason that more polar agents are less potent. However, the theory prevailed for 80 years.

In 1984 Franks and Lieb published a study showing that ethanol inhibited the function of firefly luciferase enzyme. The experiments were done with soluble protein, proving that alcohols and general anesthetics can produce action without the lipids. Soon, others followed providing evidence that ethanol affects the function of many neurotransmitter receptors. First, it was shown that ethanol enhances the function of $GABA_A$ receptors (Aguayo, 1990; Allan and Harris, 1986; Nakahiro et al., 1991; Suzdak et al., 1986; Wafford and Whiting, 1992). Next, the function of NMDA receptors was found to be inhibited by ethanol (Hoffman et al., 1989; Lovinger et al., 1989), followed by the studies showing that ethanol potentiates serotonin 5-HT_3 receptors (Lovinger and White, 1991; Machu and Harris, 1994), potentiates strychnine-sensitive glycine receptors (Aguayo and Pancetti, 1994; Celentano et al., 1988; Mascia et al., 1996), either potentiates (Aistrup et al., 1999; Bradley et al., 1984; Wu et al., 1994) or inhibits (Aistrup et al., 1999) the nicotinic acetylcholine receptors in a subunit dependent manner. Ethanol has been also shown to affect other proteins than just ionotropic receptors. Among these are adenosine (Krauss et al., 1993), norepinephrine (Lin et al., 1997), dopamine (Wang et al., 1997), and serotonin (Alexi and Azmitia, 1991) transporters and calcium channels (Camacho-Nasi and Treistman, 1986; Mullikin-Kilpatrick and Treistman, 1995). In addition, several studies show that a variety of protein kinases play a role in ethanol actions (Freund and Palmer, 1997; Harris et al., 1995; Hodge et al., 1999; Miyakawa et al., 1997; Rabbani et al., 1999; Yoshimura and Tabakoff, 1999).

Data showing that ethanol changes the function of proteins are not enough as such to prove that ethanol has a direct effect on them. Ethanol could as well affect still unknown target molecules, which then downstream affect the protein in question. More direct evidence that ethanol binds directly to the proteins came from studies where glycine and $GABA_A$ receptors were point-mutated to produce receptors that were insensitive or less sensitive to ethanol, respectively (Mihic et al., 1997). The same group also demonstrated that the size of ethanol binding pocket, or more precisely the hydrophilic cavity which ethanol can be accommodated, in glycine and $GABA_C$ ρ1 receptors can be changed by point mutation resulting in a change in cut-off point of n-alcohols (Wick et al., 1998).

Action of ethanol on its many acute targets in brain produces its unique action profile, which causes the feeling of drunkenness when alcohol drinks are consumed. In addition, ethanol also possesses features that are shared between many types of drugs of abuse. Ethanol along with other drugs of abuse and benzodiazepines seems to cause LTP-like potentiation of AMPA receptor function selectively in dopamine cells of ventral tegmental area (VTA) in midbrain after a single injection in mice (Heikkinen et al., 2008; Saal et al., 2003; Ungless et al., 2001). These data mean that drugs of abuse activate one of the brains reward pathways starting from VTA and targeting nucleus accumbens where dopamine is released. The physiological role of VTA dopamine neurons in learning was demonstrated in monkey studies measuring the firing rate of dopamine cells using intracranial recording electrode (Hollerman and Schultz, 1998). On the basis of those experiments we can conclude, that the firing rate of dopamine cells raises when an animal observes in its environment a novel object that it links to something that satisfies its bodily needs or when an animal comes across an environmental cue that has been previously linked to something that satisfies its bodily needs. It has been thought that the increase in dopamine released in nucleus accumbens enhances the memory of valuable things found in the environment by adding a feeling of satisfaction or pleasure in the memory. This has a survival value to the animal. Since ethanol and drugs of abuse activate the reward pathway, the use causes pleasurable feelings and makes the users more prone to use that particular substance again in the future.

To find an answer to the question, what makes a drug have abuse potential is one of the main goals of addiction studies. The activation of the brain reward pathway answers sufficiently to that question. Molecules that are used as drugs of abuse seem to target this pathway directly at the molecular level (reviewed in Kauer, 2004), instead of first functioning somewhere else in brain, and then downstream activating the reward pathway. In this view, the acute changes outside of the reward related areas, that are sensed during the use of drugs, are secondary and do not contribute to the need for usage, which leads to the development of dependency. To find out if this hypothesis is true, we should have a drug of abuse that only targets reward pathways. Are there such drugs? The answer obviously is no, but stimulants, especially cocaine, comes close. Cocaine is a dopamine transporter blocker, and also has effects on the uptake of other monoamines. Cocaine produces a rush that is described as a state of well being with high self esteem and alertness (Koob and Le Moan, 2005). Interestingly, ethanol also has stimulant-like effects sometimes, especially after a few drinks, even though it is a depressor of neural function and clearly sedative at high concentrations. Maybe the stimulant-like, arousing, effect of alcohol is because ethanol also activates the VTA-nucleus accumbens reward pathway.

2.10.2. Effects of Ethanol on NMDA Receptors

It is not currently clear which molecular targets of ethanol are important in ethanols acute effects, but $GABA_A$ and NMDA receptors are generally considered to be the most probable candidates. $GABA_A$ receptors are reported to be potentiated by low and moderate concentrations of ethanol (Soldo et al., 1994; Wallner et al., 2003; Weiner et al., 1997), albeit there is a controversy in the

topic (Korpi et al., 2007). Ethanol inhibits the function of all classes of glutamate receptors, and NMDA receptors have been most intensively studied.

Ethanol has been shown to inhibit the function of NMDA-type glutamate receptors in intact synapses and in heterologous expression systems (Blevins et al., 1997; Lovinger et al., 1990; Wirkner et al., 2000). Ethanol inhibits NMDA receptor currents in clinically relevant concentrations (5-50 mM), IC_{50} being around 30 mM (Weight et al., 1993). Inhibition seems to be dependent on subunit composition of NMDA receptors. A study of NR1/NR2A, NR1/NR2B, and NR1/NR2C subunit combinations expressed in *Xenopus* oocytes shows that NR1/NR2C channels were slightly less sensitive to ethanol inhibition than the other channels in Ca^{2+}-deficient, Ba^{2+}-containing medium (Kuner et al., 1993). The other *Xenopus* oocytes expression study showed that NR1 combined with NR2A was the most sensitive, NR1/NR2B a little bit less sensitive and NR1/2C combination the least sensitive to ethanol's inhibition (Mirshahi and Woodward, 1995). In the same study, the ethanol inhibition of NR1/2A receptors was reduced by substitution of the wild type NR1 with a calcium-impermeable NR1 mutant suggesting that intracellular processes activated by calcium may influence the inhibitory potency of ethanol. In native neurons, the most sensitive receptors seem to be NR2B containing, ifenprodil sensitive, NMDA receptors (Fink and Gothert, 1996; Lovinger, 1995), suggesting that subunit composition does not explain as a whole the differences observed in ethanol inhibition of NMDA receptors.

The C-tail of NR1 subunit contains the C0 domain that binds to calmodulin and actinin binding protein α-actinin-2 (Krupp et al., 1999). C0 domain has been shown to play a part in ethanol inhibition of NMDA receptors (Anders et al., 2000). Calmodulin binding to the C0 domain mediates the calcium-dependent reduction of NMDA receptor function (Krupp et al., 1999; Legendre et al., 1993). Calmodulin and α-actinin-2 seem to compete for NMDA receptor interaction, calcium concentration being the factor that determines the outcome of the competition. It is suggested that in the presence of elevated calcium NMDA receptor inactivation occurs after C0 dissociates from α-actinin-2 by two distinct but converging calcium-dependent processes; competitive displacement of α-actinin-2 by calmodulin and the reduction in the affinity of α-actinin-2 for C0 after calcium binds to it. Ethanol inhibition of NR1/NR2 combinations lacking C0 domain is reduced in HEK293 cell expression studies (Anders et al., 2000). These results show that ethanol inhibition is stronger if calcium- and calmodulin-dependent inactivation NMDA receptors are let to function properly suggesting that ethanol promotes calmodulin-dependent inactivation of NMDA receptors.

