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ALLOSTERIC REGULATION OF SYNAPTIC PROCESSES

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ALLOSTERIC REGULATION OF SYNAPTIC PROCESSES
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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In loving memory of

my aunt Sephali Guha

ABSTRACT

Glutamatergic neurotransmission is of key importance for short-term and long-term plasticity in the hippocampus, a part of the medial temporal lobe which is responsible for processes of explicit semantic and spatial memory. Short-term plasticity is mainly regulated by the presynaptic neuron and long-term plasticity is to large parts regulated by the post-synaptic neuron. In this thesis we have looked into cellular and molecular biophysical mechanisms in glutamatergic neurons mainly in the hippocampus.

We first reviewed the presynaptic mechanisms underlying short-term plasticity like assembly of the release machinery, positional and molecular priming, site preparation, calcium dynamics regulation, intrinsic vesicular fusogenicity, endocytosis, acidification and filling.

In study 1 we looked into the role of intrinsic vesicle fusogenicity on short-term plasticity by formulating a deterministic vesicular release model based on ordinary differential equations. Intrinsic vesicular fusogenicity was an allosteric property we invented in order to test the hypothesis of calcium independence. The model was able to simulate properties of resting neurons, by reproducing the spontaneous release rates and the size of the readily releasable pool. Furthermore, assuming that the heterogeneity in vesicular release probability arises due to differences in intrinsic vesicular fusogenicity, the model was able to explain depression by an imbalance between fusion and vesicular priming. It also predicted that facilitation could be due to an increase in intrinsic vesicular fusogenicity, which together with build-up of calcium gave rise to initial increase in vesicular release. Finally, we investigated the effect of three different modes of regulation of release probability on short-term plasticity. It was seen that differences in intrinsic vesicular fusogenicity gave rise to a more significant change in short-term plasticity than change in calcium sensitivity of release. All in all the results tell us that intrinsic vesicular fusogenicity has an important role in tuning short-term plasticity.

In study 2 we investigated the regulation of the postsynaptic allosteric AMPA receptor. To do this we developed a model based on the Monod Wyman Changeux framework which described the ligand concentration dependence of the conductance states by increasing affinity to conductance states. The model was able to explain thermodynamic behaviours of native and recombinant receptors when stimulated with full agonists like glutamate and quisqualate as well as partial agonists like willardiines. It was also predicted that the receptor stabilizes its large conductance state within the rise time of a so-called 'mini' post-synaptic current, providing a possible underlying mechanism for the peak of the current.

In study 3 we investigated the high-dose hook effect in allosteric proteins by first developing a combinatorical theory for how linker proteins behave under conditions of perfect binding. The theory predicted that the steady-state concentration of fully bound linker-proteins decreases at a critical concentration of initial free linker protein as the free linker protein

concentration is increased. This effect is however decreased in proteins where binding of ligand occurs in a cooperative fashion. The outcome was validated by simulations of dimeric and tetrameric linker proteins under imperfect binding. We also simulated the cooperative synaptic protein calmodulin, and it was seen to be subject to the hook effect. The hook effect was stronger in the presence of the allosteric activator Ca2+/calmodulin kinase II (CamKII). We show that increased amounts of the allosteric activator can decrease the activity of calmodulin. At 140 uM calmodulin behaved only as if the molecule only appeared in the relaxed (R) state. The relaxed state has no cooperativity, but has higher ligand affinity than the wild-type calmodulin. Even though this phenomenon may be present in many different biochemical systems, synapses contain several linker proteins that are pivotal for synaptic plasticity for instance AMPA receptors, synaptotagmin, calbinding and calmodulin.

In summary, this thesis gives insight into allosteric mechanisms in glutamatergic hippocampal neurons by using whole-cell voltage clamp and algebraic modelling. Specifically, it suggests an explanation for the important role of allosteric mechanisms in vesicular release probability and short-term plasticity. It also provides an explanation for the ligand concentration dependence of AMPA receptors and puts forward a theory for how complexes and active forms of linker proteins behave under increase of free linker protein concentration, a behaviour might contribute to pre-and postsynaptic processes.

LIST OF SCIENTIFIC STUDIES

Below I have listed the scientific publications of this thesis in the order they are discussed in the thesis.

Review Biophysical properties of presynaptic short-term plasticity in hippocampal neurons: insights from electrophysiology, imaging and mechanistic models. Ranjita Dutta Roy, Melanie I Stefan and Christian Rosenmund (published in Frontiers in Cellular Neuroscience 2014)

I. Mechanistic model predicts that intrinsic vesicular fusogenicity tunes short-term depression in hippocampal neurons. Ranjita Dutta Roy, Jens Hjerrling Leffler, Jeanette Hellgren Kotaleski and Christian Rosenmund. (manuscript)

II. Ligand-dependent opening of the multiple AMPA receptor conductance states: a concerted model. Ranjita Dutta Roy, Christian Rosenmund, Stuart Edelstein and Nicolas Le Novère. (published in PLoS One 2015)

III. Cooperative binding mitigates the high dose hook effect. Ranjita Dutta Roy, Christian Rosenmund, Melanie I Stefan (published in BMC Systems Biology 2017)

CONTENTS

LIST OF ABBREVIATIONS

AMPAR	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
NMDAR	N-methyl-D-aspartate receptor
TARP	Transmembrane AMPAR regulatory protein
STP	Short-term plasticity
ECM	Extracellular matrix
RRP	Readily releasable pool
PvR	Vesicular release probability

BACKGROUND

Synapses are the sites where one neuron connects to the next. The communication between the presynaptic and the postsynaptic neurons can either be chemical or electrical. In this thesis we have worked on the former type of synapse in glutamatergic hippocampal neurons. Many molecules are of importance in synaptic transmission. Presynaptically synaptotagmin has an important role in calcum sensitivity of vesicle release. Other calcium activated proteins like calmodulin and plasma membrane pumps are active in synapses. Also ligand-gated ion channels are regulated in a similar way to calcium binding molecules. To understand synaptic transmission in depth it is important to understand the basis of the regulation of these compounds. For instance are they orthosterically or allosterically regulated? Is there binding cooperativity, and in which case how strong is it? Can the molecule exist in spontaneously active states?

When the term allostery was coined, it was mainly used for the regulation of enzymes and eventually haemoglobin. More recently it has been used for ion channel gating. When a so-called effector molecule binds to a site which is not the active site of the molecules, it is called an allosteric regulator. In multimeric proteins, binding cooperativity is also a factor. In this thesis we have taken into account allosteric regulation and binding cooperativity of the AMPA receptor and calcium sensing in vesicle fusion, as well as calmodulin. With these three examples, we want to emphasize the importance of allosteric regulation and cooperativity in the synapse.

The physiological and pathological roles of plasticity in the hippocampus

Synaptic plasticity is the ability of neurons to alter their responses and their synaptic structures based on previous experience. Plasticity can be divided into two time scales; short-term plasticity (STP) takes place on a sub-second to minute time scale, and long-term plasticity (LTP) takes place on higher than minute scales [Zucker and Regehr 2002]. STP has been measured in all three pathways of the hippocampus; (1) the perforant pathway in which granule cells in the dentate gyrus receive input from the entorhinal cortex, (2) the mossy fiber pathway in which the granule cells in the dentate gyrus form synapses onto pyramidal neurons in the CA3 region, and (3) the Schaffer collateral pathway where CA3 pyramidal neurons form synapses onto CA1 neurons. These three pathways are involved in explicit and spatial memory consolidation. Most neurons in the hippocampus are excitatory cells and glutamatergic. Most inhibitory neurons in the hippocampus are GABAergic. In pathological states such as those where the balance between excitation and inhibition is altered, e.g. schizophrenia, epilepsy and memory related diseases such as Alzheimer's disease, have shown alterations in synaptic plasticity [Froc et al 2003, Neves et al 2008,

Witton et al 2010, Wood et al 2000]. Pathological states do not necessarily affect the single-cell firing patterns, but affect the network waves delta, theta, alpha, beta and gamma.