Certain amino acid substitutions in NMDA receptors have also been shown to affect ethanol sensitivity. In NR1 subunit, a point mutation F639A in TM3 region reduces ethanol inhibition and F639W slightly increases it (Ronald et al., 2001). In addition, point mutations M813A and L819A in TM4 of NR1 subunit enhance the ethanol inhibition (Smothers and Woodward, 2006). When the ethanol sensitivity reducing F639A (TM3) point mutation is combined with either L819A (TM4) or G822W (TM4) point mutations, the ethanol inhibition returns to the level of wild type receptor (Smothers and Woodward, 2006). The data can be interpreted such that TM3 and TM4 domains form a binding cavity for ethanol and increasing the volume of this cavity

by substituting the phenylalanine with smaller amino acids at position 639 may be compensated by the expression of the larger residues such as tryptophan at the TM4 domain.

2.10.3 Effects of Ethanol on AMPA Receptors

AMPA receptors are not generally thought to be as sensitive to ethanol as NMDA receptors. They are not much inhibited in intact synapses, when the currents are activated by synaptically released glutamate evoked by electrical stimulation of afferent axons (Carta et al., 2003; Hendricson et al., 2003; Lovinger et al., 1989; Lovinger et al., 1990; Weiner et al., 1999). The only brain areas where ethanol has been found to inhibit synaptic AMPA receptor currents to date are central amygdala (Zhu et al., 2007) and CA3 area of Hippocampus of neonatal rats (Mameli et al., 2005). Interestingly, in latter AMPA receptor currents are sensitive to ethanol and NMDA receptor currents are not, where as the opposite is true in 21-26 days old rats (Mameli et al., 2005). However, AMPA receptors seem to be equally sensitive to ethanol as NMDA receptors in experiments where currents are activated by relatively long agonist applications both in native neurons and in expression systems (Lovinger 1993; Wirkner et al., 2000). AMPA receptors desensitize strongly (about 90 %) when the drugs are applied to the cells using relatively long drug application times, common in cell culture and heterologous expression studies. However, when neurons are stimulated electrically in intact brain slices the receptors are generally non-desensitized or only slightly desensitized. Can the difference in the extent of desensitization explain the observed differences in ethanol inhibition of AMPA receptors? One aim of our research was to investigate this possibility.

As mentioned above, ethanol does not generally efficiently inhibit synaptic AMPA receptor currents. In hippocampus, 100 mM ethanol has been reported to inhibit the amplitude of AMPA receptor mediated population EPSP only by 9 %, where as in the same study 50 mM of ethanol inhibited NMDA receptor population EPSPs by 43 % (Lovinger et al., 1990). In amygdala, where synaptic AMPA receptors are significantly inhibited by ethanol, 44 mM ethanol inhibits AMPA receptor mEPSC by 30 % (Zhu et al., 2007) and in neonatal CA3 of hippocampus 50 mM ethanol inhibited EPSCs by 30 % (Mameli et al., 2005). In studies making use of drug application systems ethanol IC_{50}-values of AMPA receptor currents varied depending on subunit composition and cell types. In HEK293 cell experiments IC_{50}-value of ethanol varied from 119 to 165 mM depending on subunits expressed (Lovinger, 1993). In oocyte experiments IC_{50}-value of kainate activated AMPA receptor currents depended also on expressed subunits and varied from 170 to 238 mM (Akinshola et al., 2003). Heteromeric AMPA receptors have a higher IC_{50} than homomeric; homomeric A and D receptors had IC_{50} values of 119 and 133 mM, respectively; coexpressed A/D heteromers IC_{50}-value was raised to 165 mM (Lovinger, 1993). In cultured cortical neurons the IC_{50}-value seemed to depend on the agonist used as kainate evoked AMPA currents gave IC_{50}-value of ethanol of around 400 mM (Lovinger, 1993), whereas when AMPA was used as agonist IC_{50}-value was 162 mM (Wirkner et al., 2000).

Ethanol inhibits AMPA receptors in non-competitive manner and the ethanol inhibition is reproducible and reversible as currents return back to control level after ethanol application (Wirkner et al., 2000). Straight-chain alcohols show cut-off phenomenon in ethanol inhibition of AMPA receptors, which means that the potency to inhibit AMPA receptors grows with chain length (or hydrophobicity) of alcohol molecules, but only to certain point, which is hexanol. This suggests that ethanol has a binding site in AMPA receptor, which is reachable via hydrophobic milieu, such as lipid membrane (Akinshola, 2001).

2.10.4 Effects of Ethanol on Kainate Receptors

Kainate receptors are inhibited by ethanol in heterologous systems and neuronal cultures in concentrations greater than 25 mM (Costa et al., 2000; Dildy-Mayfield and Harris, 1995; Valenzuela and Cardoso, 1999; Valenzuela et al., 1998). Kainate receptors in synaptic transmission seem to be sensitive to ethanol in pyramidal neurons in CA3 of hippocampus even more than NMDA receptors and AMPA receptors (Weiner et al., 1999). In CA1 region of hippocampus kainate receptors are located in presynaptic terminals of interneurons, where their activation increases GABA release (Cossart et al., 2001). These kainate receptors seem to be sensitive to low, 5-10 mM, concentration of ethanol, which has been reported to decrease the interneuron firing, thus decreasing the GABA mediated inhibitory input to the CA1 pyramidal neurons (Carta et al., 2003).

Summary. Glutamate receptors are targets of several medical and recreational drugs. However, they are not generally considered as good targets for drugs, because they have too wide a role in brain functions, thus usually inducing too many adverse effects. Ethanol inhibits the function of all classes of glutamate receptors in some experimental conditions. However, in most experiments only NMDA receptors have been shown to be inhibited by clinically relevant concentration of ethanol. The ethanol inhibition of AMPA receptors seems to depend on experimental conditions, and it is not known to what degree ethanol would affect synaptic AMPA receptor currents. Ethanol effects on kainate receptors have significance in some brain areas, like in the hippocampus, where it decreases interneuron firing through the inhibition of kainate receptors.

3. Aims of the Study

The aim of this thesis work was to study the ethanol inhibition of AMPA receptors and how the cell membrane expression of AMPA receptors is regulated. These studies were done with patch clamp electrophysiology and immunostaining and immunoblotting methods. The specific aims were the following:

1. to clarify the mechanisms of ethanol inhibition of native AMPA receptors
2. to study the role of TARPs in ethanol inhibition of AMPA receptors
3. to study what properties of AMPA receptors affect the early trafficking of them to the cell membrane from ER
4. to study the state of AMPA receptor-mediated excitation in Thy1α6 mice which have altered $GABA_A$ receptor-mediated inhibition

4. Materials and Methods

4.1. Experimental Animals and Expression Systems

Male and female C57BL/J6 wild type mice were used for hippocampal neuron isolation in Study I. Wild type C57BL/J6 and Thy1-α6 mice were used in study IV. For Study IV, all experimental procedures were approved by the Institutional Animal Use and Care Committee of the University of Turku and by the Western Finland Provincial Government.

Human embryonic kidney cell line (HEK293) was used in Study I, II and III and in addition COS7 cells were used in study III.

4.2. Cell Culture (I, II, III)

HEK293 cells and COS7 cells were cultured in DMEM supplemented with 10% foetal calf serum and 2 mM L-glutamine and penicillin-streptomycin solution. Cells were kept in culture incubator at 37 °C in 5 % CO_2. Cells were split every second or third day, when the culture was confluent.