Algebraic modeling versus statistical analysis

In the field of Systems Biology, both statistical modeling and algebraic modeling are common. Below is an attempt to clarify the distinction between descriptive statistical analysis and biophysical algebraic models.

When we first get hold of a sample data set, we need to know whether it is representative of the general population. In order to do this we make a hypothesis test and see if it follows a specific distribution. A common distribution is the normal distribution, where μ is the mean and σ is the standard deviation. Coherence to the normal distribution can be tested graphically or analytically. Other commonly occurring distributions in nature are the binomial distribution and the Poisson distribution. Depending on whether the data points are quantifiable or categorical, we can choose to make either parametric distribution tests or non-parametric tests. To test the significance of the fit, we can make use of p values.

In our own studies in this thesis we have worked with electrophysiological patterns and tried to derive biophysical algebraic models which could explain the underlying mechanisms of the pattern. These biophysical models are based on reaction kinetics and developed to predict critical factors. We fitted our theoretical models to normalized extra-synaptic currents by using optimization algorithms. Coupled ordinary differential equations are often non-linear as they have unknown variables of degree higher than one in them. Such types of non-linearity give rise to non-planar bends in state space. This adds to the constrains of model fitting. Hence, simplistic linear models are almost always easier to fit to a hypothetical model compared to coupled differential equation systems.

How do we then make the fit? We can use a correlational regression factor, but we can also make use of local or global optimization algorithms. Local optimization algorithms bring the system closer to its extreme points in local concave or convex areas, whereas global algorithms use a 'broader view' to bring the system closer to its most extreme point. Regression factors on the other hand only measures the difference between the data and the theoretical curve. When biophysical models are used, parameters should be estimated by empirical methods before the optimization procedure. This requires knowledge of experimental protocols and background literature in the biological field of interest.

It has been common in the past to reduce biophysical models to get rid of complexity. A complementary method to study model behaviour versus over- or underdetermination is by using parameter identifiability analysis.

Pre-and postsynaptic mechanisms in glutamatergic neurotransmission

The role of the presynaptic transmitting neuron in a hippocampal synapse as well as the receiving postsynaptic neuron are of importance in synaptic activity. In general, the presynaptic side is thought to have a more important role in short-term plasticity and the post-synaptic side in long-term plasticity. The biophysical synaptic mechanisms underlying presynaptic short-term plasticity (STP) can be subdivided into four groups, namely (1) calcium dynamics, (2) vesicle exocytosis, (3) neurotransmitter filling and (4) vesicle and site replenishment [Zucker and Regehr 2002]. STP can take shape as depression or facilitation. The calcium hypothesis states that build-up of calcium gives rise to early facilitation, and depression is thought to be due to depletion of vesicles [Katz and Miledi 1968].

Upon depolarization of the axon, presynaptic voltage-gated calcium channels allow influx of calcium into the axon, which in turn binds to the calcium sensors of the vesicles and gives rise to exocytosis as the vesicle fuses with the plasma membrane by the help of SNARE proteins [Bischofberger et al., 2002, Meinrenken et al 2001, Neher and Sakaba 2010, Wan et al 2012]. The relationship between release and calcium concentration is not linear, and this was clear already in the early days [Dodge and Rahamimoff 1967]. A single action potential in neocortical pyramidal neurons yields a residual calcium transient of amplitude 500 nM with a decay time of 0.1 s [Koester and Sakmann 2000]. The presynaptic calcium dynamics is not only determined by the calcium released from N-type and P/Q type voltage-gated calcium channels, but it is governed by several parameters, such as calcium binding proteins, mitochondria and plasma-membrane calcium ATPases [Brini et al 2009, Carter et al 2002, Felmy et al 2003, Jouvenceau et al 1999]. Many of these proteins use cooperative binding and are regulated allosterically. Examples of a calcium binding protein, which is found in hippocampal neurons, is calbindin [Berggård et al, 2002]. It has also been suggested that different calcium channels act differently. In GABAergic neurons of the hippocampus for instance, N-type channels are thought to govern release in CCK neurons, and P/Q-channels in PV neurons [Hefft and Jonas 2005]. In addition, local calcium at the release site is regulated differently to global calcium in the synapse [Bruton et al, 1999]. We therefore refer to global and local calcium as two different transients.

Exocytosis was first captured with quick freezing in the frog neuromuscular junction [Heuser et al 1979]. It can either be evoked or occur spontaneously. Evoked release can be

blocked by magnesium without blocking spontaneous release [Katz and Miledi 1963, Vyleta and Smith 2011]. Vesicles fuse spontaneously around once per eight minutes [Murthy and Stevens 1999]**.** On the other hand, evoked release can give rise to multivesicular release from single synapses [Christie and Jahr 2006]. In conjunction with this, the question of a refractory time has been under debate [Nadkarni et al 2010, Pan and Zucker 2009, Stevens and Wang 1995]. At spontaneous fusion, only one vesicle is released and the response size of this vesicle is referred to as the quantal size [Jonas et al 1993]. The quantal size varies between neurons [Bekkers et al., 1990]. The distribution of the sizes of spontaneous exocytosis for instance in neuromuscular junction is often Gaussian, whereas in central synapses, it is often skewed [Auger and Marty, 2000]. How much neurotransmitter the vesicle fills can be dependent on the pH or membrane potential of the vesicle, which drive the ATPase coupled vesicular neurotransmitter channel. In glutamatergic vesicles the membrane potential is more important and in GABAergic vesicles the pH [Blakely and Edwards 2012, Omote et al. 2011].

After exocytosis, the membrane including its proteins and phospholipids have to be replenished [Rohrbough and Broadie 2005, Takamori et al 2006, Wang et al 2006]. Endocytosis takes place on a much longer time scale than exocytosis [Ryan et al 1993, Wu 2014, Wu et al 2014]. Clathrin-mediated endocytosis takes place on a time scale of 15-30 s outside the active zone [Granseth and Lagnado, 2008, Hori and Takahashi, 2012 and Watanabe et al 2013]. Since this was thought too slow to replenish the vesicle pool, the kiss-and-run hypothesis was suggested, which says that a vesicle touches the membrane and releases its neurotransmitters, but does not fuse fully with the membrane [Ceccarelli et al, 1973 and Gandhi and Stevens, 2003, Zhang and Tsien 2009]. Kiss-and-run endocytosis is largely debated, but it has been suggested that endocytosis on a time scale of 10-15 s is rate-limiting for replenishing the RRP when activity takes place on a longer time scale [Granseth and Lagnado 2008]. Endocytosed vesicles are significantly mobile at first, but then cluster with other vesicles and enter a low mobility state [Kamin et al 2010]. Each vesicle release site has a unique structure and dynamics [Boyken et al. 2013]. These sites also have to be replenished and primed after exocytosis.