4.2.1. Plasmids (I, II, III)

GluR-A flip plasmid used in HEK293 cell transfection was kindly donated by Dr. Kathryn Partin (I). Plasmids of GluR-D subtype of AMPA receptors and various point mutated GluR-D receptors, as well as TARP-plasmids were kindly donated by Dr. Kari Keinänen. TARP plasmids, human stargazin (γ2) and γ4 were originally from John L. Black III (Mayo Medical School, Rochester, MN).

4.2.2. Antibodies (III)

Primary antibodies used in immunofluorescence staining were monoclonal M1anti-Flag (Sigma; 5 g/ml), and Fab7 and Fab22 (both at 10 g/ml). Secondary antibody used was Cy3- conjugated anti-mouse (7 g/ml) from Jackson ImmunoResearch Laboratories. For immunoblotting, anti-GluR-BD_{LONG} (Coleman et al., 2006) or anti-stargazin (1:5000) made in-house were used. Commercial antibodies used were rabbit anti-myc (AbCam; 0.1 g/ml) and rabbit anti-NMDAR1 (Chemicon; 0.2 g/ml); secondary antibody used was anti-rabbit conjugated to horseradish peroxidase (Amersham Biosciences; 1:5000).

4.2.3. Immunofluorescence Analysis (III)

Transfected COS-7 cells were fixed and immunostained 40 h after transfection. Images were obtained and analyzed with an Olympus Provis AX70 epifluorescence microscope coupled to a Photometrics SenSys air-cooled CCD camera and Image ProPlus software.

4.2.4 Cell Surface Biotinylation, Protein Secretion, and Immunoblot Quantification (III)

Transfected HEK293 cells were biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (Pierce). Triton X-100 extracts were bound to streptavidin-conjugated Sepharose (Amersham Biosciences), and precipitated proteins were harvested and analyzed. For analysis of GluR-D S1S2 construct secretion, growth media were collected from transfected HEK293 cells 40 h after transfection, and 1 mM PMSF was added. Cell debris was pelleted by centrifugation (1500 *g*, 5 min, 4°C). Samples of media and total cell extracts prepared in Triton X-100 were analyzed by SDS-PAGE and immunoblotting. Immunoblots were scanned via Adobe Photoshop and analyzed using Image ProPlus software.

4.2.5. Endoglycosidase H Treatment (III)

Triton X-100 extracts from transfected cells were incubated with Endoglycosidase H (New England Biolabs) according to manufacturer's instructions.

4.2.6. HEK293 Cell Transfection (I, II, III)

Before the transfection the cells were replated at a density of 2×10^5 cells per millilitre into 35 mm culture dish. Cells were transfected using the calcium phosphate method (2 µg AMPA receptor and TARP plasmids and 0.7 µg EGFP DNA per 35 mm dish) (Coleman et al., 2003). The media was changed 18 hours after the transfection, and the cells were used for patch clamp experiments the following day. In each transfection in study II, both the GluR-D plasmid alone and GluR-D plus TARP plasmid were transfected in order to minimize the variation between the transfection conditions. All the cells were cotransfected with pEGFP-C1 for visualization of EGFP fluorescence.

4.3. Slice Preparation (I, IV)

C57Bl/J6 mice (10–20 days old) were decapitated and the whole brains were moved to ice-cold high-sucrose cutting solution. Coronal brain slices (400 µm in thickness) were cut using a manual vibroslice (Camden Instruments Ltd., Leicester, UK). Slices were kept at room temperature in artificial cerebrospinal fluid.

4.3.1. Isolation of Hippocampal Cells (I)

Hippocampal cells were isolated from 400 µM coronal brain slices. The brain slices were first incubated in pronase-enzyme (0.4–0.6 mg/ml; Calbiochem, San Diego, CA) in artificial cerebrospinal fluid at 37 °C for 20 to 30 min depending on age of mouse. Slices from younger animals require shorter incubation times. After incubation the slices were transferred to trituration buffer and the hippocampus was isolated from rest of the slice using a scalpel and sharp forceps. Neurons were dissociated by gentle mechanical trituration with differing sizes of fire-polished Pasteur pipettes. Cells were then allowed to settle to the bottom of a 35-mm-diameter culture dish, which was then transferred to the stage of an inverted microscope.

4.3.2. fEPSP Recordings from Hippocampal Stratum Radiatum

In a still unpublished study, we investigated the sensitivity to ethanol of AMPA receptor-mediated field excitatory postsynaptic potentials (fEPSP). fEPSP recordings of stratum radiatum of hippocampus were done using glass electrode which was filler with 1 mM NaCl, which was placed on stratum radiatum area of hippocampus. The extracellular solution was ACSF (for content, chapter 4.4.1.) The fEPSPs were evoked by stimulating the afferent Schaffer collateral axons placing the bipolar stimulation electrode few hundred µm apart from recording electrode on stratum radiatum. NMDA receptor blocker APV was used in recording solution during the experiment. Ethanol was diluted in the bath solution. fEPSP's were recorded using axoclamp 2A amplifier and pClamp 7 software (Molecular Devices, Sunnyvale CA, USA).

4.4. Patch Clamp Technique (I, II, III, IV)

Patch clamp is an electrophysiological recording technique, which allows the recording of ion current from a single cell. In patch clamp a small sized recording electrode is placed on the membrane of the cell and the cell membrane is sucked towards the electrode. This results in a seal between the electrode and the cell membrane, which has a high resistance of gigaohms. This makes it possible to record the cell membrane currents even at the single channel level. The patch clamp experiments can be done in current or voltage clamp mode. There are four different configurations in patch clamp which are; whole cell, cell-attached, outside-out and inside-out (Hamill et al., 1981). All the patch clamp studies in this thesis work have been done using whole cell voltage clamp, except one data set in the study I, which was done using outside-out membrane patches with voltage clamp mode.

4.4.1. Patch Clamp Solutions (I, II, III, IV)

Patch clamp recordings are done using solutions that resemble in their ion content physiological extracellular and intracellular milieus. Slice recordings (IV) were done in artificial cerebrospinal

fluid (ACSF), which contains (in mM) 126 NaCl, 1.6 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 18 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 D-glucose. The solution is bubbled with 95% O_2/5% CO_2 when it is used. Pipette solution in brain slice experiments contained (in mM) 120 $CsMeSO_3$, 5 NaCl, 10 TEA-Cl, 10 HEPES, 1.1 EGTA, 4 Mg-ATP, and 0.3 Na-GTP, pH adjusted to 7.2 with CsOH. Studies with acutely isolated neurons (I) and HEK293-cell (I, II, III) experiments were done in external solution containing (in mM) 150 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, pH was adjusted to 7.4 with NaOH and osmolarity was adjusted to 340 mOsM with sucrose. Pipette solution contained (in mM) 100 N-methyl-D-glucamine, 100 CH_3SO_3H, 40 CsF, 10 $MgCl_2$, 10 HEPES and 5 EGTA, pH adjusted with CsOH to 7.4 and osmolarity to 290 - 300 mOsM with sucrose.

Brain slices were cut in high sucrose cutting solution containing 194 mM sucrose, 30 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 10 mM glucose, bubbled with 95% O_2, 5% CO_2 prior to use. Hippocampal neurons were isolated in trituration buffer containing 20 mM NaCl, 130 mM N-methyl-D-glucamine, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose, osmolarity was adjusted with sucrose to 340 mOsM and pH was adjusted to 7.4 with HCl.