The recruitment of vesicles to the release sites is dependent on the actual mobilization time of the vesicle as well as the availability of the sites [Neher 2010]. Most vesicles only spend a limited time in a docked state [Toonen et al 2006, Verhage and Sorensen 2008]. They are in equilibrium between docking and undocking, and single vesicles undock approximately once

per two minutes [Murthy and Stevens 1999]. The vesicle recruitment rate however varies with temperature [Pyott and Rosenmund 2002]. While docked vesicles can be visualized by high resolution imaging techniques like FRET and electronmicroscopy, the amount of vesicles that are primed at the active zone are defined by the electrophysiological response to sucrose stimulation [Rosenmund and Stevens 1996]. Only a small fraction of vesicles is released upon action potential stimulation [Schneggenburger et al 1999]. The so-called readily releasable pool size, which comprises all vesicles that are docked and primed, varies between different hippocampal synapses. Mossy fiber terminals for instance have a readily releasable pool (RRP) size of about 1400 vesicles, whereas pyramidal hippocampal neurons can have a neuronal pool size of about 6000 [Hallermann et al 2003, Basu et al 2007]. High-frequency stimulation is another way to estimate the readily releasable pool size. In glutamatergic synapses, it however underestimates the pool size as compared to sucrose stimulation [Moulder and Mennerick 2005].

Upon exocytosis, the glutamate will diffuse through the cleft, and bind to the postsynaptic receptor [Lisman et al, 2007]. In the cleft, glutamate is cleared by the glutamate transporter within a time range of submilliseconds [Diamond and Jahr 1997]. Glutamate binds both to metabotropic and iontropic postsynaptic receptors, but for the purpose of studying STP, only the time scales of the ionotropic receptors are of relevance. The three major post-synaptic ionotropic glutamate receptors are NMDA, AMPA and kainate receptors, and are all expressed in the hippocampus. Postsynaptic receptors are generally considered less important in STP and their contribution are considered constant, however, to keep up with high-frequency stimulation, receptors must recover in time from desensitization. In order to conduct ions at a high level, the life time of the open states also have to be long lived enough to make it possible for the receptor to enter the high conductance states. Studies have showed that the ligand-binding domain alone is responsible for the lifetime of the desensitized state of glutamate receptors. Moreover, AMPARs can keep up with kilohertz stimulation, whereas native and recombinant kainate receptors are around 100-fold slower [Carbone et al 2012].

In this thesis, we have been specifically interested in two classes of synaptic mechanisms of glutamatergic neurons, namely vesicle fusogenicity and post-synaptic AMPA receptor activation kinetics. We have constructed a first mechanistic model to investigate how intrinsic vesicular fusogenicity affects STP and a second model to describe the mechanism behind AMPA receptor activation. With the first model we found that intrinsic properties of

membrane lipids and proteins have a more important role in regulating vesicular fusogenicity and thereby STP than previously thought. This is supported experimentally by published imaging and electrophysiological data. In the model we have treated these lipid and protein properties as a biophysical entity called intrinsic vesicular fusogenicity, but below we discuss possible biochemical identities of the entity. With the second model we found that AMPA receptor activation by partial and full agonists can be explained by increased affinity of the receptor to higher conductance states. Moreover, given this mechanism the large conductance state of synaptic AMPA receptors would be stabilized within the rise time of a spontaneous EPSC. In the section below we discuss the biophysical basis of AMPAR activation. In a third and final study of the thesis we looked into allosterically regulated linker proteins and the prozone effect i.e. proteins with several binding sites for ligands. Such proteins are common in synapses both on the presynaptic and postsynaptic sides, e.g. the AMPA and NMDA receptors, calmodulin and calbindin.

Heterogeneity of vesicular fusogenicity

Vesicular release probability is dependent on local calcium at the synaptic vesicular release site, which cannot presently be measured due to spatial and temporal limitations of existing imaging and electrophysiological techniques. Hence, the role of release probability in short-term plasticity has been studied previously in the crayfish neuromuscular junction, Calyx of Held and in hippocampal synapses by using mechanistic modeling.

Under prolonged stimulation in the Calyx of Held, vesicles release with distinct kinetic constants [Schneggenburger et al 2002]. Two time scales of release are seen also in hippocampal neurons. Synchronous release take place on a ms time scale, whereas asynchronous release takes place on a tens of ms time scale [Scheuss et al 2007]. To explain the presence of asynchronous and synchronous release upon depolarization and STP, a heterogeneity in vesicular release probability is often assumed. The main source of heterogeneity in STP dynamics have been suggested to lie in differences in the initial release probability and in vesicle recruitment rate between synapses [Hanse and Gustafsson 2001, Trommershauser et al 2003]. It is the origin of this heterogeneity that we investigate in study I.

One explanation of the heterogeneity can be differences in distance to clusters of voltage-gated calcium channels between groups of vesicles, and thereby a difference in calcium concentration at the fusion site [Trommershauser et al 2003]. Nanodomain coupling

between the calcium sensor and the voltage-gated calcium channels has been shown, but the exact distance between the voltage-gated calcium channels and the active zone, as well as the exact number of calcium channels are under investigation [Nadkarni et al 2012, Eggermann et al 2011, Herman and Rosenmund 2015]. It has also been shown that reluctant vesicles can be recruited to the fast vesicle pool in an actin-dependent manner [Lee et al 2012].

A second possible explanation for the heterogeneity is different calcium sensors of synchronous and asynchronous release. To support this hypothesis, studies show two release components with different Ca2+ sensitivity [Goda and Stevens 1994]. In the case of vesicle fusion, the main trigger for fast release is thought to be calcium binding to synaptotagmin [Sun et al 2007]. For synchronous release, synaptotagmin is the sensor, but the calcium sensor for asynchronous and spontaneous release have been subject to much debate [Kaeser and Regehr 2014, Sorensen et al 2013, Sun et al 2007, Walter et al 2011, Weber et al 2014].

As a third explanation, in addition to calcium dependency, there are also intrinsic vesicular and synaptic factors that affect the fusogenicity of vesicles. These are the factors we refer to as intrinsic vesicular fusogenicity in this thesis. Improper assembly of the release machinery involving SNARE complexes and associated proteins is one example that can affect the intrinsic fusogenicity either of the synapse or of individual vesicles depending on where the proteins are situated. There are several molecular determinants of intrinsic vesicular fusogenicity linked to SNARE proteins [Rosenmund and Rizo 2008, Rosenmund et al 2003]. One such example is Munc-13, the C1 domain of which facilitates fusion of vesicles [Basu J. 2007]. Another example is complexin, which regulates both spontaneous and evoked release [Xue et al 2008]. Some of these molecules are calcium dependent [Shin et al 2010]. In addition to SNARE associated proteins, another factor that can affect vesicle fusogenicity is membrane composition in terms of phospholipids and cholesterol. For instance the phospholipid phosphatidyl inositol promotes fusion [Martin 2001]. The amount of cholesterol in the synapse has also shown to have an important role in spontaneous release [Wasser and Kavalali 2009]. Also, the expression level of vesicular glutamate transporter affects vesicle fusogenicity [Herman et al 2014]. Thirdly, release probability has seen to correlate with the size of the active zone [Holderith et al 2012].