4.4.2. Patch Clamp Electrophysiology of Isolated Neurons and HEK293 Cells (I, II, III)

Patch clamp recordings were done from the cells adhering to the bottom of 35 mm culture dish, which was mounted in a recording chamber under the microscope. During the experiments the cells were continuously superfused with recording solution. Experiments were carried out at room temperature. Whole-cell (I, II, III) and outside-out (I) patch-clamp recording techniques were used (Hamill et al., 1981). Pipette resistance was 2 to 4 MΩ, when filled with an internal solution. Drugs and ethanol were diluted in recording solution and applied to whole cells with stepper motor driven solution application system (Warner Instrument, Hamden, CT). In the majority of experiments, neurons were lifted clear of the bottom of the culture dish to facilitate solution exchange. AMPA receptor agonists and drugs were applied simultaneously in most experiments. Solution exchange time of the drug application system was 150 ± 60 ms for isolated hippocampal neurons sitting in the bottom of the dish and 22 ± 5 ms for lifted cells, when measured by applying 1 mM kainate to the cells to activate the AMPARs and then changing the standard solution to the Na^+ lacking solution, which almost completely eliminated the kainate-evoked current.

4.4.3. Miniature EPSC Recordings from Hippocampal Slices (IV)

Slice electrophysiology was done from CA1 pyramidal neurons of the hippocampus in 400 µm thick coronal slices. The brain slice containing the hippocampus was prepared as described above. After the cutting, the slices were transferred to the recording chamber under an upright Olympus BX51WI (Olympus Imaging Europa GmbH, Hamburg, Germany) microscope. The recording

solution was constantly flowing to the recording chamber at flow rate of 2 ml/min. Patch clamp electrode was 2-4 MΩ in resistance when filled with intracellular solution. Pyramidal neurons were identified on the basis of location in the dense CA1 pyramidal neuron layer and morphology. The miniature EPSC recordings were done with whole cell voltage clamp technique at the holding potential of -60 mV. In order to get enough miniature EPSCs the neurons were recorded at least for 15 min at the presence of $GABA_A$ antagonist picrotoxin and NMDA antagonist APV.

4.5. Maximal Electroshock (IV)

Maximal electroshock (MES) convulsion test was done in study IV to find out the convulsion sensitivity of wild type and Thy1α6 mice. Electroshock-induced convulsions were produced by delivering rectangular unipolar positive current pulses (100 Hz, duration 1.0 sec; width 0.5 msec) via ear-clip electrodes from an ECT unit (Ugo Basile 7800, Comerio, Italy). Animals received a single electroshock (5-13 mA), after which they were observed for appearance of tonic hindlimb extension (THE) convulsion. The number of animals exhibiting THE was recorded and expressed as a percentage of the mice tested for each line and current intensity.

4.6. Statistical Tests (I, II, III, IV)

All statistical tests were done with Prism 3.0 software (GraphPad Software, Inc La Jolla, CA, USA) and SPSS 15.0.1 for Windows (SPSS Inc., Chicago, IL, USA). The analysis used to calculate statistical significances in the studies of the effect of ethanol was two-way ANOVA with post hoc comparisons using Bonferroni's test (I) and repeated measures ANOVA (II). Miniature EPSCs (IV) were analysed with Kolmogorov-Smirnov test in Mini Analysis Program 6.0.3 (Synaptoft, Inc., Decatur, GA, USA). To determine the threshold for MES induced THE (IV), nonlinear regression fit with a sigmoidal four-parameter logistic equation in Prism software was used to determine the electroconvulsive threshold EC_{50} (current intensity sufficient to induce THE in 50% of animals).

5. Results and Discussion

5.1 The Effect of Ethanol on AMPA Receptors

5.1.1. Ethanol Inhibits AMPA Receptor fEPSPs

Ethanol inhibited the fEPSP's recorded from hippocampal slices from stratum radiatum (unpublished data). The inhibition was reproducible and reversible as depicted in Figure 7D. Interestingly, the inhibition was age dependent as there was a clear difference in ethanol inhibition when the animals were pooled into two groups, young mice (age under 1 month) and adult mice (age over 2 months) (**Fig. 7 A,B**). Ethanol did not affect paired pulse ratio of fEPSPs, which indicates that ethanol did not alter the release probability of glutamate (**Fig.7C**). In some recordings picrotoxin was present in bath solution to be sure that $GABA_A$ receptors were not contributing to observed ethanol inhibition. Picrotoxin application did not affect the extent of ethanol inhibition.

Aforementioned results were consistently seen in one laboratory during the whole period of experiments. However, we have not been able to repeat these findings in a different laboratory indicating that the phenomenon might be dependent on experimental conditions or the animal strain differences between the laboratories. Nevertheless, the results clearly show that also AMPA receptors along with NMDA receptors (Lovinger et al., 1990) can be very sensitive to ethanol in brain slice experiments. To date, we have not been able to clarify further what the exact conditions are, which make the AMPA receptors sensitive to ethanol in field potential recordings.

5.1.2. Desensitization of AMPA Receptors Affects Ethanol Inhibition

In patch clamp recordings from acutely isolated hippocampal neurons, cultured cortical neurons and HEK293 cells ethanol was found to inhibit AMPA receptor currents in a concentration-dependent manner. The receptor desensitization was found to affect the ethanol inhibition, as there was a correlation between receptor desensitization and ethanol inhibition (I). The correlation was shown with several different methods, which are summarized in Table 1. In summary, when high concentration of AMPA was used to activate currents in isolated hippocampal neurons, the steady state component of the current, which originate from receptor pool that contains mostly desensitized receptors, was inhibited more than the peak current, which originate from receptor pool containing mostly non-desensitized receptors. There was no difference in ethanol inhibition between the peak and steady-state current when low (10 μM) concentration of AMPA was used to activate currents, probably because under these conditions both the peak and the steady state current components originate from a receptor pool containing large amount of desensitized receptors. When kainate (which produces less desensitization than AMPA) was used to activate the currents, ethanol inhibition was decreased as compared to AMPA activated currents. However, ethanol inhibition of

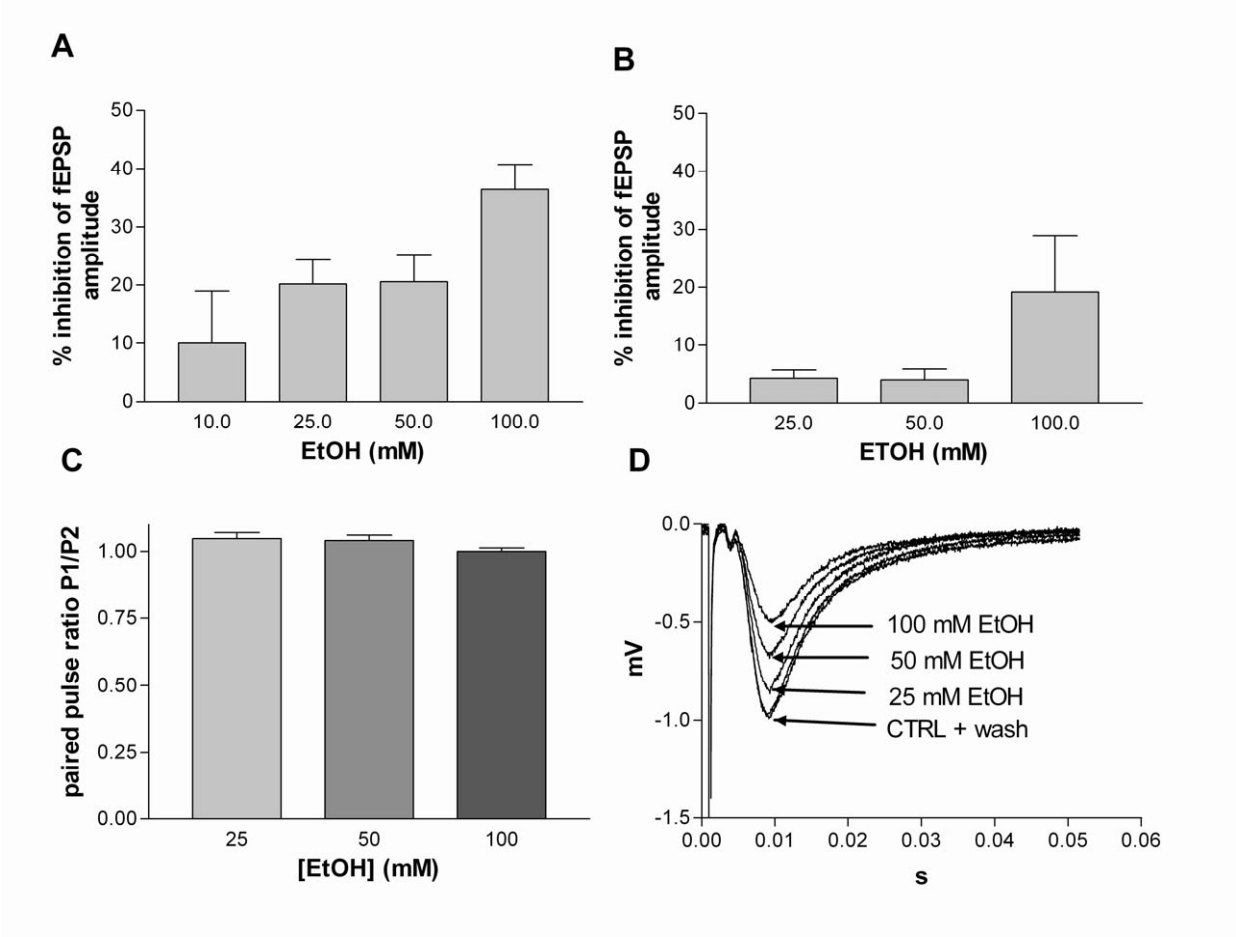

Figure 7. Ethanol inhibits AMPA receptor mediated fEPSP's in stratum radiatum of hippocampus. Ethanol inhibition of fEPSP amplitude in the presence of APV in young (age under 1 month) **(A)** and in adult mice (age over 2 months) **(B)**. Ethanol did not affect paired pulse ratio of fEPSP amplitude of young mice **(C)**. Representative traces of EPSP's of young mice in the presence of 25-100 mM ethanol are shown in **(D).** Note that control and wash traces are overlapping.

kainate activated current was found to depend on kainate concentration, as low concentration (30μM) activated currents were markedly more inhibited by ethanol; previously observed with oocyte expression system (Dildy-Mayfield and Harris, 1992). In addition, when cyclothiazide (which blocks the desensitization of AMPA receptors) was used together with AMPA, ethanol inhibition was decreased. Ethanol inhibition was also lower in GluR-A L497Y point-mutated homomeric receptors expressed in HEK293 cells as compared to wild type GluR-A. Finally, we used faster piezoelectric applicator and out-side-out membrane patches to better resolve the peak current of non-desensitized receptors, and found that it was not significantly inhibited by ethanol. In all experiments ethanol inhibition of AMPA currents was concentration-dependent, reproducible and reversible. In addition, ethanol inhibited the currents with or without 1 min pre-application of ethanol similarly suggesting that the effect of ethanol is very fast.

In order to measure ethanol effects on the rate of desensitization, the time course of the exponential decay of 100 μM AMPA-activated currents was determined using nonlinear curve fitting. Decay was well fitted with a single exponential function both in the absence and presence of

ethanol. The time constant (τ) for decay of current during agonist exposure was similar in the presence and absence of ethanol (for 50 mM ethanol experiments, baseline = 42 ± 7 ms, ethanol = 42 ± 7 ms; for 100 mM ethanol experiments, baseline = 35 ± 4 ms, ethanol = 33 ± 5 ms).

We also examined recovery from desensitization of 100 μM AMPA-induced current in the absence and presence of 100 mM ethanol in isolated hippocampal neurons (I; Fig. 5). This was accomplished using a two-pulse protocol in which AMPA was applied for 100 ms followed by a wash period of 200 to 1000 ms, and subsequent reapplication of AMPA. ethanol was applied throughout the duration of the recording. The peak amplitude of AMPA-induced current recovered with a time constant of 189 ms (95% confidence interval 175–204 ms, n = 9 cells) and this value increased to 233 ms (95% confidence interval 217–250 ms, n = 9 cells) in the presence of ethanol. Thus, ethanol produced a modest slowing of the rate of recovery from desensitization, which indicates that ethanol keeps the receptors in a desensitized state for a longer time than in control situation.

Table 1. Ethanol inhibition of AMPA receptors depends on desensitization (I) EtOH = ethanol; Ethanol inhibition % (inh. %) was calculated comparing current ampliture of ethanol application to the average of current amplitude of control applications before and after the given ethanol application. The bolded text in comment column is a conclusion made from the results supported with statistical significance.

Current activation condition	EtOH inh. % 50 mM	EtOH inh. % 100 mM	Comment / Conclusion
10 μM AMPA I_{PEAK}	21	36	EtOH inhibition is not different between 10μM AMPA activated peak and SS current
10 μM AMPA I_{SS}	22	36	
100 μM AMPA I_{PEAK}	7	16	EtOH inhibits peak less than SS current. **⇒ Desensitized receptors inhibited more by ethanol.**
100 μM AMPA I_{SS}	21	37	
30 μM kainate	34	44	Low concentration of kainate activated currents are highly sensitive to EtOH. High concentration of kainate activated currents are less inhibited by EtOH as compared to AMPA activated currents. **⇒ Non-desensitized receptors less inhibited.**
1 mM kainate	12	23	
10 μM AMPA	21	39	cyclothiazide decreases EtOH inhibition **⇒ Inhibition of desensitization decreases EtOH inhibition.**
+ cyclothiazide (same cells)	12	19	
HEK cells: GluR-A_i WT	26	46	**less EtOH inhibition with non-desensitizing mutant receptor**
GluR-A_i L497Y	5	19	
ultrafast appl. 100 μM I_{PEAK}		0	Pure peak current is not inhibited by EtOH. **⇒ EtOH does not inhibit non-desensitized AMPA receptors**
I_{SS}		40	

5.1.3 Ethanol Accelerates the Desensitization of GluR-D_i Receptors Coexpressed with TARPs in HEK293-cells

To study the effects of TARPs on AMPA receptor currents, a saturating concentration of glutamate (10 mM) was applied for 1 s to the HEK293 cells expressing GluR-D_i receptors. Coexpression of TARPs increased steady-state current/peak current-ratio, slowed the 10 to 90 % rise time of the peak current slightly and slowed the onset of the desensitization, measured as τ-value of decay of the peak current (II; Fig. 1).

Ethanol inhibited the recombinantly expressed GluR-D_i AMPA receptor currents, either with or without coexpressed TARPs, in a concentration-dependent manner. The steady-state current was inhibited more than the peak current (II; Fig. 2)

Ethanol decreased the τ-value of desensitization in GluR-D_i receptors (II; Fig. 3). The coexpression of either stargazin or γ4, further increased the effect of ethanol on desensitization. The highest ethanol concentration tested, 200 mM, reduced the τ-value by 16.5 ± 4.9 % (n=5) in GluR-D flip receptors, and by 29.3 ± 9.3 % (n=6) in GluR-D_i with stargazin. With γ4 coexpression 200 mM ethanol reduced the τ-value by 34.2 ± 5.0 % (n=7) and in the matching control experiments with D_i by 14.4 ± 5.8 % (n=5).