Trommerhauser's algebraic model of short-term plasticity in the Calyx of Held

One of the first models that was published for short-term plasticity and heterogeneity of vesicular release probbility was the one of Trommershauser et al 2002. We have made use of this model in study I, and will therefore describe the different steps in detail below. What was special about the model was that it included a recruitment rate for both readily and reluctantly releasable vesicles. In addition, it assigned vesicle pool heterogeneity to closeness of calcium channel clusters.

In study I we have formulated a kinetic model in order to look into the effect of intrinsic vesicular fusogenicity on STD in hippocampal neurons, assuming that there are two groups of vesicles with different intrinsic vesicular fusogenicity, which could underly asynchronous and synchronous release.

In Trommershauser's model there are two types of vesicles, namely readibly releasable vesiles and reluctantly releasable vesicles. Reluctantly releasable vesicles are recruited from a reserve pool with the rate constant k_r. They travel back to the reserve pool by the rate k_{-r}. The amount of reluctantly releasable vesicles n_1 is hence only dependent on these two forward and backward rates to the storage pool.

$$\frac{dn_1}{dt} = -k_r n(t) + k_r$$

The readily releasable vesicles are dependent on the availability of free release sites n_{-2}. The rate k_s that drives vesicles into the readily releasable pool is calcium dependent.

$$k_s(t) = k_0 + k_s \frac{[Ca^{2+}]_{gl}}{[Ca^{2+}]_r}$$

$$k_s = k_0 + k_s \frac{[Ca^{2+}_{gl}]}{[Ca^{2+}]_r}$$

$$\frac{dn_2}{dt} = -k_t n_2(t) + k_s(t) n_0(t)$$

We have based our system on these sets of equations and extended them with an allosteric factor that we refer to as intrinsic vesicular fusogenicity (see study three). The two groups of vesicles have different values of release probability, which contributes to the amplitude I of the EPSC with the formula I=npq, where n=number of releasable vesicles, p is equal to the release probability and q equals the number of neurotransmitters per vesicle, i.e. the quantal number.

AMPA receptor activation

Single-channel measurements have shown that the AMPA receptor visits multiple conductance states in a ligand concentration dependent manner [Rosenmund et al 1998, Jin et al 2003, Gebhardt et al 2006, Smith and Howe 2000, Smith et al 2000]. In CA1 neurons of the hippocampus, native AMPA receptors showed increased number of conductance states with increased agonist concentration. It showed up to four conductance levels between 5 and 11.2 pS [Gebhardt and Cull-Candy 2006]. In GluA3/GluK2 constructs, three active states were seen at conductances between 5 to 23 pS [Rosenmund et al 1998]. In study II, we have developed a mechanistic allosteric model of how the conductance states are regulated by ligand concentration when the ligand is a full or partial agonist. The fact that quisqualate, NMDA and kainate open glutamate channels with different subconductance levels was found early. In this pioneering study it was shown that NMDA activated conductances above 30 pS, and quisqualate and kainate activated conductances below 20 pS [Cull-Candy and Usowicz 1987].

The AMPA receptor can be found in resting, active, desensitized and deactivated states [Hansen et al 2007]. The receptor has been caught in a quasi active form in a crystal structure of a GluA2 ligand-binding domain tetramer in a configuration that involves a 30 degree rotation of the LBD dimer relative to the crystal structure of the full receptor, which is stabilized by a disulphide bond. The configuration corresponds to an intermediate state of AMPAR activation [Lau et al 2013]. The existence of desensitization indirectly regulate cell death by excitotoxicity as it hinders excess of ions into the neuron. The ligand binding domain of the AMPA receptor is thought to regulate the recovery from desensitization [Carbone and Plested 2012]. Conformational restriction by crosslinking of a disulphide bond in GluR5-GluR7 receptors hinders desensitization in kainate and AMPA receptors [Weston et al 2006]. Several factors on the postsynaptic side in addition to the kinetics of AMPAR states determine the size and kinetics of the EPSC. For instance the density and sizes of receptors, the ligand binding probability and the time inbetween collisions of ligand and receptors [Ventriglia and Maio 2013]. Quantal size however depends more on the density of AMPARs

than the number [Raghavachari and Lisman 2004]. The free glutamate concentration in the synaptic cleft, which also is a determinant of EPSC kinetics cannot be measured directly, but its peak concentration and decay time have been approximated to 1.1 mM and 1.2 ms respectively [Clements et al 1992]. Despite the multitude of underlying mechanisms of EPSC kinetics, activation kinetics could have a potentially important role in STP, which has not been thoroughly investigated to date.

The AMPA receptor is a tetrameric receptor with one binding site for glutamate per subunit. Each subunit consists of four parts [Gouaux 2004]. The initial segment on the outside of the plasma membrane is the N-terminal and is followed by the ligand binding domain. The ligand-binding core has a clam-shell like structure and consists of the S1 and S2 subunits. At an apo state of the receptor, the ligand binding core is maximally open, whereas it is closed when it is bound to an agonist. The antagonist NBQX stabilizes the open state of the ligand binding core, and the agonists, glutamate and AMPA, give rise to a cleft closure of similar degree i.e. the cleft closure is 21 degrees more closed compared to the open state. The partial agonist kainite gives rise to a cleft closure that is smaller [Gouaux 2003]. The ligand-binding core, and more specifically the C-terminal end of the S2 domain, has a stretch of 38 amino acids, the sequence of which determine whether it is a so-called flip or flop splice type. These two splice types exist for all four types of subunits, however, for GluA1 both flip and flop splice types have the same channel opening and closing times. For the GluA2-4 subunits on the other hand, the opening rates are the same, but the desensitization rates are 3 times faster for the flop slice types than their corresponding flip variants. In addition, the flop splice types recover slower from desensitization [Pei et al 2009].

Apart from subunit composition of the receptor and splice types of subunits, auxiliary subunits have been implicated in AMPAR conduction. Examples of these are transmembrane AMPAR regulatory proteins (TARPS), cornichons, CKAMP44 and GSG1L. TARPs are the most studied of these auxiliary subunits and can be subdivided into types 1a, 1b and 2. TARPS have shown to regulate gating and desensitization kinetics of AMPA receptors via interactions with the N terminal domain of the AMPA receptor [Cais et al 2014].

Another question is whether different modulators affect AMPAR conductance in different ways [Stern-Bach et al 1994]. For instance the three agonists AMPA, glutamate and

quisqualate interact differently with the AMPA receptor, especially in the region of a hydrophobic pocket that is close to the anionic gamma-substituents [Jin et al 2002]. It is unknown how full and partial agonists act with different efficiency on the AMPA receptor conductance even though the full receptor has been crystallized in a willardiine bound state as well as an antagonist bound state [Sobolevsky et al 2009, Sobolevsky 2015, Yelshanskaya et al 2014]. One structural and single-channel study showed that the GluA2 binding domain binds to the partial willardine agonists in different conformations of the ligand binding domain than glutamate [Jin et al 2003]. Positive allosteric modulators increase the probability for the receptor to dwell in the open state. On a general basis, environmental factors such as pH, ionic environment and lipid composition of the membrane may be of importance as mentioned earlier. Chemically, several positive modulators have been characterized e.g. benzothiazides, benzamides, benzoxainones and biarylsulfonamides. Nevertheless, apart from modulating the active state positively, they also modulate desensitization kinetics [Weeks et al 2014].

It is also of interest whether ligand binding is required to open the ion channel and whether increasing conductance depends only on increased ligand binding. Single-molecule fluorescence resonance energy transfer studies of the wild-type AMPAR and the T686S mutant showed that the extent of activation depends on the probability of the ligand-binding domain entering a closed-cleft conformational state [Landes et al 2011]. Dynamical simulations of glutamate-gated chloride channel from Caenorhabditis elegans from the same family, emphasized the importance of the relationship between the quaternary twisting and the opening/closing of the ion pore [Calimet et al 2013]. In one of the early papers on the topic of multiple conductance states of the AMPAR it was hypothesised that binding of two agonists give rise to the lowest conductance level, three to the second lowest and four to the highest [Rosenmund et al 1998]. In contradiction to this, at a pH of 4.6 the bacterial Gloeobacter violaceus pentameric ligand-gated ion channel homologue, which is a pentameric ligand-gated ion channel from the Cys-loop family, has been found in an apparently conformation in an X-ray structure [Bocquet et al 2009]. Moreover, in mouse muscle cells, patch-clamp recordings have shown evidence of spontaneously open nicotinic acetylcholine receptors [Jackson 1984]. Single-channel recordings have also shown spontaneously opened NMDA receptors [Turecek et al 1997].

The methods of finding the correlations between ligand binding and conductance are few. One such method is covalently tethering one ligand at a time, which has been done to single

rod cyclic nucleotide-gated channels [Ruiz and Karpen 1999]. This is however cumbersome and an artificial way of producing a conductive receptor. To this end simulators are available to differentiate between single-channel openings and ligand-binding events [Edelstein et al 1997]. Two common ways to describe allosteric proteins are by the Koshland Nemethy Filmer theory 1966 and the Monod Wyman Changeux theory [Koshland et al 1966, Monod et al 1965, Changeux and Edelstein 1998, Stern-Bach 2004]. The kinetic scheme of the MWC model has previously been used to describe the transitions of the nicotinic acetylcholine receptor between resting, active and desensitized states [Edelstein et al 1996].

Monod Wyman Changeux framework

The Monod Wyman Changeux (MWC) framework was originally used for haemoglobin and enzymes. In study II we have used the MWC framework in order to describe AMPA receptor gating. The main message is that the number of bound sites does not correspond to a specific conductance state. On the contrary, the receptor may be found spontaenously open without ligands bound to it.

The model if of allosteric type. For this function it uses an allosteric parameter known as L. L is a measure of how prone the model is to be found unliganded in an active conductance state.

The model also has a dissociation constant called K. This K is specific for each conductance state. In our model the affinity of the ligand to higher conductance states was higher.

Another parameter of this framework is the cooperativity constant c. This constant is a ratio between the K value of a high conductance state and the K value of a low conductance state.

There are two types of functions usually used to compare with empirical data. One is the state function. This describes the fraction of receptors at a specific conductance state.

$$A_L = \frac{\frac{1}{L_L}\left(1+\frac{X}{K_L}\right)^4}{\frac{1}{L_S}\left(1+\frac{X}{K_S}\right)^4 + \frac{1}{L_M}\left(1+\frac{X}{K_M}\right)^4 + \frac{1}{L_L}\left(1+\frac{X}{K_L}\right)^4 + \left(1+\frac{X}{K_B}\right)^4}$$

Another function is the binding function which shows the fraction of occupied binding sites with the concentration of ligand.

$$Y = \frac{\frac{X}{K_B}\left(1+\frac{X}{K_B}\right)^3 + \frac{1}{L_S}\frac{X}{K_S}\left(1+\frac{X}{K_S}\right)^3 + \frac{1}{L_M}\frac{X}{K_M}\left(1+\frac{X}{K_M}\right)^3 + \frac{1}{L_L}\frac{X}{K_L}\left(1+\frac{X}{K_L}\right)^3}{\frac{X}{K_B}\left(1+\frac{X}{K_B}\right)^4 + \frac{1}{L_S}\frac{X}{K_S}\left(1+\frac{X}{K_S}\right)^4 + \frac{1}{L_M}\frac{X}{K_M}\left(1+\frac{X}{K_M}\right)^4 + \frac{1}{L_L}\frac{X}{K_L}\left(1+\frac{X}{K_L}\right)^4}$$

AIMS AND SPECIFIC OBJECTIVES

The aim of this thesis is to investigate the regulatory mechanisms in glutamatergic hippocampal neurons to get a better understanding of what drives short-term plasticity in these neurons. More specifically, we are looking at the following questions:

1. What is the role of intrinsic vesicular fusogenicity in hippocampal STP? (Study I)

2. How does ligand concentration govern AMPA receptor activation? (Study II)

3. How does the high-dose hook effect express itself in allosteric receptors (Study III)?

4. How are dynamical and quantitative properties underlying synaptic short-term plasticity measured, and how much is known at this stage? (Review)

MATERIALS AND METHODS

Measuring synaptic responses in vitro

In vitro, synaptic plasticity is evaluated by measuring the response to trains of depolarization, either by field stimulation or whole-cell patch-clamp in mass cultures or slice cultures, or by whole-cell patch-clamp in autaptic neurons. The advantage of using the latter system is that network effects are avoided, and release from a single neuron can be measured. Such measurements have shown heterogeneity of STP in hippocampal neurons, and the overall purpose of this thesis is to understand the origin and function of the heterogeneity.

Autaptic cultures

Hippocampal neurons were cultured on astrocyte islands as previously described. Single-electrode whole-cell voltage-clamp measurements were done on embryonic neurons, growing individually on an island between days 12 to 18 after culture. These neurons have preserved electrophysiological properties, even though they do not preserve developmental properties [Bekkers and Stevens 1991, Rose et al 2013].

Electrophysiology

Cells were voltage-clamped at -70 mV and depolarized to 0 mV for 2 ms to evoke an unclamped action potential. Short-term plasticity was measured by estimating the EPSC amplitudes upon each stimulation as no change in kinetics of the EPSC trace was seen over the stimulation train. Cells with less than 500 pA response amplitude were excluded. The release probability of the readily releasable pool was measured by dividing the charge of an evoked response with the charge of response to 500 mM sucrose. For measurements we used an Axopatch 200B amplifier (Molecular Devices) and the Clampex 10.0 software from Molecular Devices. Data were acquired at a sampling rate of 10 kHz and low-pass filtered at 3kHz. We compensated for the series resistance, which ranged between 7 and 15 MOhm.

The extracellular medium contained 136 mM NaCl, 2.5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$, 4 mM $MgCl_2$ and the internal solution contained 140 mM potassium gluconate, 10 mM HEPES, 1 mM EGTA, 4.6 mM $MgCl_2$, 4 mM Na-ATP, 15 mM creatine phosphate, and 50 U/ml phosphocreatine kinase. To identify excitatory neurons 3 mM KA

was applied for 2 s, and to measure the charge of the readily releasable pool hypertonic sucrose solution diluted to 500 mM in the external solution was used.

Kinetic simulations and optimization

The kinetic ODE models in study I and study II were simulated using the deterministic LSODA algorithm in COPASI and STOIC respectively [Hoops et al 2006]. The thermodynamic model in study 2 as well as the data analysis algorithm in Study 2 were encoded in Matlab. In both studies 1 and 2, the parameters were estimated by using the genetic algorithm encoded in COPASI, and refined with the steepest descent algorithm.

Statistical considerations

Standard error estimates of experimental STP data was done in Excel. Pearson's R correlation coefficients were calculated in Excel in order to compare experimental data and model predictions. Calculation of the median release probability as well as standard deviation measurements for the release probability were calculated using R.