Next, we measured the effect of ethanol on the recovery from desensitization of GluR-D_i receptors expressed with or without TARPs. Coexpression of stargazin did not affect the time constant of recovery from desensitization, τ_{rec}, but coexpression of γ4 increased it. 100 mM ethanol did not affect the τ_{rec}. The results differ from the results of study I where 100 mM ethanol was found to increase the time of recovery from desensitization in isolated hippocampal neurons. τ_{rec}-values between the studies also varied substantially, being 189 ms in neurons (I), and 31 ms for GluR-D_i expressed in HEK-cells (II). This difference might reflect the different AMPA- and TARP-subunit composition between the studies. TARPs seem to affect the time of recovery from desensitization, since we observed that the coexpression of γ4 increased the τ_{rec} to 59 ms (II).

5.1.4. General Discussion of Ethanol Inhibition of AMPA Receptors

Studies on the inhibition of AMPA receptors by ethanol have produced controversial results. Studies in brain slices, where the neurotransmission is intact, have produced to date mainly data showing that ethanol does not inhibit AMPA receptor-mediated function (Carta et al., 2003; Hendricson et al., 2003; Lovinger et al., 1989; Lovinger et al., 1990; Weiner et al., 1999), rare exceptions being neonatal CA3 area of the hippocampus (Mameli et al., 2005) and central amygdala (Zhu et al., 2007). On the other hand, numerous studies done with isolated neurons or heterologous expression systems, like *Xenopus laevis* oocytes or HEK293 cells, have shown that AMPA receptors are sensitive to ethanol at clinically relevant concentrations (Dildy-Mayfield and Harris, 1992; Lovinger, 1993; Wirkner et al., 2000). In these experiments, AMPA receptors have been found to be inhibited by ethanol as much as NMDA receptors, which are, along with $GABA_A$ receptors, considered to be the most important targets of ethanol in the brain. Our data give a possible explanation to this discrepancy as ethanol mainly affects desensitized AMPA receptors (I).

The extent of receptor desensitization differs radically between brain slice experiments and cell experiments, because synaptic AMPA receptor currents are thought to originate from non-desensitized channel openings, whereas in experiments with isolated or cultured cells the receptor currents are evoked by applying agonist onto the cells for such a long time that the currents originate from the receptor pool containing mostly desensitized receptors. In cell experiments, currents originating from non-desensitized receptors can be activated only if high enough concentration of agonist is applied to the receptors, faster than the onset of desensitization. We did this using piezoelectric applicator and outside-out membrane patches pulled from cultured cortical neurons, and showed that the pure peak current is not significantly inhibited by ethanol.

Interestingly, our results show that ethanol decreases the τ-value of desensitization of GluR-D_i receptors expressed in HEK293 cells. When either stargazin or γ4 are coexpressed with the GluR-D_i, this effect of ethanol on desensitization is enhanced (II). Our data suggest that ethanol inhibits AMPA receptors by speeding up the transition to desensitized state. The involvement of TARPs in ethanol inhibition of AMPA receptors may be significant in the brain where putatively the majority of AMPA receptors are combined with TARPs in synapses (Hashimoto et al., 1999; Menuz et al., 2008; Milstein et al., 2007; Rouach et al., 2005; Tomita et al., 2003). GluR-D_i which we used in HEK293-cell expression studies is strongly expressed during development (Gallo et al., 1992; Mosbacher et al., 1994; Zhu et al., 2000), as is the γ4 subunit of TARPs, which is the only TARP expressed during development (Tomita et al., 2003). Ethanol was found to speed up very efficiently the transition to desensitized state of GluR-D_i+γ4 combination, which might increase the sensitivity of developing nervous system to ethanol. Our fEPSP recordings from hippocampal slices of young animals also suggest that AMPA receptor-mediated synaptic function is sensitive to ethanol. Therefore, AMPA receptors in developing brain might be sensitive to ethanol in some conditions. Inhibition of AMPA receptors by ethanol could thus contribute to the impairments induced by fetal alcohol exposure, e.g. in fetal alcohol syndrome (Calhoun and Warren, 2007).

As discussed above, ethanol has been shown to inhibit AMPA receptor currents in native synaptic conditions only rarely (Mameli et al., 2005; Zhu et al., 2007). This might be because, generally speaking, the desensitization does not participate in synaptic AMPA receptor currents. Miniature EPSCs and currents activated by electrical stimulation decay at the rate of deactivation, rather than at the rate of desensitization. However, there are brain areas where the desensitization has been shown to participate in synaptic AMPA receptor currents, such as in cerebellum and at a calyceal synapse (Barbour et al., 1994; Maguire, 1999; Otis et al., 1996; Trussell et al., 1993; Wall et al., 2002). In these brain areas, clinically relevant concentrations of ethanol might inhibit the AMPA receptors. The lowest concentration affecting AMPA receptors in electrophysiological studies is around 20 mM, which is equivalent to 1 per mille of blood alcohol, achievable during moderate drinking. Some of the effects of high ethanol intoxication, like loss of memory and motor coordination problems could well be caused by the effect of ethanol on AMPA receptors along with NMDA receptors.

Our studies on ethanol inhibition of AMPA receptors were carried out in acutely isolated native neurons (I) and HEK293 cell expression system (II). The results between the studies were generally similar, albeit with some minor differences. The peak current was inhibited less than

steady-state current in both studies, and the extent of ethanol inhibition of these currents was similar between the studies. However, in native hippocampal neurons ethanol did not affect the rate of desensitization, whereas in HEK293 cells it did. The other difference was that ethanol increased the recovery from desensitization in native neurons, but not in HEK293 cells. One possible explanation for these discrepancies is different combinations of AMPA and TARP subunit expression between the experimental preparations. Hippocampal neurons express extensively γ8 and also smaller amounts of other TARPs (Tomita et al., 2003) and GluR-A, -B and -C subunits of AMPA receptors (Keinänen et al., 1990). The other major difference between native neurons and expression systems is that the receptors are differently regulated by other proteins. For instance, in acutely isolated neurons part of the receptors are still attached to the postsynaptic density protein complex, whereas in HEK293 cells they are on the cell membrane in non-organized manner.

5.2. Agonist Binding Capability Determines AMPA Receptor Trafficking

In order to study the trafficking of AMPA receptor from the ER, point mutated GluR-D_i receptors were expressed in HEK293 and COS7 cells and the surface expression of them was measured using electrophysiology, immunofluoresence and surface biotinlation assay. Receptors used in this study were: GluR-D_i R507K and E727Q, which do not bind glutamate (Jouppila et al., 2002); E727D, which preserves binding although with somewhat lowered affinity for glutamate; and L505Y, which does not desensitize to the same extent as the wild type counterpart but retains binding.

To verify the functional status of the mutant receptors, whole cell patch clamp recordings were made from transfected HEK293 cells. L-Glutamate (10 mM) elicited rapidly desensitizing currents in both wild-type and E727D receptor channels, whereas no measurable current was observer with R507K or E727Q mutants. A substantial non-desensitizing steady-state current was observed with the E727D mutant but not with WT GluR-D_i (III; Fig 2A). Glutamate triggered a robust non-desensitizing current in GluR-D L505Y receptors. EC_{50}-values for glutamate and AMPA were calculated from dose response curves and are reported in Table 2.