Ethical considerations

Ethical permits for using mouse material was obtained from the ethics committee in Berlin (Landesamt für Gesundheit und Soziales).

RESULTS

All figures referred to in this section can be found in the original publications.

Review: Kinetic and quantitative synaptic properties underlying presynaptic short-term plasticity

I began by making a literature review to identify the known physical parameters for study I. In this review we look into biophysical synaptic mechanisms underlying short-term plasticity such as vesicle recruitment, fusion, endocytosis, neurotransmitter filling, trans- and postsynaptic mechanisms. The purpose is to find out how such properties are measured, which magnitudes and time scales they have, and which properties can presently not be measured, and require mechanistic modelling in order to be elucidated. The main factors behind short-term plasticity, which are discussed in the introduction, are summarized in figure 1 in the published review.

Study I: Intrinsic vesicular fusogenicity determines short-term plasticity in hippocampal neurons

Resting states with heterogeneity in intrinsic vesicular fusogenicity exist

To look into the role of intrinsic fusogenicity under activity, we first needed to know the states of vesicles and vesicle fusion sites when the neuron is not being stimulated. Since undocked sites cannot be detected with imaging techniques at this stage in addition to docking and priming not being entirely defined, we used a kinetic model as this allowed us to characterize the state of each vesicle biophysically. This model had three different modules, namely vesicle dynamics, site dynamics and calcium dynamics. In hippocampal neurons, it is known that release occurs at both millisecond time scales and tens of millisecond time scales. To account for both of these time scales, we introduced two groups of vesicles with low and high intrinsic vesicular fusogenicity. The rationale for introducing this lumped biophysical entity called vesicular intrinsic fusogenicity is that several electrophysiological and imaging studies have shown that calcium independent factors such as lipids, cholesterol and synaptic proteins, for instance vesicular glutamate transporters and complexin increases the fusogenicity of synapses, and we find it likely that such fusogenicity increasing factors are unequally distributed over populations of vesicles. With the kinetic model and its parameters, we first wanted to see if we could describe the resting state of a hippocampal neuron with this setup of two vesicular groups of different intrinsic vesicular fusogenicity. To do this, we

estimated parameters of recruitment such as priming, docking and site-preparation as well as spontaneous fusion rates from literature. With these constraints we were able to reproduce a readily releasable pool (the vesicles closest to the stage of fusion) of around 4000, which is a typical readily releasable pool (RRP) size for an autaptic hippocampal glutamatergic neuron. Thus, the model could reproduce the size of the RRP under resting as well as spontaneous fusion rates and recruitment parameters with two groups of vesicles with different intrinsic fusogenicity.

Depletion of vesicles because of slow vesicular priming can explain synaptic depression

Next, we measured the STP of autaptic glutamatergic hippocampal neurons at 1 Hz and 20 Hz with whole-cell voltage-clamp under an external calcium concentration of 2 mM. From our STP measurements, we observed a depression at 20 Hz (figure 3), but no change in plasticity at 1 Hz (figure 3). This gave rise to the question why there is depression at 20 Hz, but not at 1 Hz. So, using our kinetic model of STP with two groups of vesicles with difference in intrinsic vesicular fusogenicity as defined above, we firstly investigated whether we could describe the differences in STP at the two frequencies. Secondly, if the depression could be explained, what was the mechanistic explanation for it? The model could indeed explain STP at 1 and 20 Hz (see figure 3). In order to investigate the underlying cause of why there is depression at 20 Hz, but not at 1 Hz, we first simulated the dynamics of the readily releasable pools over the stimulation trains at both frequencies. This showed us that the RRP could not keep up with the fusion rate at the stimulation frequency of 20 Hz (figure 4). The relatively slow production of the RRP could have a number of explanations; (1) too low priming rate, (2) too low docking rates or (3) too low number of sites available for vesicles to dock to and as an effect of the two last points, the number of vesicles feeding into the RRP would be limiting. When we simulated the docked but unprimed vesicles, i.e. the vesicle pool which feeds into the primed pool, they decreased initially at both stimulation frequencies but eventually attained a plateau. The initial decrease in docked vesicles was however not due to lack of available sites, since simulating these showed a constant increase from the initial stimulation. Hence, the initial decrease must have been due to relatively low docking rate. Nevertheless, the size of this docked and unprimed vesicle pool which feeds into the readily releasable pool was never the limiting factor of production of readily releasable vesicles. The only plausible explanation for why the readily releasable pool cannot keep up with the fusion rate must be the slow priming rate. In order to confirm our conclusion, we performed sensitivity analysis of the model with respect to the calcium dependencies of the recruitment

rates. This also predicted that the main regulating step in depression was the calcium dependency of vesicle priming, since only a change in this parameter gave a significant change in the steady-state short-term plasticity (see figure 5). In essence, the presence of two pools of vesicles with different intrinsic vesicular fusogenicity could explain depression by slow priming being the limiting factor in order for the size of the readily releasable pool to keep up with the fusion rate.

Calcium dependency of molecular priming contributes to facilitation

We also wanted to see whether the model with two different intrinsic fusogenicity groups of vesicles could explain facilitation. Given our previous results, we hypothesized that the priming rate could also be a contributor to facilitation. Opposed to the case of depression where the slow priming rate keeps the readily releasable pool from growing, we predicted that tuning up the priming rate would increase the pool and make it possible for increasing release over the first few stimulations in a train. Accordingly, when we speeded up the priming rate to a similar level as the calcium dependent docking and site-preparation parameters it resulted in a facilitation at 20 Hz, 10 Hz and 5Hz (see figure 6). From this we concluded that increased molecular priming produces more readily releasable vesicles, and together with the build-up of calcium that exists at these frequencies, gives facilitation of short-term plasticity. Hence, the same mechanism that hinders the population of readily releasable vesicles to grow and keep up with release at depression, will give rise to an increasing readily releasable pool over the first stimuli and together with build-up of calcium will contribute to facilitation.

STP behaviour changes significantly with heterogeneity in intrinsic vesicular fusogenicity

The initial release probabilities of each of the intrinsic fusogenicity groups of vesicles determine the global initial release probability and thereby the STP. The initial release probabilities of the subgroups are not only dependent on the biophysical entity we refer to as intrinsic vesicular fusogenicity, but obviously they also depend on calcium sensitivity of fusion and the global fusogenicity of the synapse. We saw that if the difference in intrinsic vesicular fusogenicity increases in a way that the higher intrinsic fusogenicity is increased, and the gap between the two vesicle groups becomes larger, the STP after 20 stimuli depressed significantly at both frequencies. When the initial release probability changed to the same level by a switch in calcium sensitivity or global intrinsic fusogenicity, the STP also showed depression, but not as much as in the case of a change in difference in intrinsic

vesicular fusogenicity (see figure 7). In conclusion, this tells us that a greater spread in the heterogeneity of intrinsic vesicular fusogenicity has a different effect on STP than a global change in synaptic fusogenicity either by a mutation of the calcium sensitivity of synaptotagmin I or changes in the levels or efficiency of a global fusogenicity protein such as complexin.