Next, we wanted to know the expression levels of AMPA receptors and what state of the trafficking process the receptors undergo using immunofluorescence microscopy, endoglycosidase H (EndoH) treatment and LBD-secretion analysis. The results are presented in Table 2. In summary, the overall protein expression levels did not differ between the GluR-D_i receptors indicating that the cells translate all the cDNAs to proteins and that the cells are not degrading any mutant receptor faster. Only WT GluR-D_i and E727D mutant showed intense surface binding indicating that they are transported to cell surface in large quantities. L505Y staining was lower than with wild type GluR-D_i, even thought in patch clamp recordings with this mutant receptor had large currents of hundreds of pA which is comparable to wild type GluR-D_i currents (III: Fig. 2A). The possible reason for this discrepancy is that non-desensitizing receptors produce large currents even when their number is low on the cell surface. Interestingly, inclusion of competitive antagonist NBQX increased the surface expression dramatically (III: Fig. 3C). The reason for this may be that the antagonist function as molecular chaperone which facilitate the

trafficking of receptor or that it was able to prevent the possible cell toxicity that might result from expression of non-desensiting mutant. R507K and E727Q mutant receptors were largely absent from cell surface. E727Q, R507K and L505Y were sensitive to EndoH-treatment, which indicates that they contained unprocessed glycans that are added in ER, but have not been processed further in Golgi apparatus. Therefore, the receptor mutants are primarily retained in the ER. Immunoblot analysis of media and cell extracts of transfected HEK293 cells revealed S1S2-LBD of GluR-D_i and E727D mutant were efficiently secreted into the culture medium indicating that LBD is translated and trafficked independently of other domains of AMPA receptors. LBD of L505Y mutant was secreted at intermediate level, whereas all nonbinding mutant LBDs were not secreted. Interestingly, the coexpression of stargazin was able to rescue the surface expression of nonbinding R507K and E727Q receptors.

Table 2. Summary of the results of Wild type GluR-D_i and point mutated receptors. + = strong; +/- = intermediate/detectable; - = not detectable; EndoH = endoglycosidase H enzyme (endoglycosidase H cleaves the glycans which have been added in the ER, but not if processed further in the Golgi apparatus. Resistance to EndoH thus reveals if the proteins have been trafficked out from the ER.); Perm. im.staining = permealized immunostaining; non-perm im.staining = non-permealized immunostaining. LBD = ligand binding domain (Cell lines secrete constructed S1-S2-ligand binding domains to the cell culture media. The corresponding point mutations that were in full length receptors were made to LBD).

construct	phenotype	Glutamate EC_{50}	AMPA EC_{50}	perm. im.staining	non-perm. im.staining	resist. to EndoH	LBD secretion
D_i (WT)	Wild type	2 mM	86 µM	+	+	+	+
R505K	non binding for AMPA and Glu	-	-	+	-	-	-
E707Q	non binding for AMPA and Glu	-	-	+	-	-	-
E727D	lower affinity to Glu	64 mM	100 µM	+	+	+	+
L505Y	non-desensitizing	49 µM	30 µM	+	+/-	-	+/-

Next, we investigated how point mutated AMPA receptors are trafficked in neurons, which natively express GluR-D and other AMPA receptor subunits, as well TARPs. For this purpose, the cortical neurons were co-transfected with GFP and GluR-D cDNAs at 10 *d.i.v.* and visualized for GFP expression and anti-Flag surface staining at 14 *d.i.v.* Clear surface labeling was seen for recombinant GluR-D_i and GluR-D_o receptors. In contrast, the nonbinding GluR-D_i mutants R507K and E727Q, which were not trafficked to the cell membrane in cell lines (in the absence of stargazin), did not show any surface expression in transfected neurons. As cortical neurons express stargazin and other TARPs (Tomita et al., 2003), the specific trafficking defect observed for the

non-binding GluR-D mutants implies differences in the quality control mechanisms and regulation of trafficking between neurons and heterologous cells.

The results show that agonist binding capability is an important determinant of whether ER quality control will allow AMPA receptors trafficking to cell membrane. Quality control checking is independent of other domains and tetrameric structure of the receptor, since the extracellular secretion of S1S2-LBD is regulated similarly to the full length receptors surface expression. Our results confirm further the influence of stargazin (or other TARPs) in the trafficking of AMPA receptors because its coexpression rescued the non-glutamate-binding mutants. However, in neuronal cultures AMPA receptors, which are unable to bind glutamate, do not enter the cell surface, although the neurons express endogenous TARPs, including stargazin. The trafficking machinery between expression cell lines and cultured neurons differs in this respect, being apparently more complex and selective in neurons. This indicates that still unknown proteins or protein modifications could participate in AMPA receptor quality control in mature neurons. The role of TARPs in the control of AMPA receptor trafficking may increase during the development. Neurons seem to express more flop-isoforms of AMPA receptors as the nervous system matures (Gallo et al., 1992; Mosbacher et al., 1994), and particularly the flop forms seem to require the assistance of TARPs in trafficking to the cell membrane (Coleman et al., 2006; Penn et al., 2008).

Desensitization (or the ability of the receptor to transition between non-desensitized and desensitized state) has also been proposed to have a role in trafficking of AMPA receptors through ER (Fleck, 2006). The non-desensitizing GluR-B mutant (L504Y) is expressed poorly on surface of neurons, whereas a mutant (N768D) with accelerated desensitization has increased surface expression (Greger and Esteban, 2007; Greger et al., 2006). Also in kainate receptors, desensitization plays a role in receptor trafficking as mutant GluR6 receptors locked in a non-desensitizing conformation are poorly transported to cell surface (Priel et al., 2006). Consistent with these studies, we found that the non-desensitizing GluR-D_i mutant L505Y has reduced cell surface expression as compared to wild type receptor (III: Fig. 3C). However, in our study, the inclusion of NBQX increased the surface expression of L505Y receptor, and improved the condition of cultured cells indicating that expression of L505Y is toxic to the cells. Part of the observed decrease in the surface expression might, therefore, be result of the death of the cells expressing high levels of this particular mutant.

5.3. Maximal Electroshock Sensitivity and Synaptic AMPA Receptor Function Are Decreased in Thy1α6 Mice

To determine the electroconvulsive sensitivity of Thy1α6 and wild-type mice, they were subjected to electroshocks of different intensities. The occurrence of tetanic hind limb extension (THE) was observed. When the proportion of animals having THE was plotted as a function of current, electroconvulsive threshold EC_{50} of the wild-type mice was 8.2 mA ($R^2 = 0.9618$; 95% confidence interval 7.9 - 8.6), which was about 2 mA ($P < 0.001$) lower than the EC_{50} of Thy1α6

mice (10.0 mA; $R^2 = 0.9548$; 95% confidence interval 9.7 - 10.4). Thus, the transgenic mice displayed a higher threshold to electroconvulsions.

In order to find the explanation for the observed differences in electroshock thresholds the state of AMPA receptor-mediated excitation was studied using mEPSC recordings of hippocampal CA1 pyramidal neurons. When the individual mEPSCs from the complete recording of WT and Thy1α6 mice were averaged, and the traces reproduced, there was a difference in the peak amplitude with the shapes remaining identical (IV; Fig. 2). The wild-type mice had significantly higher average amplitude of 10.3 ± 2.5 pA (mean ± SD, n=13) as compared to that of Thy1α6 mice, which was 8.4 ± 2.2 pA (n = 16; $P < 0.05$, Student's *t*-test). This can also be seen in a relative cumulative frequency histogram-plot (IV; Fig. 2B). AMPA receptor mEPSCs from wild-type and Thy1α6 mice had a similar frequency (0.25 ± 0.19 Hz and 0.20 ± 0.22 Hz, respectively) and τ-value of decay (4.7 ± 1.8 and 4.7 ± 2.5, respectively).