Study 2: Ligand-dependent opening of the multiple AMPA receptor conductance states: a concerted model

Full and partial AMPAR agonist effects explained by conductance dependent ligand affinity

In this study we wanted to investigate how the multiple agonist concentration dependent conductance states of the AMPA receptor are regulated mechanistically. To do this we formulated an allosteric model using the Monod Wyman Changeux framework (see figure 1). The theoretical model gave us the ability to simulate the frequency of receptors in each of the conductance states as a function of the ligand concentration, but also as a function of two more mechanistic parameters. The first parameter L describes the likelihood of finding the receptor in a non-liganded closed state compared to a non-liganded open state at any of the other conductances. The second parameter K describes the dissociation constant of the ligand for the receptor at each of the conductance states. In other words, what we investigated was whether we could explain the conductance state with receptors that could attain different conductances independent of liganded state, but with some conductance states being more likely at some liganded states. These receptors also will have different affinity to the ligand depending on which conductance state they are in at the moment. Another assumption of the model is that all changes in conductance occur by concerted change between the subunits. The model predicted that GluA3/GluK2 receptors expressed in HEK cells stimulated with quisqualate and native receptors expressed in granule cells stimulated with glutamate bind to ligands with different affinities in the different conductance states, and stabilize the large conductance state. It was also predicted that stimulation of homomeric GluR2 constructs with the partial agonist iodowillardiine stabilized the intermediate conductance state (see figure 2). From this we concluded that full agonists stabilize the large conductance state and partial agonists stabilize intermediary conductance states in a ligand dependent manner with increasing ligand affinity with conductance states. As a consequence of the good fit to data under relatively constrained conditions, the model also supports existence of AMPA receptors in non-liganded conductive substates, which are decreased in frequency with enhanced conductance level.

The large conductance state is stabilized during the rise phase of a synaptic event

Given the results above, that it is indeed possible to explain the ligand concentration dependence of the AMPA receptor conductance states by increasing ligand affinity to conductance states, we wanted to see how populations of AMPA receptors behave over the time course of a spontaneous postsynaptic current in glutamatergic hippocampal neurons. To this end we extended the formulation of the thermodynamic model above to a kinetic model of the conductance state dynamics. Since we did whole-cell voltage-clamp recordings under high magnesium concentration (4 mM) in the external medium and thereby blocking NMDA receptors, we assume that the postsynaptic current upon depolarizing autaptic neurons is mediated by AMPA receptors. We collected single mini EPSC traces from glutamatergic hippocampal autaptic neurons, and averaged in order to estimate the average rise time of 0.53 ms (see figure 3). The model simulations showed that the large conductance state of the AMPA receptor was stabilized during the rise time both in the deterministic and the stochastic cases (see figure 3). Thus, the stabilization of the large conductance state could provide an explanation for the mini EPSC peak.

Study 3: Cooperativity antagonizes the high-dose hook effect in linker proteins

The prozone or so-called high-dose hook effect was first discovered by immunologists many decades ago, but it was not until the late nineties that it was established as a general phenomenon for biochemical systems with linker proteins [Bayne-Jones 1917, Bray and Lay 1997]. In layman's terms the effect means that addition of multimeric proteins to a mixture of ligand initially leads to increase of fully bound complexes, but begins to decrease at a certain concentration of multimeric proteins. A few years back it was suggested that the prozone effect might have less impact on cooperative proteins compared to non-cooperative proteins. In this study we have investigated this claim mathematically under perfect and imperfect binding.

Absence of allostery gives rise to decrease of fully bound complexes

The simplest reaction system, which we employed in order to investigate our hypothesis, was a linker protein (L) with two binding sites, one for ligand A and one for ligand B (see figure 1). We initially considered a case with perfect binding, thus having an infinitely small dissociation constant. In the case where the number (b) of ligand B is limiting, the expected number of LAB complexes that will be formed are b. The same principle holds when the

number (l) of linker proteins L is limiting, since the expected number of LAB complexes will be l. On the other hand when the number of L is substantially larger than both the numbers of A and B, the situation becomes slightly different. The probability that A will be bound will be a/l and the probability that B will be bound is b/l. Hence, the probability that A will bind first and B later or vice versa is a/l multiplied by b/l, and the number of L in LAB complexes are this factor multiplied by the number of free L in the beginning. Thus, when the l is varied over a range of values in a situation when b < a , the number of complexes will initially rise until the amount has reached b upon which b becomes the limiting factor. At a critical point it then starts decreasing and breaks into incomplete complexes, so the number of LABs starts decreasing with increasing l. (It should be noted that this effect is not seen when the initial amount of ligand is increased.) When we ran simulation with imperfect binding with same (see figure 1) and different initial amounts of A and B, the results were the same as in the theoretical case. Moreover, running the same simulations for a tetrameric complex, also yielded that the fully bound complex decreases upon increase of the linker concentration from a critical point.

Cooperativity gives less decrease of fully bound complexes

As in the case without cooperativity, at the presence of cooperativity we first considered perfect binding. The same reasoning can be applied when cooperativity is taken into account, except that a cooperativity factor c will increase the affinity to the binding of the second ligand. As an effect we find that the probability of having a fully bound complex is a/l multiplied by bc/l. In other words, the probability of LAB formation increases with the c factor compared to the case of absence of cooperativity (see figures 2 and 3). Thus, the steady-state concentration of LAB upon increase of L does not decrease as much as it does at the absence of cooperativity. This was also found in the simulations of dimeric proteins with imperfect binding.

Cooperativity and allostery affect the high-dose hook effect in calmodulin

In order to investigate the high-dose hook effect in a synaptic protein, we chose the tetrameric calcium binding molecule calmodulin. Calmodulin can be found in a relaxed state (R) and a tense state (T), and the transition between the two states give rise to binding cooperativity. Hence, we pushed in the molecule in the R state to get rid of the cooperativity. In both the wild-type and the R state we observed the high-dose hook effect. However, the R state reached a higher peak and had a lower tail.

We tested for the natural concentration range of calmodulin which micromolars to tens of micromolars. No only R state mutant of calmodulin is known to date. However, with high concentrations of its allosteric regulator CaMKII the molecule can be stabilized in its R state. At lower concentrations of the allosteric modulator both molecules in the T and R states were present. This showed that the prozone effect is lowered in the presence of allosteric modulator (see figures 4 and 5).

DISCUSSION

In this thesis our three major conclusions were (1) intrinsic vesicular fusogenicity tunes short-term plasticity in glutamatergic hippocampal neurons, (2) ligand concentration dependence of AMPA receptor conductance states are regulated by concerted activity of its subunits and increased affinity to ligands with conductance and (3) allosteric linker proteins, which are common in synapses, can balance the prozone effect.