In Thy1α6 mice the overexpression of $GABA_A$ receptor α6 subunit is induced in various forebrain areas including CA1 area of the hippocampus. In this brain region the overexpression of α6 subunit leads to a five-fold increase of extrasynaptic $GABA_A$ receptor-mediated tonic inhibition (Wisden et al., 2002). There is also 30 % less synaptic $GABA_A$ receptor-mediated inhibition as measured by mIPSC analysis (Wisden et al., 2002), probably because overexpression of α6 diverts $GABA_A$ receptor subunits to an extrasynaptic location from synaptic location. Interestingly, our results also show that synaptic AMPA receptor function is decreased in these animals. This suggests that synaptic scaling processes reduce the synaptic excitation as a response to decreased synaptic inhibition. The difference in electroshock sensitivity between Thy1α6 mice and wild types could be due to decreased AMPA receptor function, as AMPA receptor blockers are also reported to decrease MES induced convulsions (Micale et al., 2002; Yamashita et al., 2004). The other explanation for decreased MES sensitivity in Thy1α6 mice may be the increased extrasynaptic inhibition. However, this seems unlikely. Thy1α6 mice are more sensitive to $GABA_A$ receptor antagonists, picrotoxinin and DMCM-induced convulsions (Sinkkonen et al., 2004) indicating that phasic inhibition is more important in resistance to convulsions than tonic extrasynaptic inhibition. DMCM antagonises mainly synaptic $GABA_A$ receptors (Knoflach et al., 1996), and might even have a positive effect on extrasynaptic $GABA_A$ receptors in Thy1α6 mice (Crestani et al., 2002).

We observed that only the amplitude of mEPSCs was decreased in Thy1α6 mice, the frequency and time constant of decay being at the same level as in wild types (IV). This indicates that there are changes in AMPA receptors at the post synaptic membrane, such as decrease in number, change in phosphorylation or subunit composition. It has been shown that phophorylation increases the opening frequency and the mean open time of AMPA receptors (Greengard et al., 1991). Decay kinetics of AMPA receptor currents has also been shown to depend also on subunit composition of AMPA receptors (Geiger et al., 1995). Since the time constant of mEPSC decay did not differ between the mouse lines in study IV, AMPA receptor populations presumably had the same subunit composition. This implies that the number of receptors in synapses was lower in Thy1α6 mice. The removal of AMPA receptors from synapses is a dynamic process involving complex interaction between many proteins. For instance, in cerebellar Purkinje cell LTD GluR-B containing receptors are taken out from synapses through protein kinase C phosphorylation of

ser859 in intracellular C-tail of GluR-B (Chung et al., 2000; Matsuda et al., 1999). This leads to a decrease of AMPA receptor binding to GRIP and actin-binding proteins, but increases the binding to PICK1, which promotes the removal of AMPA receptors from synapses. Phosphorylation of ser859 has been shown to increase the removal of AMPA receptors from synapses in hippocampus (Chung et al., 2000; Perez et al., 2001); albeit the phosphorylation is not executed by PKC, but by some still unknown kinase (Kim et al., 2001). Recent studies have discovered one possible intracellular signalling mechanism in regulation of number of AMPA receptors on neural membrane; immediate early gene Arc (Shepherd et al., 2006). High neural activity increases the levels of Arc protein, which leads to the endocytosis of AMPA receptors, causing a decrease in AMPA receptor surface expression and thus a decrease in AMPA receptor mEPSC. In contrast, low neural activity decreases Arc levels and increases AMPA receptor surface expression and mEPSC amplitude. However, the role of Arc in the regulation of synaptic AMPA receptors is not as straightforward as mentioned above, because Arc is also induced in LTP consolidation, which requires net insertion of AMPA receptors (Guzowski et al., 2000).

5.4 Future Directions in the Research of AMPA Receptors

In this thesis (study I, II) it was shown that ethanol inhibits AMPA receptors and the mechanism of inhibition was found to be the increase of desensitization of the receptor. It is still unclear in what conditions AMPA receptors participate in the actions of ethanol in the brain. Our fEPSP recordings showed that in young mice AMPA-mediated synaptic function can be sensitive to ethanol. However, it has not been widely reported that AMPA receptors are markedly inhibited by ethanol in intact synaptic transmission. Therefore, more studies in this field are required. These experiments should target the brain areas where desensitization participates in synaptic AMPA currents like cerebellum (Barbour et al., 1994; Wall et al., 2002). The effect of ethanol on synaptic AMPA function should also be studied in different conditions. For instance, train stimulation of axons could elevate the extracellular glutamate concentrations and lead to the situation where AMPA receptors would be partially desensitized and thus sensitive to ethanol. This would mimic the burst firing of neurons (Swensen and Bean, 2003) and hyperexcitability related to brain pathology (Chattipakorn and McMahon, 2003). The effect of TARPs on ethanol inhibition of other GluR subunits than GluR-D should to be studied also in order to clarify the exact role of TARPs in the effects of ethanol. Whether TARPS play a role in some of the rare instances where ethanol inhibits AMPA receptors (Mameli et al., 2005; Zhu et al., 2007) should also be clarified.

Studies of AMPA receptors have increased our understanding of how the brain works. The control of AMPA receptor function and trafficking are now considered one of the most important mechanisms in many aspects of brain function, such as learning and memory, as well as brain pathology. In recent years, many proteins involved in various stages of AMPA receptor trafficking and regulation of function have been discovered. Great deal of advances has also been made in determining the important domains and amino acids in the AMPA receptors involved in protein interactions. However, there are still many unknown proteins and regulation mechanisms in

AMPA receptor trafficking. For example, the known protein interactions do not sufficiently explain all the mechanisms of quality control taking place in ER and how the insertion and removal of synaptic AMPA receptors is regulated. Our results (III) indicate that glutamate binding capability is an important feature of GluR-D receptors in trafficking out of ER. Is the glutamate binding also important in the trafficking of other GluR subunits? In study III the non-desensitizing mutant receptor was trafficked to cell membrane less efficiently than wild type receptors. Does this also relate to agonist binding capability, since desensitized receptors bind glutamate with highest affinity?

One fundamental weakness in many of the studies on regulation of AMPA receptor trafficking and function has been that they make use of heterologous expression cell lines instead of native conditions. The regulation of AMPA receptors may differ substantially between these two conditions, as became evident in study III where the surface expression of non-binding mutants was rescued by stargazin in HEK293 cells but not in cultured neurons. AMPA receptor regulation is very complex and may require many proteins that are not present in expression cell lines. In addition, the difference in morphology between dendritic tree containing neurons and more-or-less round and flat shape expression cells may contribute to the differences in experimental conditions and results. As the research methods develop in future, research should concentrate more on studies in native conditions in order to clarify how brain function is really regulated. This is especially important if we wish to find treatments to pathological conditions involving AMPA receptors. Knowing the exact molecular regulation mechanisms would not only help to develop new medical treatments, but also help to explain possible adverse effects and weaknesses in them. For instance, from the study IV and studies with AMPA antagonists (Micale et al., 2002; Yamashita et al., 2004) one can conclude that reducing AMPA receptor function could be used as an anticonvulsion treatment. Whilst this approach could work in some types of acute convulsions, it would probably not be a good strategy for a long term medical treatment, such as convulsion prevention, since synaptic scaling would likely increase the excitation back to the level it use to be before the treatment.

6. Conclusions

1. Ethanol inhibition of AMPA receptors depends heavily on the receptor desensitization. The more receptors that are desensitized the higher is the ethanol inhibition. Ethanol speeds up the rate of desensitization of GluR-D_i receptors expressed in HEK293-cells.

2. When TARPs are coexpressed with GluR-D_i, increase in the rate of desensitization by ethanol is greater than with GluR-D_i alone. Accelerating the rate of desensitization is a mechanism of ethanol inhibition of AMPA receptors which leads to an increase in the time the receptors channel spend in closed state.

3. Ligand binding capability is important determinants of the early trafficking of GluR-D_i AMPA receptors. Desensitization plays a minor role in it. The TARP stargazin is able to rescue the trafficking to the cell surface of non-ligand binding receptors in expression cell lines, but endogenous TARPs fails to do so in primary neuronal cultures.

4. Hippocampal synaptic function of AMPA receptors is decreased in a transgenic mouse line which has less synaptic $GABA_A$ receptor-mediated inhibition. This mouse line is also less sensitive to electrical convulsions. The results demonstrate that synaptic excitatory and inhibitory neurotransmission systems tend to be in balance.

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