Calcium dependent release probability versus intrinsic vesicular fusogenicity and regulation of presynaptic STP

We saw that depression occurred at 20 Hz because the slow rate of priming could not keep up with the fusion rate. This is similar to a previous model of the Calyx of Held, where it was predicted that vesicles with vicinity to calcium channel clusters and therefore a higher calcium concentration upon membrane depolarization as opposed to those that dock further away from the channel clusters, become depleted due to slow priming. Our model questions that only calcium regulation should be of importance for depression of STP. We made the assumption that there is heterogeneity in calcium independent biochemical factors (mentioned in the introduction and results) that underlie the biophysical entity intrinsic vesicular fusogenicity and due to the small size of hippocampal synapses differences in distances to voltage-gated calcium channels can be neglected. Vesicles are therefore subjected to essentially the same calcium concentration levels upon stimulation. To support the differences in action between global calcium sensitivity and intrinsic vesicular fusogenicity of the subgroups, we showed that increasing the differences in fusogenicity between the groups has a higher impact on the steady-state level of STP than increasing global calcium sensitivity of fusion despite the same intial global vesicular release probability. Calcium concentration and sensitivity versus intrinsic vesicular fusogenicity can be tested by making mutations that target these modes of fusion regulation mechanisms, not changing the initial release probability and doing patch-clamp to see if the STP pattern becomes different in the end. This can for instance be validated by looking into studies where synaptotagmin has been mutated versus for instance complexin knockouts under the same external calcium concentrations. As an example, synaptotagmin 1 with enhanced calcium sensitivity at initial release probabilities of around 5.5 % (wt), 8%, 10% and 14% gave steady-state 10 Hz depressions around 0.75, 0.5, 0.4 and 0.25. At the same external calcium concentration wild-type neurons with initial release probabilities of around 6.5 % and

complexin knockouts of 3% had a steady-state STP of around 0.4 and 0.9 respectively [Rhee et al 2005, Xue et al 2008]. Comparing the change in STP with the change in release probability, we find that increase of release probability by augmenting calcium sensitivity has a lower impact on steady-state depression compared to global intrinsic fusogenicity. Thus, in line with our predictions, the output on short-term plasticity is different depending on which mechanism the synapse employs to regulate fusion.

The physical basis of the thermodynamics and kinetics of AMPAR conductance

The model developed in study II can to some extent be considered a black box model, where the electrical conductance of the ion pore is described as a function of ligand concentration. Nevertheless it does gives us mechanistic information about liganding and affinity. The advent of the patch-clamp technique in the seventies increased our ability to measure electrical single-channel behavior, and the cloning of AMPARs in the nineties have given us insight into biochemical functional domains [Bezanilla 2008]. Yet, there is limited structural information about what causes increasing conductance in the ion channel pore of AMPA receptors [Traynelis et al 2010]. In our diagram we represent this ability of the ion channel to increase its conductance symbolically by a dilating iris diaphragm, however, a physical expansion like this is not likely to underlie increasing conductance since the selectivity of the ion channel is partly regulated by the size of its pore. A much more plausible explanation is the movement of lining proteins in the pore, which will make it less or more energetically favorable for sodium, potassium and calcium to permeate the channel.

In the ligand-dependent conductance model, we make a generalized model for the AMPA receptor conductance states. The aim was to see whether we could describe the ligand dependence by increased affinity to conductance states, which is an important assumption of the Monod Wyman Changeux theory. Nevertheless, as mentioned in the introduction, the activity kinetics of AMPA receptors depends on factors like subunit composition, auxiliary proteins, splice types and post-translational modifications.

TARPs as mentioned in the introduction are a relatively well-studied family of AMPA receptor auxiliary proteins. Single-channel studies have shown that co-expression of members of the TARP I family (gamma 2, gamma 3, gamma 4 and gamma 8) together with GluA1 and GluA4 slow down the deactivation and desensitization. Furthermore, they increase the channel opening rate, bring the channels to an initial state of high probability of opening and enhance the duration of bursts of channel openings. What is most interesting in

relation to our model in study II is that TARP associated AMPARs due to the slowing down of deactivation and desensitization spend more time in the high conductance states above 30 pS. This makes the model for physiologically relevant since receptors non-associated with auxiliary proteins usually do not have the time to enter the higher conductance states before becoming deactivated or desensitized [Zhang et al 2014, Howe 2015]. Concerning molecular properties that affect channel kinetics, we have already mentioned above that the flop splice forms of the GluA2-GluA4 subunits have a three times faster desensitization rate than their corresponding flip isoforms, and they also recover slower [Pei et al 2009].

In general, our model presents a possible regulation mechanism for the conductance states by increased ligand affinity to conductance states, but opening and desensitization kinetics of specific subtypes, splice types and auxiliary proteins need to be integrated to describe single-channel, gating and population currents and to gain a more detailed understanding of whether the suggested regulation mechanism is plausible.

The prozone effect in synapses

In study 3 we looked into the prozone effect in allosteric proteins, and learnt that the cooperativity factor of allosteric interaction contributes to balancing the amount of fully bound protein complexes. As an effect the fully bound complex concentration does not decrease as much as in non-allosteric cases.

Since it was investigated in a thesis on biophysical properties of glutamatergic neurons, a natural question to ask is how the decreased prozone effect in allosteric proteins applies to synapses. One example of a synaptic linker protein and its ligand concentration dependence has been discussed in this thesis, namely the AMPA receptor. We know now that the conductance state of the receptor increases with ligand concentration, but not necessarily with ligand binding. In the synapse, ligand concentration presumably increases at the beginning of an EPSC because of more and more release from the presynapse, until it reaches a peak and starts decreasing. The decay phase is said to be attributed to factors like receptor desensitization and ligand buffering from the synaptic cleft. Over a train of stimulations, we saw in study I that there is presynaptic depression because of slow vesicle priming. For the postsynaptic side, it is known that AMPARs are inserted into the membrane upon activation of the synapse. The time scale at which the insertion occurs is dependent on the location from which they are recruited; the spine extra-synaptic membrane, exocytotic vesicles released to the spine or from the dendritic shaft to the spine neck, but an upper limit for the half-time of

scaffold protein occupancy at the membrane is 710 ms [Tolle and Le Novère 2010]. Hence, AMPA receptor trafficking is an underlying factor of long-term plasticity, but studies have also shown that removal of the extracellular matrix (ECM) causes AMPAR trafficking to affect short-term plasticity in central neurons [Shepherd and Hugar 2007, Gundelfinger et al 2009]. More specifically, paired pulse depression is decreased at the absence of ECM at interstimulus intervals between 10 and 300 ms, after which it is fully recovered. This has a relevance for juvenile brains since the extracellular matrix is fully established only 3-5 weeks postnatally when the synaptic circuitry is already developed. To sum up this means that the active linker concentration increases over a train of high frequency stimulation of for instance 20 Hz, while the ligand concentration decreases due to slow vesicle priming and glutamate buffering in the synaptic cleft. Depending on the density of receptors and ligand during the time course, we expect a possible critical point where the increase of active AMPAR density balances the ligand concentration to start decreasing the concentration of receptors at higher conductances, even though the total conductance is increased by enhanced levels of receptors at low conductance states.

Another example of possible implication of the prozone effect is the presynaptic calcium transient and its impact on short-term plasticity. In its rise phase, the concentration increases by influx from voltage-gated calcium channels upon depolarization of the axon hillock and possibly by calcium induced calcium release. Its decrease pertains to several factors like calcium buffers, mitochondrial buffering, plasma membrane extrusion pumps and diffusion. An example of a calcium buffer that is important in hippocampal neurons is calbindin, which has four binding sites for calcium and is therefore also a linker protein. Another calcium binding protein that functions as a linker is calmodulin. Mitochondria are arrested in the bouton upon elevation of calcium, and it has been seen in hippocampal synapses that motility of mitochondria decreases the variability in EPSC amplitude of trains of presynaptic stimulation [Sheng 2014]. Moreover, calcium binding buffers and proteins bind calcium with cooperativity, and plasma membrane pumps increase their activity with calcium concentration. Similarly to the possible scenario in AMPA receptors, the increased activation of calcium regulators might reach the critical point where the number of fully active calcium bound proteins and pumps decrease instead of increasing despite increasing concentration of the free proteins. This could have an impact on the time length and amplitudes of short-term plasticity.

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